**Effects of the levonorgestrel-releasing intrauterine system on cell proliferation, Fas expression and steroid receptors in endometriosis lesions and normal endometrium**

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**BACKGROUND:** The objectives of this study were: (i) to evaluate the effects of the levonorgestrel-releasing intrauterine system (LNG-IUS) on both proliferation and apoptosis markers and hormone receptors of the eutopic and ectopic endometrium of women experiencing pain related to endometriosis and (ii) to compare the results with those obtained with GnRH agonist (GnRHa) injections.

**METHODS:** Pre- and post-treatment endometrium and endometriosis specimens were obtained from 22 women experiencing pain related to endometriosis who were treated with LNG-IUS (n = 11) or GnRHa (n = 11) for 6 months. Changes in the expression of proliferating cell nuclear antigen, Fas, progesterone receptor (PRA) and estrogen receptor α (ER-α) were analyzed by immunohistochemistry.

**RESULTS:** The cell proliferation index was significantly reduced in the epithelium and stroma of both the eutopic and the ectopic endometrium after treatment with the LNG-IUS and GnRHa. Only LNG-IUS users showed an increased H-score for Fas in the epithelium of the eutopic and ectopic endometrium (P < 0.05). Expression of ER-α and PRA by the glandular epithelium was lower in the eutopic endometrium after both treatments, but this reduction was noted in the ectopic endometrium only after LNG-IUS treatments (P < 0.05). No difference was detected between groups for any of the markers.

**CONCLUSIONS:** LNG-IUS reduced both cell proliferation and the expression of PRA and ER-α and increased Fas expression in the eutopic and ectopic endometrium of patients with endometriosis. Some of these actions were not observed with GnRHa.

**Key words:** endometriosis / levonorgestrel-releasing intrauterine system / GnRH agonist / apoptosis / steroid receptor

**Introduction**

The use of the levonorgestrel-releasing intrauterine system (LNG-IUS) has gained popularity as a contraceptive method (Luukkainen and Toivonen, 1995), which has also been used to control chronic pelvic pain (CPP) in patients with endometriosis (Vercellini et al., 2003; Lockhat et al., 2005; Petta et al., 2005). The efficacy of the LNG-IUS in controlling pain in patients with endometriosis is similar to that of GnRH agonists (GnRHa), but the LNG-IUS offers the advantages that it does not provoke hypoestrogenism and requires only a single medical intervention every 5 years for its insertion (Petta et al., 2005). LNG-IUS reduces serum levels of CA-125, a marker of the activity of endometriosis, with effects similar to that of GnRHa (de Sá Rosa e Silva et al., 2006). The molecular mechanism by which the LNG-IUS acts on the eutopic and ectopic endometrium to control pain in patients with endometriosis is still a matter of speculation.

The imbalance between cell proliferation and apoptosis plays an important role in the pathogenesis of endometriosis (Fujisita et al., 1999; Dmowski et al., 2001; Braun et al., 2002). Studies have observed
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a reduction and loss of the cyclical nature of apoptosis in both eutopic and ectopic endometrium samples of patients with endometriosis that are not observed in those of normal women (Gebel et al., 1998; Dmowski et al., 2001). Reports indicate that one of the mechanisms by which some drugs act on the clinical control of endometriosis is by a reduction of cell proliferation and an increase of apoptosis. It was shown that only one month of a combined oral contraceptive was sufficient to significantly reduce markers of cell proliferation and increase the apoptotic index in the eutopic endometrium of patients with endometriosis, as observed with GnRH-a (Imai et al., 2002; Meresman et al., 2002, 2003). It has been proposed that the antiproliferative effect of GnRH-a may be mediated by the activation of the Fas-Fasl system, which stimulates cell death by apoptosis (Imai and Tamaya, 2000).

The effect of the LNG-IUS on cell proliferation and apoptosis in the endometrium has been previously demonstrated in women with hemorrhage due to adenomyosis (Maruo et al. 2001). The LNG-IUS reduced endometrial proliferation and stimulated apoptosis in both the glandular epithelium and the endometrial stroma.

Since the imbalance in tissue homeostasis plays a role in the pathogenesis of endometriosis, and since ovarian steroids may control endometrial apoptosis (Rotello et al., 1992; Otsuki et al., 1994; Tabibzadah, 1995; Critchley et al., 1999), questions have been raised regarding the effect of the LNG-IUS on the treatment of endometriosis. For example, what is the effect of the LNG-IUS on cell proliferation and apoptosis in the eutopic and ectopic endometrium of patients with endometriosis? Does the LNG-IUS modify the expression of ovarian steroid receptors in the eutopic and ectopic endometrium? Is there a correlation between the effects of the LNG-IUS on cell proliferation and apoptosis and the modification of hormone receptor expression?

The objectives of the present controlled and randomized clinical study were: (i) to investigate the effect of 6 months of use of the LNG-IUS on the proliferating cell nuclear antigen (PCNA), the apoptosis marker Fas, and the expression of the estrogen receptor (ER) type α (ER-α) and progesterone receptor (PR) type A (PRA) in the eutopic and ectopic endometrium of patients with chronic pelvic pain related to endometriosis and (ii) to compare the results to those obtained after 6 months of treatment with GnRH-a.

Patients and methods

Patients

Twenty-two patients aged 18–40 years were included in the present controlled clinical study between December 2003 and May 2005. All patients were under treatment at the Pelvic Pain Outpatient Clinic of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo (HCFCRP-USP). Patients with CPP and endometriosis confirmed by laparoscopy and histology were included in the study. An additional inclusion criterion was a pain score ≥ 5 on the visual analog scale (VAS; Woodforde and Merskey, 1972) during the pretreatment phase. The study was approved by the Research Ethics Committee of HCFCRP-USP, and all patients gave written informed consent before participating.

All patients had regular menstrual cycles (24–35 days of interval), and none had received any hormonal treatment for at least 3 months or had received treatment with depot-medroxyprogesterone acetate or GnRH-a during the previous 9 months. No patient had a history of osteoporosis, clotting disorders or contraindications for the use of the LNG-IUS as defined by the World Health Organization (2004). Exclusion criteria were the presence of uterine fibroids, diabetes mellitus, cardiovascular disease, dyslipidemia, systemic lupus erythematosus or other rheumatological diseases, HIV infection, any active infection or a smoking habit.

The patients were randomly categorized into two groups by means of a computer-generated randomization scheme using opaque sealed envelopes in a ratio of 1:1 and were admitted by order of entry into the study. They were allocated to either a group receiving 6 months of treatment with the LNG-IUS (Mirena®; Schering Oy, Helsinki, Finland) or a group receiving 6 months of treatment with GnRH-a (Lupron® depot 3.75 mg; TAP Pharmaceuticals, Lake Forest, IL, USA); the latter was administered in six doses (1 ampoule every 28 ± 3 days). Each group consisted of 11 patients. Both treatments were started (the LNG-IUS was inserted) within the first 7 days of the menstrual cycle. The patients were instructed not to use any medication in addition to those provided in the study. Follow-up visits were scheduled every 28 ± 3 days after the beginning of treatment for a total of at least six complete visits during the study period.

Subjects with endometriosis indicated by laparoscopy who had pain scores ≥ 5 after surgical treatment were eligible for randomization. These patients were offered a second laparoscopy in the follow-up. On both occasions (basal laparoscopy and second-look), the disease was staged according to the criteria proposed by the American Society for Reproductive Medicine (Revised American Society for Reproductive Medicine classification of endometriosis, 1997) under video documentation. Surgical treatment of endometriosis was performed in all procedures. Clinical follow-ups and the pre- and post-treatment surgical procedures were performed by different doctors. Thus, the surgeon who performed the second-look laparoscopy did not know the previous stage of endometriosis for those patients. The basal laparoscopy was performed during the early follicular phase of the menstrual cycle (within the first 5 days), and the second-look laparoscopy was performed immediately after 6 months of treatment. During the surgical procedure, biopsies of both the endometriotic lesions and the endometrium were obtained; the latter was performed with a Novak curette. The samples obtained from the eutopic and ectopic endometrium were fixed in 10% formal and routinely processed for paraffin embedding.

Immunohistochemistry

Immunohistochemical processing was performed in 4-μm sections of endometrial tissue. The immunohistochemical reactions were performed by means of an antigen-antibody reaction followed by development of the reaction with a marker visible under the microscope. Deselectionized and hydrated slides were recovered antigenically by incubation in a steam pot in buffered medium (citrate, pH 6.0) for 40 min. After the material cooled, endogenous tissue peroxidases were removed via the addition of hydrogen peroxide. Non-specific binding of the primary antibody was prevented by the addition of horse serum. The slides were then incubated with a primary antibody in a humid chamber for 12 h; this was followed by incubation with the secondary antibody and finally an avidin-biotin step. The reaction was identified after development with 3,3-diaminobenzidine (DAB, Sigma-Aldrich Inc., USA) for 5 min and counterstaining with Harris hematoxylin, and the material was mounted on slides.

Table I lists the antibodies, clones and cellular locations of labeling, and the dilutions used in the study. Primary antibodies were purchased from Novocastra Laboratories Ltd. (Newcastle-upon-Tyne, UK). PCNA was used to assess cell proliferation, and Fas was used to assess apoptosis. Estrogen and PR levels were evaluated using estrogen receptor type α (ER-α) and the PRA, respectively.

A tonsil section was used as a positive PCNA control, and small bowel tissue served as a positive Fas control. An endometrium sample
Criteria for immunohistochemical evaluation

A quantitative method, in which the number of immunohistochemically labeled cells in 1000 cells was counted on the four quadrants of the slide, was used for PCNA evaluation. On the basis of this calculation, we obtained the cell proliferation index (CPI), which is the number of PCNA-labeled cells in the 1000 glandular and stromal cells that were counted. The two cell types were analyzed separately.

The results of immunolabeling with Fas, PRA and ER-α were analyzed semiquantitatively using an immunohistochemical histological score (H-score) that evaluates both the distribution and intensity of specific labeling. The H-score is defined by the formula $H = \sum (P_i \times i)/100$, where $P_i$ is the percentage of labeled cells and $i$ is the labeling intensity ranging from 1 to 3 (Huang et al., 1996). Scores of 1, 2 and 3 indicate weak, moderate and strong immunoreactivity, respectively. Glands and stroma were classified separately for each specimen. The slides were studied in regions of intense immunolabeling (hot spots) with an area of 1.0 cm in diameter (magnification: 40 ×). When less than 10% of the cells were labeled, labeling was considered to be negative and cells were assigned a score of 0. When more than 10% of the cells were labeled, labeling was considered to be positive; the percentage of labeled cells was then analyzed by the H-score formula. The analyses were performed by a trained pathologist who was unaware of the original treatment groups or their responses to the medications used. Glands and stroma were analyzed on the same slide.

Statistical analysis

Variables are reported as means ± standard errors of the mean (SEM). For intergroup and intragroup analysis, ordinal variables involving scores were analyzed by the two-tailed Mann–Whitney test, and categorical variables were analyzed by the $\chi^2$ test or the Fisher’s exact test. Correlations were calculated by the Spearman test. The level of significance was set at $P < 0.05$ in all analyses.

Results

A flow chart for the study is shown in Fig. 1. Among the 22 women initially selected for the study, 4 (one in the LNG-IUS group and three in the GnRHa group) were excluded due to refusal of the second-look laparoscopic surgery. There was no significant difference between groups with regard to basal characteristics such as age, staging of endometriosis, parity and the basal pain score at VAS. The mean ages of the patients who completed the study were 29.2 ± 5.5 and 32.6 ± 5.3 years for the LNG-IUS and GnRHa groups, respectively. Fifty percent of the patients in both groups were nulliparous. In the LNG-IUS group, three patients scored between 5 and 7 on the pretreatment VAS, whereas seven patients scored > 7. In the GnRHa group, six scored between 5 and 7, whereas two scored > 7 on the pretreatment VAS. At the pretreatment laparoscopy, the numbers of patients with stage of endometriosis I and II, III or IV were 3, 3 and 4 in the LNG-IUS group and I, 3, 4 in the GnRHa group, respectively.

Data regarding clinical improvement and laparoscopic findings have been published elsewhere recently (Gomes et al., 2007). Pretreatment expression of the markers studied, including PCNA, Fas, ER-α and PRA, in the glandular epithelium and the stroma were similar for the two groups ($P > 0.05$). Figure 2 illustrates the immunolabeling of some samples of eutopic and ectopic endometrial tissue for all markers studied. A comparison of marker expression by eutopic endometrium versus ectopic endometrium showed similar labeling for most markers in both the epithelium and stroma. The sole exception to this trend was the $H$-score for Fas; despite weak pretreatment labeling, the score for Fas was greater in the eutopic endometrium (epithelium, $P = 0.0008$) and stroma ($P = 0.0217$) than the ectopic samples (data not shown).

Improperly labeled sections and sections containing insufficient material for quantification of labeled cells were discarded, which meant that the numbers of specimens available for analysis with the different antibody markers were different.

Effect of treatment on PCNA expression in the ectopic and eutopic endometrium

Among the samples of eutopic endometrium from the LNG-IUS group, six pretreatment and eight post-treatment samples met the requirements for analysis. Among the samples from the GnRHa group, six pretreatment and seven post-treatment samples met the requirements for the analysis.

The epithelium of the endometrium from both groups revealed a reduction of cell proliferation evaluated by the CPI (cell proliferation index) after treatment (LNG-IUS: $35.5 ± 3.8$ versus $18.9 ± 1.5$, $P = 0.001$; GnRHa: $32.8 ± 5.3$ versus $19.8 ± 0.8$, $P = 0.005$). In the stroma, the CPI was also reduced after exposure to both treatments.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Product code</th>
<th>Clone</th>
<th>Cellular location</th>
<th>Dilution</th>
</tr>
</thead>
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<tr>
<td>PCNA1</td>
<td>NCL-PCNA</td>
<td>PC10</td>
<td>Nuclear</td>
<td>1:200</td>
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<tr>
<td>Fas2</td>
<td>NCL-FAS-310</td>
<td>GM20</td>
<td>Membrane</td>
<td>1:100</td>
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<tr>
<td>Progesterone receptor (type A)3</td>
<td>NCL-PGR-312</td>
<td>16</td>
<td>Nuclear</td>
<td>1:100</td>
</tr>
<tr>
<td>Estrogen receptor (type α)4</td>
<td>NCL-ER-6F11</td>
<td>6F11</td>
<td>Nuclear</td>
<td>1:50</td>
</tr>
</tbody>
</table>

1PCNA—Proliferating Cell Nuclear Antigen was used as a cell proliferation marker since it is an antigen that functions as a DNA polymerase co-factor in the S phase of the cell cycle and is also associated with mechanisms of DNA repair. 2Fas, also known as CD 95 antigen, is a transmembrane glycoprotein that mediates apoptosis by binding to its own receptor. 3This progesterone receptor mediates the actions of progesterone and can be one of two types: progesterone receptor type A (PRA) and progesterone receptor type B (PRB). Only PRA was evaluated here. 4This estrogen receptor mediates the actions of estradiol and can be one of two types: estrogen receptor type α (ER-α) and estrogen receptor type β (ER-β). Only ER-α was evaluated here.
Women assessed for eligibility: 33

Women who did not meet inclusion criteria: 8
  Refused to participate: 3

Randomized: 22

Assigned to receive LNG-IUS: 11
  Received LNG-IUS: 11

Included in primary analysis: 10
  Excluded: 1 for refusal of the second-look laparoscopic surgery

Assigned to receive GnRH analogue: 11
  Received GnRH analogue: 11

Included in primary analysis: 8
  Excluded: 3 for refusal of the second-look laparoscopic surgery

Figure 1 Study flow chart.

Figure 2 Photomicrograph of the immunohistochemical reactions. A, PCNA labeling of ectopic endometrium before treatment (arrows), 400 x; a, negative control for PCNA labeling, 400 x. B, Fas labeling in the eutopic endometrium before treatment (arrow), 200 x; b, negative control for Fas labeling, 400 x. C, Estrogen receptor type α (ER-α) labeling in the ectopic endometrium before treatment (arrow), 400 x; c, negative control for ER-α labeling, 400 x. D, Progesterone type A receptor (PRA) labeling in the ectopic endometrium after treatment with a GnRH agonist (arrow), 400 x; d, negative control for PRA labeling, 400 x. S, stroma; E, epithelium. (*) Note: the field in the figure represents the immunohistochemical reaction and not the labeling quantified.

(LNG-IUS: 19.0 ± 1.4 versus 10.5 ± 0.8, P = 0.0008; GnRHα: 14.6 ± 1.6 versus 9.9 ± 1.3, P = 0.0350). There was no difference between groups regarding this CPI reduction (Fig. 3A).

Six samples of endometriotic lesions prior to the use of the LNG-IUS and five samples after its use, as well as five samples before and after the use of GnRHa, were considered suitable for
Both treatments reduced the CPI of both epithelial (LNG-IUS: 36.8 ± 3.4 versus 20.2 ± 3.5, \( P = 0.005 \); GNRHa: 49.5 ± 3.7 versus 17.9 ± 2.2, \( P = 0.002 \)) and stromal (LNG-IUS: 16.5 ± 1.8 versus 10.2 ± 1.0, \( P = 0.014 \); GNRHa: 20.1 ± 0.6 versus 8.8 ± 0.8, \( P = 0.002 \)) cells in the ectopic endometrium (Fig. 3B). There was no difference between groups regarding this CPI reduction.

**Effect of treatment on Fas expression in the eutopic and ectopic endometrium**

Fas was tested in seven samples of eutopic endometrium before treatment with the LNG-IUS and in 10 samples after treatment; Fas tests were also possible in seven and six samples of eutopic endometrium before and after GnRHa, respectively. There was a significant increase in the H-score for Fas in the epithelium of eutopic endometrium during treatment with the LNG-IUS (0.8 ± 0.0 versus 1.3 ± 0.2, \( P = 0.019 \)). In contrast, this increased expression of Fas was not observed in the epithelium of the eutopic endometrium of the group treated with GnRHa (H-score: 0.8 ± 0.1 versus 0.9 ± 0.3, \( P = 0.710 \)). In the stroma, neither treatment induced a significant increase in the H-score for Fas (LNG-IUS: 0.1 ± 0.1 versus 0.2 ± 0.1, \( P = 0.743 \); GNRHa: 0.1 ± 0.0 versus 0.1 ± 0.0, \( P = 0.754 \); Fig. 4A).

Six and seven samples of endometriotic lesions were analyzed before and after treatment with the LNG-IUS, respectively, while
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Effect of the treatments on ER-α expression in the eutopic and ectopic endometrium

ER-α was evaluated in five samples of eutopic endometrium before and after treatment with the LNG-IUS. The corresponding values for treatment with GnRHα were both equal to five. A significant reduction of the H-score for ER-α was observed in the eutopic endometrium after treatment with both agents; these reductions were observed in the glandular epithelium (LNG-IUS: 1.9 ± 0.2 versus 0.3 ± 0.1, P = 0.004; GnRHα: 2.4 ± 0.3 versus 0.3 ± 0.0, P = 0.008) and stroma (LNG-IUS: 1.1 ± 0.2 versus 0.2 ± 0.0, P = 0.004; GnRHα: 1.5 ± 0.2 versus 0.1 ± 0.0, P = 0.008), respectively (Fig. 5A).

Five samples of endometriotic lesions were analyzed before and after treatment with the LNG-IUS, while three and two samples were analyzed before and after treatment with GnRHα, respectively. The scarcity of material from the endometriotic lesions impaired the quality of labeling. In the eutopic epithelium, there was a significant reduction in the H-score for ER-α only in the LNG-IUS group with respect to both the epithelium (1.6 ± 0.3 versus 0.2 ± 0.1; P = 0.024) and stroma (0.9 ± 0.2 versus 0.2 ± 0.0; P = 0.024). In the GnRHα group, this reduction was not significant (P = 0.052). In the stroma of the eutopic endometrium, the basal expression of the apoptosis marker was low and did not change after either treatment (LNG-IUS: 0.0 ± 0.0 versus 0.1 ± 0.0 P = 0.059; GnRHα: 0.1 ± 0.0 versus 0.0 ± 0.0 P = 1.000). There was no difference in the H-score for Fas between treatments with respect to the epithelium or the stroma and the eutopic or ectopic epithelium.

Five samples were analyzed before and four after treatment with GnRHα, respectively. In the ectopic endometrium, Fas expression was similar to that observed in the eutopic endometrium (Fig. 4B). A significant increase in the H-score for Fas was observed only in the glandular epithelium of endometriotic lesions after treatment with the LNG-IUS (0.2 ± 0.0 versus 0.9 ± 0.2, P = 0.035). In the GnRHα group, the H-score for Fas in the ectopic endometrium increased from 0.3 ± 0.1 to 0.8 ± 0.1, but the difference was not significant (P = 0.052). In the stroma of the ectopic endometrium, the basal expression of the apoptosis marker was low and did not change after either treatment (LNG-IUS: 0.2 ± 0.0 versus 0.2 ± 0.0 P = 0.2; GnRHα: 2.4 ± 0.2 versus 0.3 ± 0.1, P = 0.001). In the stroma of the eutopic endometrium, the reduction of Fas expression was significant only in the LNG-IUS group (0.7 ± 0.2 versus 0.0 ± 0.0, P = 0.001). In the GnRHα group, this reduction was not significant (0.6 ± 0.2 versus 0.2 ± 0.0, P = 0.1709; Fig. 6A). When the two groups were compared, only the reduction induced by the LNG-IUS in the stroma of the endometrium was greater than the reduction induced by GnRHα (P = 0.003).

Six samples of endometriotic lesions before and five samples after treatment with the LNG-IUS were analyzed, while five samples before and three samples after GnRHα treatment were also analyzed. The scarcity of material from endometriotic lesions impaired the quality of labeling. There was a significant reduction in the H-score for Fas in the ectopic endometrium for the LNG-IUS group. This reduction was present for both the epithelium (2.0 ± 0.3 versus 0.0 ± 0.0, P = 0.002) and stroma (0.5 ± 0.1 versus 0.0 ± 0.0, P = 0.045). However, no reduction was observed in the GnRHα group (epithelium: 1.4 ± 0.3 versus 0.3 ± 0.2, P = 0.064; stroma: 0.5 ± 0.2 versus 0.2 ± 0.1, P = 0.714; Fig. 6B). As observed for the

Figure 5 Effect of the levonorgestrel-releasing intrauterine system (LNG-IUS) and GnRH agonist (GnRHα) on the estrogen receptor α (ER-α) in the eutopic (A) and ectopic (B) endometrium of patients with endometriosis. Note: the left side of each graph represents the H-score of ER-α-labeled glands before and after treatment, and the right side represents the H-score of ER-α-labeled stromal cells before and after treatment. Values are expressed as means ± SEM. *P < 0.05 versus pretreatment; # indicates insufficient ‘n’ for statistical analysis.
eutopic endometrium, LNG-IUS induced a greater reduction than GnRHa only in the stroma of the endometriotic lesion \( (P = 0.036) \). There was no correlation between the changes in the CPI and \( H \)-score for Fas and the changes in the expression of ER-\( \alpha \) or PRA.

**Discussion**

The present study showed that the LNG-IUS acts by modifying the homeostasis of the eutopic and ectopic tissues of women with CPP related to endometriosis. After 6 months of treatment with the LNG-IUS, several effects were observed: there was a significant increase in the expression of an apoptosis marker in the epithelium of the eutopic and ectopic endometrium; a reduction in the expression of cell proliferation markers (PCNA); and ER-\( \alpha \) and PRA were observed in the glandular and stromal epithelia of the eutopic and ectopic endometrium. We are not aware of any previous prospective, randomized and controlled study that reported these findings in the eutopic and ectopic endometrium of patients with endometriosis.

The eutopic endometrium of patients with endometriosis presents fundamental differences from that of women without the disease. These differences are at the basis of the idea that the endometrium plays an important role in the pathogenesis of this disease (Meresman et al., 2000; Sharpe-Timms, 2001). The increased cell viability in the endometrium samples of these patients as a consequence of both reduced apoptotic cell death and increased cell proliferation indicates that this condition may facilitate the invasive behavior in this endometrium (Johnson et al., 2005).

Both apoptosis and cell proliferation are targets of treatments habitually used to control pain in patients with endometriosis. In the present study, GnRH-a users exhibited reduced cell proliferation evaluated by PCNA in both the stromal and glandular cells of the eutopic and ectopic endometrium. Similar results showing that GnRH-a reduced cell proliferation were observed in endometriotic glands (Mizutani et al., 1999), eutopic endometrial cultures from patients with endometriosis (Meresman et al., 2003) and in human leiomyoma cell cultures (Wang et al., 2002). In patients treated with the LNG-IUS, we observed changes similar to those observed in patients treated with GnRH-a. Cell proliferation was reduced after 6 months of treatment in both the eutopic and the ectopic endometrium. Maruo et al. (2001) demonstrated an expressive reduction of PCNA labeling in the eutopic endometrial stroma and epithelium 3 months after the use of the LNG-IUS in patients with menorrhagia due to adenomyosis.

Experimental studies have suggested that apoptosis is at least one target that is important to the therapeutic action of GnRH-a in the treatment of endometriosis (Imai and Tamaya, 2000; Meresman et al. 2003). The mechanism by which GnRH-a stimulates apoptosis is still a controversial subject. Since the activation of apoptosis occurs through two main mechanisms, the methods of analysis vary across studies. One of the mechanisms of activation involves the mitochondrial pathway. The balance between anti- and pro-apoptotic molecules such as Bcl-2 and Bax defines susceptibility to apoptosis. This intrinsic pathway utilizes organelles such as the mitochondria to amplify the cell death signal, which results in the activation of caspases that provoke apoptosis (Chinnaiyan et al., 1997; Danial and Korsmeyer, 2004). By means of this interaction, the Bax protein antagonizes the function of Bcl-2 and cancels its effect on prolonging cell survival. The second pathway of apoptosis activation occurs via the death receptor. This pathway, which involves the Fas-Fas ligand (FasL) system, is the most extensively studied and the primary mechanism of apoptosis induction in cells and tissues (Curtin and Cotter, 2003). Fas (also known as CD95, Apo-1 and TNFRSF6) is the major representative of the family of tumor necrosis factor (TNF) receptors (Locksley et al., 2001) and induces apoptosis by binding to FasL. This binding triggers cell death characterized by nuclear condensation and DNA fragmentation (Watanabe et al., 1997).

Fas expression was observed in glandular cells, but not in the stroma of the eutopic and ectopic endometrium of women with...
endometriosis (Watanabe et al., 1997). In this study, we observed no cyclic variation, but other studies have demonstrated greater Fas expression during the secretory phase (Yamashita et al., 1999; Atasoy et al., 2003). In the present study, the samples of eutopic and ectopic endometrium were obtained in the early proliferative phase before the treatments presented weak Fas expression in the epithelium and virtually no expression in the stroma.

In agreement with the data reported by Bilotas et al. (2007), our study did not demonstrate an increase in apoptosis by means of the Fas marker in GnRHa users. It has been previously demonstrated that GnRHa increases Fas expression and induces FasL expression in cultures of leiomyoma cells (Wang et al., 2002). The limited analysis of only one apoptosis marker and the small sample size provide explanations for the present findings of the present investigation.

In contrast, the effect of the LNG-IUS on Fas expression differed from the effect of GnRHa. Despite the weak expression of the marker in the sample before the insertion of the device, a significant increase in Fas expression was observed for the first time in the glandular epithelium of both the endometrium and endometriotic lesion 6 months after insertion. No corresponding increase occurred in the stroma. A similar effect was reported by Maruo et al. (2001), who demonstrated greater Fas expression in the stroma of endometrial biopsies after 3 months of LNG-IUS use. This group also demonstrated a reduction of Bcl-2 expression associated with the increase in apoptosis. Critchley et al. (1999) confirmed the inhibitory effects of progestogens on endometrial proliferation and proposed that these hormones may act by stimulating apoptosis in the endometrium.

Controversial results have been reported in studies examining the pattern of ER and PR expression during the menstrual cycle in the eutopic and ectopic endometrium of women with endometriosis (Bergqvist et al., 1993; Jones et al., 1995; Nisolle et al., 1997). However, the expression of these steroid receptors does not seem to differ between the eutopic endometrium of normal women and that of patients with endometriosis (Jones et al., 1995). Cyclic hormonal variations in a normal endometrium are associated with changes in ER and PR expression, and progesterone inhibits both of these (Lessey et al., 1988).

After menopause, the expression of ER and PR is less intense than that in women of reproductive age. Down-regulation (inhibition) of ER-α and ER-β expression was observed in the atrophic endometrium compared with the proliferative endometrium, and this change was likely secondary to hypoestrogenism. While PRB was significantly reduced in the atrophic endometrium, greater PRA expression in comparison to the late secretory endometrium was detected (Mylonas et al., 2007). As observed in the present study, this pattern of expression, secondary to hypoestrogenism, appears to be similar to that observed with the use of GnRHa in patients with endometriosis. In the eutopic endometrium, GnRHa reduced the expression of ER-α in both the epithelium and stroma. It reduced the expression of PRA only in the epithelium.

Although Matsuzaki et al. (2000) detected a reduction of ER-α, but not of ER-β mRNA in endometriomas after a long period of GnRHa treatment, it was not possible to observe significant changes in ER-α or PRA expression in either epithelial or in stromal endometriotic lesions after 6 months of GnRHa in the present study. It is important to point out that only a small number of samples in this group met the requirements for the analysis, and the samples that were considered appropriate for analysis showed weak labeling that consequently impaired data analysis. This may constitute a bias in this study if we consider that only lesions which presented greater resistance to GnRHa effects could be analyzed, all the others were completely eliminated. On the other hand, in terms of disease treatment, one can speculate that, in clinical practice, some lesions may present a certain resistance to GnRHa effect, allowing earlier recurrence and influencing the effectiveness of this specific treatment.

The present findings regarding LNG-IUS users agree with findings in the literature (Janne and Ylostalo, 1980; Lu, 1991; Pengdi et al., 1999; Vereide et al., 2006). It was possible to demonstrate that the reduced expression of ER-α and PRA also extends to the epithelium and stroma of the ectopic endometrium. The LNG released by the IUS does not mimic the effect of progesterone on the normal endometrium. Receptors in the normal endometrium are reduced during the late secretory phase, and this reduction differs between the epithelium and stroma. In contrast, LNG-IUS users exhibit a uniform reduction regardless of cell type (Pengdi et al., 1999).

The reduced expression of steroid receptors by LNG-IUS and GnRHa is believed to influence the control of apoptosis and cell proliferation, although no correlation was detected in the present study. The reduction of the apoptosis-repressing protein Bcl-2, and possibly the increase in FasL expression in the late secretory phase, is positively correlated with weak ER and PR expression during the same phase (Otsuki et al., 1994). On the basis of the present findings, therefore, we suggest that hypoestrogenism among GnRHa users may be responsible for the reduction of steroid receptors in the endometrium. This would stimulate apoptosis and reduce cell proliferation in both the eutopic and ectopic endometrium. However, no hypoestrogenism occurs in LNG-IUS users (Nilsson et al., 1984). On this basis, the high local concentrations of LNG may inhibit the expression of the receptors, causing the endometrium to become insensitive to circulating estradiol (Luuikainen and Toivonen, 1995). This insensitivity may stimulate apoptosis, with a predominance of the antiproliferative effect being present in the endometrium (Pengdi et al., 1999). This mechanism may explain the action on the eutopic, but not the ectopic, endometrium.

Hypotheses have been generated to explain how the LNG-IUS acts on the ectopic endometrium (Bahamondes et al., 2007). One hypothesis suggests a direct action on the lesions. However, the fact that LNG concentrations are high only in the eutopic endometrium contradicts this hypothesis. Oral LNG administration at a dose 10 times higher than that released by the IUS resulted in endometrial LNG concentrations similar to those of adjacent tissues. Further, the atrophic effects on the eutopic endometrium were much less intense than those promoted by the LNG-IUS (Nilsson et al., 1982). In our opinion, as well as that reported by Bahamondes et al. (2007), the most likely hypothesis is that the LNG-IUS acts directly on the lesion in a manner similar to that of progestogens in the normal endometrium. The ectopic endometrium, when constantly exposed to LNG concentrations (even a low but non-physiological one), may reduce ER and PR expression as observed in the present study. The endometriotic lesions may become insensitive to circulating estradiol, an event that also seems to occur in the eutopic endometrium. Consequently, this insensitivity to estradiol allows an antiproliferative effect to predominate in the ectopic endometrium exposed to LNG-IUS treatment. This antiproliferative effect may be consistent with the present findings of reduced cell proliferation and increased apoptosis.
Finally, the present study demonstrates that 6 months of LNG-IUS treatment reduces the expression of the cell proliferation markers ER-α and PRA and increases the expression of apoptosis markers in both the glandular epithelium and stroma of the eutopic and ectopic endometrium of patients with pelvic pain related to endometriosis. Some of these modifications were similar to those observed in the group treated with GnRHa. These findings explain at least part of the mechanism of the clinical improvement (Vercellini et al., 2003; Lockhat et al., 2005; Petta et al., 2005) and absence of worsening of laparoscopic staging (Gomes et al., 2007) observed in endometriosis patients using the LNG-IUS. Several other factors (e.g. immunological, angiogenic and hormonal) are definitely involved. For the first time, it was possible to demonstrate antiproliferative changes in the ectopic endometrium of patients with pelvic pain and endometriosis treated with the LNG-IUS. The clinical improvement of endometriosis in LNG-IUS users, which has also been observed by others, has now been supported by histology. The present results should be evaluated with caution since the number of samples available for analysis was small. However, these results provide a basis for additional investigations of the action of the LNG-IUS on endometriotic lesions.

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References


Vereide AB, Kaino T, Sager G, Arnes M, Orbo A. Effect of levonorgestrel IUD and oral medroxyprogesterone acetate on glandular and stromal progesterone receptors (PRA and PRB), and estrogen receptor (ER-alpha and ER-beta) in human endometrial hyperplasia. Gynecol Oncol 2006;101:214–223.


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