Evaluation of the efficacy of a danazol-loaded intrauterine contraceptive device on adenomyosis in an ICR mouse model

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BACKGROUND: Danazol, a synthetic steroid with antigonadotrophic properties, has been widely used for the treatment of endometriosis and adenomyosis. However, the local application of danazol to the uterus to treat adenomyosis is controversial. The objective of this study is to develop an effective treatment for adenomyosis using danazol via intrauterine contraceptive device (IUCD) delivery. METHODS: An adenomyosis animal model was established using Institute for Cancer Research, Swiss-derived (ICR) mice, grafted with a single pituitary gland (n = 30). Four months after grafting, IUCDs with three different quantities of danazol were prepared and used to treat the ICR mice with adenomyosis. After 2 months of treatment with a danazol-loaded IUCD, the number of adenomyosis nodules and the hematoxylin-eosin staining scores were measured and compared with mice given daily oral danazol and controls (no adenomyosis). RESULTS: As the danazol dose increased, the nodule number decreased reaching significance at a dose of 2.0 mg per 20 g body weight (P = 0.002). When compared with oral administration, the plasma danazol concentrations with IUCD delivery were low and stable. CONCLUSIONS: These results suggest that an IUCD loaded with an appropriate dose of danazol may be an effective treatment for adenomyosis and that human trials are warranted.

Keywords: danazol; adenomyosis; mouse; animal model; intrauterine contraceptive device

Introduction

Adenomyosis is defined as the presence of endometrial glands and stroma in the myometrium of the uterus. The exact etiology and pathogenesis of adenomyosis remains unclear. Clinical symptoms include dysmenorrhea, hypermenorrhea and infertility, and treatment frequently involves hysterectomy (Ferenczy, 1998). However, this option is inappropriate in young or nulliparous women who wish to preserve their uterus. In this situation, current treatment options include GnRH analogs, oral danazol or conservative surgery where a localized area of adenomyosis can be demarcated. This type of surgery is technically difficult and of no benefit in the common situation where adenomyosis is diffusely distributed through the myometrium. The available drugs have frequent side effects and require long-term administration (Wood, 1998). Compared with current drug treatments, increased efficacy and reduced side effects might be achieved by sustained delivery to the uterus itself.

Igarashi et al. (2000) reported that adenomyosis could be treated with 300–400 mg of danazol loaded onto an intrauterine contraceptive device (IUCD). In their study, they treated 14 adenomyosis patients with dysmenorrhea, hypermenorrhea or infertility. During the treatment, blood danazol levels were undetectable, ovulation was not inhibited and no side effects were reported. The danazol-loaded IUCD was effective at reducing uterine size and dysmenorrhea in 9 of 10 cases. Pregnancy occurred in three cases after removal of the IUCD. A danazol-loaded vaginal ring (Igarashi et al., 1998) and IUCD (Cobellis et al., 2004) have also been tested in patients with endometriosis with encouraging results that local application of danazol via vaginal or intrauterine drug delivery represents an appealing option.

Danazol is a derivative of the synthetic steroid ethisterone. It has strong antigonadotrophic properties (Dmowski, 1990) and has been widely used as a treatment for endometriosis (Farquhar, 2007) and adenomyosis (Matalliotakis et al., 2003). Oral danazol has been shown to suppress the pituitary-ovarian axis by inhibiting the production of both pituitary gonadotrophins and suppressing ovulation (Franchimont and Cramilion, 1997; Nomura et al., 2006). As danazol is relatively insoluble in water, undergoes hepatic metabolism and has low oral bioavailability, high oral doses are required to achieve effective blood
concentrations (Badawy et al., 1996a,b). Severe side effects are common and length of treatment is limited. Recent accumulated evidence suggests that danazol can act directly on endometrial or endometriotic tissue in vitro to inhibit DNA synthesis and induce apoptosis, exert direct effects to suppress the growth of endometriotic implants and decrease macrocyte-enhanced endometrial proliferation in peripheral blood in vivo (Surrey and Halme, 1992; Braun et al., 1994; Igarashi et al., 1994; Tamaoka et al., 2000). In addition, danazol has been shown to inhibit aromatase activity in endometriotic cells in vitro (Murakami et al., 2006). On the basis of these direct in vitro and in vivo actions of danazol on endometrial or endometriotic tissue and the clinical findings described above, we hypothesized that a danazol-loaded IUCD would be a more effective and better tolerated treatment for adenomyosis than current oral formulations.

In the present study, an Institute for Cancer Research, Swiss-derived (ICR) mouse adenomyosis model was established, and a suitably sized and loaded danazol IUCD was developed. The danazol-loaded IUCD was placed into the adenomyosis mouse uterus and the efficacy and pharmacokinetics were investigated. In addition, we compared the IUCD-loaded dose with the concentration of danazol in the uterine cavity to determine an appropriate clinical dose of danazol for the treatment of adenomyosis.

Materials and Methods

Animals and experimental procedures of adenomyosis model

Virgin female ICR mice maintained at Department of Biological Sciences, Zhejiang University, were used in this study (Lu et al., 2006). They were housed in plastic cages with wood chips under controlled conditions (12 h of light from 6 A.M. to 6 P.M.) in accordance with the principles outlined in the Guide for Animal Care and Use of the Committee of the Graduate School of Science, Zhejiang University. All mice had free access to a commercial diet and tap water. All the experimental procedures conformed to the regulations described in the Guide to the Care and Use of Laboratory Animals of the U.S. National Institutes of Health.

Validation of ICR mice as a suitable model for adenomyosis, similar to SHN mice

The mammary tumor prone SHN mouse strain is known to develop adenomyosis spontaneously, especially after pituitary grafting (Sakamoto et al., 1992). The SHN mouse strain, which has a high incidence of mammary cancer, was originally developed by inbreeding and selection from Swiss stock mice by Dr H. Nagasawa et al. in 1976. However, SHN mice are only available in Japan (Nagasawa et al., 1976; Nagasawa and Kusakawa, 2001). To determine whether ICR mice can be used as an experimentally induced animal adenomyosis model like the SHN mice, we followed the experimental procedures described by Kawahara et al. (2003b). We implanted a single pituitary gland from age-matched male ICR mice into the right uterine lumen of 27-week-old virgin female ICR mice. The mice were sacrificed by cervical dislocation 4 months after implantation and the uterine tissue investigated by naked eye observation and histology using hematoxylin-eosin (HE) staining. The histology confirmed that adenomyosis had developed in 19 of 20 ICR mice (95%) and demonstrated that ICR mice, like SHN mice, can be used as an animal model for adenomyosis.

Preparation of the danazol-loaded IUCD

Silicone gel (Shanghai Medical Rubber Institution, Shanghai China) and its vulcanizing agent were mixed at the ratio of 9:1:1. A specific quantity of danazol (Jiangsu Lianhound pharmaceutical Co., Ltd., Jiangsu China) was added and the final mixture coated onto a rod of 0.2 cm diameter polyvinyl chloride (College of Pharmaceutical Sciences, Zhejiang University, Zhejiang China). The coated rod was vulcanized for 24 h at room temperature.

Preparation of the oral danazol suspension

Danazol of 100 mg was suspended in phosphate-buffered saline (pH 7.2) at the concentration of 10 mg/ml. This suspension was homogenized by ultrasound in a water bath before intragastric (oral) administration.

Danazol-loaded IUCD release in vitro

To test the release rate of danazol from the IUCD in vitro, 1 cm of IUCD loaded with danazol was cut and placed into 1000 ml of dissolution medium containing 0.1% HCl and isopropanol at a ratio of 6:4, and stirred at 60 rpm at 37°C. After 30 min, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, 144 h, 7, 8, 9, 10, 11, 12, 13, 14, 15 days, and 3 months, 1 ml of sample was removed and assayed for danazol after filtration through a 0.45 μm membrane. The danazol concentration was calculated using the high-performance liquid chromatography (HPLC) method described below, according to a standard curve with a range from 2.5 to 500 μg/ml (Fig. 1).

The HPLC conditions for danazol assay were as follows. Mobile phase: methanol:water = 78:22; chromatographic column: agilent Zorbax Eclipse XDB-C18 (250 mm x 4.6 mm, 5 μm); flow rate: 1 ml min⁻¹; detection wavelength: 285 nm; column temperature: 25°C; injection volume: 20 μl.

The results showed an initial release of ~20% of the loaded amount within the first several days. The release then followed zero-order kinetics at a slower release rate. The release rate decreased as the danazol:carrier ratio decreased. Once this ratio reached 1:3, zero-order release occurred at a rate of 1.4% per day for almost 2 months. This formula was chosen for the in vivo studies.

Release of danazol-loaded IUCD in vivo

Twenty-four ICR female mice were used for the release test in vivo. The mice were anaesthetized using i.p. injection of chloral hydrate. The abdomen was disinfected, opened and the right uterus separated. A small incision was made 0.5 cm above the junction of the right and left uteri. A 1 cm long danazol-loaded IUCD (1.0 mg danazol/20 g body weight) was inserted into the uterus through the incision and

Figure 1: The accumulated release in vitro of danazol from the different danazol-loaded IUCDs.
the abdomen closed. The mice were sacrificed by cervical dislocation 1, 2, 4, 7, 14, 28 and 56 days after surgery. The IUCD was removed and placed into a centrifuge tube with 3 ml chloroform to extract the remaining danazol. After the extraction, 0.5 ml water was added to precipitate the silicon gel. The organic phase was transferred to another tube after centrifugation at 750g. The solvent was evaporated under a nitrogen blow, and the residue dissolved with 100 µl methanol for the HPLC assay of danazol. The release rate of danazol from the danazol-loaded IUCD was calculated.

Figure 2 shows the typical chromatogram of the HPLC assay. It can be seen that there was no interference from the IUCD to the peak of danazol, which demonstrates the assay method is good for this in vivo release study. The drug release was determined by the measurement of the residual danazol on the IUCD. This residue continually reduced over time, and reached 16.4% of the initial danazol load at the 56th day of the administration (Fig. 3). The results demonstrate that danazol release from the IUCD followed a controlled and sustained release pattern, suggesting that side effects due to high drug concentration release from the IUCD are unlikely and that the treatment effect may last for 2 months.

Plasma pharmacokinetics of the danazol-loaded IUCD
Blood samples were taken from the mice used in the release study immediately before and 1, 2, 4, 7, 14, 28 and 56 days after IUCD insertion. The blood was collected in tubes with heparin sodium, the plasma separated by centrifugation, and the sample stored at −20°C. The danazol concentration in the plasma was assayed using the HPLC method described above. The results show that the plasma danazol concentration increased gradually, to reach a steady-state level and a peak concentration of ~70 ng/ml on the 4th day after insertion (Fig. 4).

Treatment of adenomyosis with danazol-loaded IUCD in ICR mice
After the successful establishment of an experimentally induced ICR mouse adenomyosis model, 34 ICR mice were recruited to investigate the efficacy of a danazol-loaded IUCD on adenomyosis. Adenomyosis was induced in 30 mice using the procedures described above. The remaining four control ICR mice underwent surgery without pituitary gland grafting. The 30 adenomyosis mice were randomized into six groups 4 months after pituitary grafting. Group 1: five mice with no IUCD inserted; Group 2: five mice treated with a blank IUCD; Group 3: five mice treated with an IUCD containing 0.5 mg danazol per 20 g body weight; Group 4: five mice treated with an IUCD containing 1.0 mg danazol per 20 g body weight; Group 5: five mice treated with an IUCD containing 2.0 mg danazol per 20 g body weight; Group 6: five mice treated with 1.0 mg danazol per 20 g body weight by intra-gastric administration each day. The dose of danazol coated on IUCD was determined by calculating body surface area (BSA). Using BSA, 1 mg danazol per 20 g body weight for an ICR mouse each day equates to 390 mg danazol daily in a 70 kg woman. All mice were sacrificed 2 months after the medication, and the uteri and ovaries weighed. The right uterus was examined under ×20 magnification for the extrauterine nodules characteristic of advanced adenomyosis. Adenomyosis was confirmed histologically using HE staining, and graded according to Mori’s classification (Mori et al., 1998, 2001). Briefly, Grade 0, normal uterus; Grade 1, uterus with an invasion of endometrial stromal cells into the inner layer of myometrium; Grade 2, uterus with an invasion of endometrial stromal and gland cells into the inner layer of myometrium; Grade 3, uterus with an invasion of endometrial stromal cells in the connective tissue space between the inner and outer myometrial layers; Grade 4, uterus with cystic hyperplasia of the endometrial glands and small nodules beneath the serosa; Grade 5, uterus with cystic hyperplasia of the endometrial glands and a large number of subserosal nodules. The plasma concentrations of danazol in the treated mice were monitored according to the method described above.

Statistical analysis
All data are expressed as the mean ± SE. Differences between two groups were evaluated using Bonferroni for non-paired data. One-way analysis of variance was used for the evaluation of the differences among multiple groups of non-paired data [11.5 Statistical Package for the Social Sciences (SPSS) software; SPSS Co., USA]. Values of P < 0.05 were regarded as statistically significant.
Results

The uteri of the control group (without induction of adenomyosis) showed normal contour and no congestion. The uteri of Group 1 (no treatment) and Group 2 (treated with blank IUCD) showed distorted contour and obvious adenomyotic nodules extruding from the serosal surface. The uteri in the groups treated with the danazol-loaded IUCD showed improved contour, with increased benefit with increasing danazol dose. The uteri in Group 5 (2.0 mg/20 g) showed an almost smooth contour with no obvious adenomyotic nodules. In contrast, the uteri in Group 6 (treated with a daily oral dose of 1.0 mg/20 g) still showed distortion of the uterine contour and adenomyotic nodules extruding from the serosal surface, although these were thinner and less congestive than those in Group 1 (Figs 5 and 6).

The uterine wet weight and the final body weight of the control group mice and the treated groups mice were not significantly different (F = 0.524, P = 0.785; Table I). However, the ratio of uterine wet weight to total body weight (U/B) was significantly different between the control group and the treated groups (F = 7.835, P < 0.001). The U/B ratio was significantly lower in the control group than in Groups 2 (P = 0.001), 3 (P = 0.001) and 4 (P = 0.026). There was no difference in the U/B ratio between the control group and Group 5 (P = 1.000), 6 (P = 1.000) or 7 (P = 0.136). Moreover, the ratio of U/B was significantly higher in Group 2 than Group 5 (P = 0.015) or 6 (P = 0.006). The ratio of U/B in Group 3 was also significantly higher than that in Group 5 (P = 0.016) or 6 (P = 0.007).

The number of adenomyosis nodules and the HE scores in Group 1 were 11.5 ± 2.4 and 3.3 ± 0.3, respectively (Table I); in Group 2, 11.0 ± 2.7 and 3.1 ± 0.4; and in Group 3, 10.0 ± 2.2 and 2.9 ± 0.4. Although the number of adenomyosis nodules and the HE scores progressively decreased as the dose of danazol incorporated in the IUCD was increased, only Group 5 (5.6 ± 1.1) was significantly different from Group 1 (P = 0.002). The decreased HE scores only reached significance in Groups 4 (2.5 ± 0.4, P = 0.044) and 5 (2.0 ± 0.2, P < 0.001) when compared with Group 1. In Group 6, neither the nodules nor the HE scores were significantly different compared with Group 1.

Figure 5: Treatment efficacy of danazol-loaded IUCD on adenomyosis in an experimentally induced Institute for Cancer Research, Swiss-derived (ICR) mouse model.

(A) Normal animal controls, normal uterine contour, smooth walls, no congestion; (B) Group 1: adenomyotic animal controls without treatment, distorted uterine contour, adenomyosis nodules extruding from the surface of the uterus with the implant; (C) Group 2: adenomyotic animals treated with blank IUCD, distorted uterine contour, adenomyosis nodules extruding from the surface of the uterus with the implant; (D) Group 3: adenomyotic animals treated with 0.5 mg danazol IUCD/20 g body weight, slight improvement of the distorted uterine contour and extruding adenomyosis nodules; (E) Group 4: adenomyotic animals treated with 1.0 mg danazol IUCD/20 g body weight, marked improvement of the distorted uterine contour and extruding adenomyosis nodules; (F) Group 5: adenomyotic animals treated with 2.0 mg danazol IUCD/20 g body weight, smooth uterine contour, rare and small adenomyosis nodules; (G) Group 6: adenomyotic animals treated with 1.0 mg danazol/20 g body weight by daily intragastric (oral) administration, marked improvement of the distorted uterine contour and extruding adenomyosis nodules. Arrows: site of the IUCD.
Two months after treatment, the mean levels of plasma danazol in Groups 3, 4 and 5 were 42.1 ± 3.9, 50.1 ± 5.9 and 73.1 ± 8.9 ng/ml, respectively (Fig. 7). The concentrations were significantly higher in Group 5 than those in Groups 3 (P = 0.002) and 4 (P = 0.015). However, there was no difference in plasma concentration between Groups 3 and 4 (P = 1.000). The mean plasma danazol concentrations in the mice treated with oral danazol was 480.0 ± 18.7 ng/ml, which was substantially higher than the levels from mice treated with the danazol-loaded IUCD (P < 0.001).

Table 1. Comparisons of the uterine wet weight, final body weight, adenomyosis nodules and HE score among the controls and the treated groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uterine wet weight (mg)</th>
<th>Final body weight (g)</th>
<th>Ratio (U/B*)</th>
<th>Nodules (N)</th>
<th>HE** score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>79.3 ± 9.6</td>
<td>36.3 ± 2.5</td>
<td>2.2 ± 0.1</td>
<td>/</td>
<td>0</td>
</tr>
<tr>
<td>Group 1#</td>
<td>93.0 ± 5.7</td>
<td>37.3 ± 2.2</td>
<td>2.5 ± 0.1</td>
<td>11.5 ± 2.4</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Group 2</td>
<td>91.8 ± 5.6</td>
<td>37.0 ± 1.3</td>
<td>2.5 ± 0.1</td>
<td>11.0 ± 2.7</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>Group 3</td>
<td>88.6 ± 4.4</td>
<td>36.0 ± 1.8</td>
<td>2.4 ± 0.1</td>
<td>10.0 ± 2.2</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>Group 4</td>
<td>86.0 ± 5.7</td>
<td>37.2 ± 1.3</td>
<td>2.3 ± 0.1</td>
<td>7.8 ± 1.5</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>Group 5</td>
<td>82.2 ± 5.9</td>
<td>36.6 ± 2.2</td>
<td>2.2 ± 0.1</td>
<td>5.6 ± 1.1</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Group 6</td>
<td>89.6 ± 6.1</td>
<td>38.0 ± 1.2</td>
<td>2.4 ± 0.1</td>
<td>8.6 ± 1.1</td>
<td>2.4 ± 0.4</td>
</tr>
</tbody>
</table>

U/B*, uterine wet weight/final body weight; HE**, hematoxylin-eosin; Group 1#, normal animals without IUCD; Group 2, adenomyotic animals treated with blank IUCD; Group 3, adenomyotic animals treated with 0.5 mg danazol IUCD/20 g body weight; Group 4, adenomyotic animals treated with 1.0 mg danazol IUCD/20 g body weight; Group 5, adenomyotic animals treated with 2.0 mg danazol IUCD/20 g body weight; Group 6, adenomyotic animals treated with 1.0 mg danazol/20 g body weight by daily intragastric (oral) administration.
Discussion

To our knowledge, this is the first study investigating the efficacy of local application of danazol in an experimentally induced ICR mouse adenomyosis model. The establishment of an animal adenomyosis model is a prerequisite for investigating the treatment efficacy of the danazol-loaded IUCD on adenomyosis. Although a variety of animals can be used as models for adenomyosis (Kida, 1994; Kojjyo et al., 1998; Kawahara et al., 2003a; Zhou et al., 2003; Greaves and White, 2006), only SHN mice grafted with a pituitary gland are currently well-established for this purpose (Mori et al., 1984; Singtripop et al., 1993). Our results using ICR mice showed that adenomyosis was developed and confirmed histologically in 19 (95%) of the 20 ICR mice 4 months after grafting with a pituitary gland. This incidence of adenomyosis is comparable with the SHN mouse model (Singtripop et al., 1992). It is therefore suggested that ICR mice, like SHN mice, are a good animal model for adenomyosis.

The in vitro release test showed that danazol was continually and steadily released from the danazol-loaded IUCD over 3 months when the ratio of danazol/carrer was 1:3. The in vivo release test showed that the residue of danazol on the silicon bar continually decreased over time to 16.4% of total danazol dose at the 56th day of administration. Moreover, it was found that the concentration of plasma danazol gradually increased to a peak level of ~70 ng/ml on the 4th day, which was then maintained for 2 months, indicating an effective treatment period of 2 months. These results demonstrate that a danazol-loaded IUCD would be an effective local drug delivery system for the treatment of adenomyosis.

Oral danazol therapy is accepted as an effective treatment for adenomyosis. However, due to its frequent side effects, it is rarely used clinically for this purpose. In the present study, the distorted uterine contour and adenomyosis nodules of the ICR mice gradually resolved over the treatment period and with increasing danazol dose. Maximal effect was achieved with an IUCD loaded with 2.0 mg danazol per 20 g body weight and this was more effective than intragastric administration. Although the number of adenomyosis nodules and the HE scores were decreased as the dose of danazol coated on IUCD increased, this only reached significance in the mice treated with a 2.0 mg danazol per 20 g body weight IUCD. However, as neither the nodules nor the HE scores were significantly decreased in mice treated with intragastric danazol, it can be concluded that local application of danazol via an IUCD had at least equivalent and possibly higher efficacy in treating adenomyosis when compared with oral delivery in this ICR mouse model.

Recent evidence from in vitro and in vivo studies has demonstrated that danazol acts directly on endometrial or endometriotic tissue to inhibit DNA synthesis and induce apoptosis. It also suppresses the growth of endometriotic implants (Surrey and Halme, 1992; Braun et al., 1994; Tamaoka et al., 2000). Although the pathogenesis of adenomyosis remains unclear, the theory of endo-myometrial junction invagination of the endometrium is well accepted. It is suggested that intrauterine danazol exerts its treatment effect when absorbed to the sites of adenomyosis loci through the endo-myometrial junction.

Our in vivo release test confirmed that release from the danazol-loaded IUCD resulted in local and plasma danazol concentrations remaining stable for 2 months, which ensures a sustained local treatment effect. Moreover, the results showed that plasma danazol levels were all <80 ng/ml, although the levels did increase as the dose of danazol incorporated in the IUCD increased. Mice treated with intragastric danazol had mean plasma levels over six times higher than those with the highest danazol IUCD dose. It is therefore reasonable to predict that local treatment with a danazol IUCD would have fewer side effects than oral treatment. This is supported by previous reports from small clinical trials (Igarashi et al., 1998, 2000; Cobellis et al., 2004).

In summary, we have demonstrated that ICR mice are a suitable animal model for adenomyosis, that a danazol-loaded IUCD is a reliable method of administration for danazol and that a danazol-loaded IUCD is an effective way to treat adenomyosis in ICR mice. These results are encouraging and pave the way for an alternative route of danazol administration in humans. Once an appropriate dose of danazol has been determined, the local application of danazol via an IUCD may represent an appealing treatment option for women with adenomyosis.

Author’s contribution

X.Z. is responsible for writing this manuscript and experimental design. H.Y. is responsible for the preparation and release test in vivo of danazol-loaded IUCD. L.D. is responsible for the establishment of adenomyosis ICR mouse model.

F.H. is responsible for the release test in vitro of danazol-loaded IUCD. J.M. is responsible for collecting data. J.L. is chief designer.

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