Long-term regression of experimental endometriosis in a rat model treated with local application of levonorgestrel-loaded biodegradable microspheres

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BACKGROUND: A previous study demonstrated that local application of levonorgestrel-loaded polylactic acid microspheres (LNG microspheres) resulted in significant regression of endometriotic cysts in a rabbit model for 6 months without disturbing the metabolic parameters or ovarian function. In order to investigate the feasibility of local application of LNG microspheres as a long-term maintenance treatment for endometriosis, the suppressive effect of a single intra-cystic injection of LNG microspheres was studied for 1 year in a rat model.

METHODS AND RESULTS: Twenty four rats with experimental endometriotic cysts were randomized to be treated with a single intra-cystic injection of LNG microspheres (n = 8); 6-month GnRH agonist (GnRHa, n = 8) or control (n = 8). Intra-cystic injection of LNG microspheres and GnRHa treatment caused comparable regression and atrophy in endometriotic cysts in the first 6 months. Compared with the control, the wet weight of the endometriotic cysts was significantly lower in both groups at Month 6 but by Month 12 only remained low in the LNG microspheres group (P < 0.01). The immunostaining of estrogen receptors (ERs) in both the epithelium and stroma and progesterone receptors (PRs) in the stroma was significantly weakened in the LNG microspheres group at Month 6 and was not fully restored at Month 12 (P < 0.01). Metabolic parameters and estrous cycle were not disturbed by local application of LNG microspheres.

CONCLUSIONS: In a rat endometriosis model, the suppressive effect of a single intra-cystic injection of LNG microspheres was comparable to that of GnRHa, and was maintained for 1 year. The down-regulation of ERs and PRs might serve as possible mechanism of long-term effectiveness.

Key words: endometriosis / levonorgestrel / local application / microsphere / rat

Introduction

Since there is no complete cure for endometriosis, women treated with surgery or drugs experience recurrences frequently. Regrowth of in situ residual endometriotic lesions or cells is believed to be the main reason of recurrence (Guo, 2009). It is reported that endometrial cells can survive at the implanted site even after apparent complete morphological regression and may grow into an implant if estradiol is present (Rajkumar et al., 1990).

Medical therapy, therefore, needs to be maintained for years rather than months to successfully prevent endometriosis recurrence. Unfortunately, most of the patients cannot tolerate the side effects associated with the long-term maintenance therapy. For this background, an effective medical therapy, which has fewer side effects and can be administered over a long time period, will be valuable in avoiding endometriosis recurrence.

We have previously devised an injectable controlled release system for progestogen, levonorgestrel-loaded polylactic acid microspheres (LNG microspheres), for the treatment of endometriosis by local application (Yuan et al., 2010a,b). A study in a rabbit model has demonstrated that intra-cystic injection of LNG microspheres resulted in significant regression of the endometriotic cysts for 6 months without
disturbing the metabolic parameters or ovarian function (Yuan et al., 2010b). The experiment ended 6 months after the injection because the serum level was below the limit of assay. Unfortunately, we found the microspheres were still present 6 months after the injection. According to previous studies, it takes them ~1 year to biodegrade completely (Beck et al., 1979). This became our concern because the polymer will accumulate following repeated injections every 6 months.

In order to investigate the feasibility of local application of LNG microspheres as a long-term maintenance treatment for endometriosis, the suppressive effect of a single intra-cystic injection of LNG microspheres was studied for 1 year in a rat model. GnRH agonist (GnRHa) is the gold standard for the pharmacological treatment of endometriosis (Prentice et al., 2000). The treatment is usually limited to 6 months, and over time should be used with add-back therapy. Several animal studies have shown that GnRHa caused significant regression and atrophy of endometriotic lesions (Dogan et al., 2004; Altintas et al., 2008). So we chose 6-month GnRHa therapy as a positive control. Another option of this study was to analyze whether down-regulation of estrogen receptors (ERs) and progesterone receptors (PRs) was related to therapy response.

Materials and Methods

Animal model of endometriosis

Approval was obtained from Animal Ethics Committee of the Fourth Military Medical University. Twenty-five female 12-week-old Sprague–Dawley rats were anesthetized by using pentobarbital sodium and laparotomized. The left uterine horn was trimmed and opened longitudinally and the outer layer of myometrium was peeled away. The endometrium was sectioned into four pieces (5 × 5 mm) and implanted subcutaneously between the abdominal muscle and skin on both sides of the midline incision. The implants grew into dark blue fluid-filled, ovoid cysts by 2 months (Fig. 1). Animals’ model were included in the study if they showed growth of at least two cysts whose major diameters were ≥5 mm (n = 24). For each rat, only two large cysts were left for further study. The remnant cysts and the implants with unsuccessful induction were removed. This model adequately mimicked the human endometriosis. Furthermore, locating the implants subcutaneously made it convenient to measure changes in their size.

Study interventions

The animals were randomized into three groups: each rat in the LNG microspheres group (n = 8) received an intra-cystic injection of 3.5 mg LNG microspheres (containing 66.5 μg of LNG) per cyst. The LNG microspheres were suspended in 0.1 ml of 0.9% saline solution containing 2% (w/w) sodium carboxymethylcellulose and 1% (w/w) Tween-80. The preparation and characteristics of LNG microspheres were described in our previous study (Yuan et al., 2010b). The rats in the GnRHa group (n = 8) were given a subcutaneous injection of depot leuprorelin acetate (1 mg/kg, Takeda, Japan) at 4-week intervals for six times (Dogan et al., 2004). The rats in the control group (control, n = 8) were given no medication.

Since the major diameter of the endometriotic cyst and its volume correlate perfectly (Vernon and Wilson, 1985), size changes of the cysts were evaluated by monthly measurements of the major diameter. Vaginal smears were examined daily for evaluation of estrous stage. One endometriotic cyst was removed from each rat, 6 and 12 months after the initial treatment, for weighing and histological evaluation. Blood samples were obtained by cardiac puncture and stored at −80°C for analysis of liver function, serum lipids and glucose (Fig. 2).

Histological evaluation

The formalin-fixed endometriotic cysts were embedded in paraffin wax, sectioned at 5 μm thickness, stained with hematoxylin and eosin and examined under a light microscope. In order to investigate the response of the endometrial epithelium to the treatment, the persistence of epithelial cells in each endometriotic cyst was evaluated semi-quantitatively as follows: 3, well-preserved epithelial layer; 2, moderately preserved epithelium; 1, poorly preserved epithelium and 0, no epithelium. This evaluation was based on a previous rat endometriosis study (Keenan et al., 1999).

Immunohistochemistry

Tissue sections, 5 μm thick 10% formalin fixed and paraffin-embedded, were deparaffinized in xylene and hydrated in a graded series of ethanol solutions. Antigen retrieval was subsequently performed in a microwave oven with boiling citrate buffer for 15 min followed by cooling and washing. Then the sections were immersed in 3% hydrogen peroxide solution for 10 min and washed. The detection of ERs and PRs was carried out by applying monoclonal rabbit antibodies to rat ERs and PRs (Zhongshan Goldenbridge Biotechnology Co.), secondary antibody (goat anti-rabbit IgG/HRP; Zhongshan Goldenbridge Biotechnology Co.) and DAB.
kit sequentially, according to the instructions from the manufacturer. The sections were then counterstained with hematoxylin, dehydrated and cleared for evaluation.

The immunostaining for ERs and PRs was evaluated semi-quantitatively by the immunohistochemical histological score (H-score) which incorporates both the intensity and the distribution of immunostaining. The H-score has been calculated by the formula: 

\[ H\text{-score} = \frac{\sum (P \times I)}{100} \]

where \( P \) denotes the percentage of stained cells and \( I \) denotes the intensity of the staining ranging from 1 to 3 (Vereide et al., 2006). The intensity scale of 1, 2 and 3 indicated weak, moderate and strong staining, respectively. The epithelium and stroma were classified separately for each specimen. The evaluation was performed blinded to the treatment by a trained pathologist.

Statistical analysis

Data were expressed as mean ± SD. The Student–Newman–Keuls \( q \) test was used to assess the significance of differences between groups in multiple comparisons. The results of arbitrary ordinal scale measurement were presented as median and quartile range. Non-parametric multiple comparisons were performed using the Kruskal–Wallis \( H \)-test and the Nemenyi test. Values of \( P \leq 0.05 \) were considered significant.

Results

Size changes of endometriotic cysts

There were no differences between groups when considering the mean major diameter of the endometriotic cysts at the time of randomization; the groups were homogeneous prior to the treatment. Cysts in the control group did not show any significant changes from the beginning of the experiment to the end. One rat in the control group died of anesthetic overdose before completing the investigation protocol. Cysts in the LNG microspheres group showed significant regressions from Month 2 onwards. The size of the cysts reached a minimum at 6 months after the treatment and then was stabilized without significant changes until Month 12. For the GnRHa group, cyst regression was similar to the LNG microspheres group for first 6 months. However, the size of the cysts began to increase after Month 6 and regained pretreatment values at Month 9 (Fig. 3). The wet weight of the cysts (mean ± SD) at Month 6 was 308 ± 199 mg in the control group but significantly lower in the LNG microspheres group and GnRHa group (17 ± 5 and 17 ± 3 mg, respectively, \( P < 0.01 \)). There was no significant difference between the LNG microspheres group and GnRHa group at Month 6. At Month 12, the wet weight of cysts was significantly lower in the LNG microspheres group (26 ± 6 mg, \( P < 0.01 \)) than GnRHa group (247 ± 110 mg) and the control (276 ± 157 mg).
Histological changes of endometriotic cysts

On histological examination, in the control group the wall of the endometriotic cysts was composed of endometrial epithelium and stromal tissue. Intra-cystic injection of LNG microspheres induced highly atrophic changes with decreased cytoplasm and condensed nuclei of cells in the epithelium and stroma at Months 6 and 12. The microspheres were visible as ovoid open spaces inside the cysts at Month 6, but had disappeared at Month 12, indicating that the microspheres had been biodegraded between Months 6 and 12. The endometriotic cysts were also highly atrophic after 6-month GnRHa treatment. However, the suppressive effect was not maintained to Month 12 (Fig. 4). Semi-quantitative evaluation of the persistence of endometrial epithelial cells in the endometriotic cysts showed a significantly lower score in the LNG microspheres group (both Months 6 and 12, \( P < 0.01 \)) and GnRHa group (only Month 6, \( P < 0.01 \)), when compared with the control (Fig. 5).

Immunostaining of ERs and PRs

As shown in Fig. 6, ERs were localized in the nuclei of epithelial and stromal cells while progesterone receptors were localized mainly in the nuclei of stromal cells in the control group. In comparison with the control, the immunostaining of ERs in both the epithelium and stroma and PRs in the stroma was significantly weakened in the LNG microspheres group at Month 6 and was not fully restored at Month 12 (\( P < 0.01 \)). For the GnRHa group, the immunostaining of ERs was significantly intensified in both the epithelium and stroma (\( P < 0.01 \)), and the staining of PRs in the epithelium was also enhanced at Month 6 (\( P < 0.01 \)). However, at Month 12, the immunostaining of ERs and PRs in the GnRHa group was similar to the control. The differences in the H-score for ERs and PRs among groups were shown in Figs 7 and 8.

Metabolic parameter and estrous cycle

Six and 12 months after the treatment, neither local application of LNG microspheres nor GnRHa treatment had any significant influence on body weight, liver function, serum lipids and glucose. There was no significant difference among these groups (data not shown). In this study, we examined vaginal smears every morning for the evaluation of estrous cycle in the first 7 months. The duration of the estrous cycle of the rats in the control group was 4–5 days. No disturbance of estrous cycle was noted in the LNG microspheres groups. Approximately 5 days after the initial injection of GnRHa, the rats had disorder of estrous cycle and the phase of pro-estrus, estrus and metestrus did not appear until 6–7 months later in the GnRHa group.

Discussion

In the present study, we used a rat endometriosis model to demonstrate that the suppressive effect of a single intra-cystic injection of
LNG microspheres on endometriotic cysts was comparable to that of GnRHa, and the suppressive effect was maintained for 1 year. We observed in our previous studies in rabbits that the LNG serum level fell below the limit of assay 6 months after local application of LNG microspheres, yet the intra-cystic level remained high (275 ng/ml). Thus, the release of LNG from the microspheres is still ongoing.

**Figure 6** The immunostaining of ERs (A) and PRs (B) in endometriotic cysts for each group at 6 and 12 months after the treatment.

**Figure 7** The immunohistochemical histological score (H-score) of ERs in the endometrial epithelium (A) and stroma (B) of the endometriotic cysts for each group (n = 8) at Months 6 and 12. H-score = \( \sum (P_i \times I) / 100 \), where \( P_i \) is the percentage of cells stained and \( I \) the intensity on a scale from 1 (weak) to 3 (strong). Results are presented as median and quartile range. *\( P < 0.01 \) when compared with the control (Kruskal–Wallis H-test and Nemenyi test).
after 6 months, indicating that endometrial cysts could still remain in the control of local high level of LNG (Yuan et al., 2010b). Nevertheless, intra-cystic LNG concentrations will be at a relatively low level during the final few months before biodegradation of the microspheres is completed.

The most likely hypothesis is that intra-cystic LNG in low concentration acts directly on the lesion in a manner similar to that of LNG-IUS on the ectopic endometrium. Non-contraceptive use of LNG-IUS, which release 20 μg of levonorgestrel per day directly into the uterine cavity, is reported to be effective for women with endometriosis (Fedele et al., 2001; Petta et al., 2005). However, the mechanism by which the LNG-IUS acts on the ectopic endometrium is still unknown. It is proposed that the ectopic endometrium constantly exposed to low and non-physiological LNG concentrations may reduce the expression of ERs and PRs and thus become insensitive to circulating estradiol (Bahamondes et al., 2007; Gomes et al., 2009). The same could have happened during the last few months of the present study since we observed that down-regulation of ERs and PRs was still significant in the LNG microspheres group 12 months after intra-cystic injection. The down-regulation of ERs and PRs and thus insensitivity to estradiol may exert anti-proliferative effects in the ectopic endometrium exposed to LNG of low concentration.

In this study, the suppressive effect of intra-cystic LNG microspheres on endometriotic cysts was comparable to that of GnRHs, but the suppressive effect of LNG microspheres lasted much longer. Moreover, effective doses of local LNG microspheres have no deleterious influence on metabolic parameters or the estrous cycle, while GnRHs should be used with add-back therapy during a long-term treatment in order to minimize the hypoestrogenic side effects (Surrey and Hornstein, 2002). Therefore, repeated local application of LNG microspheres is potentially preferable to GnRHs as a long-term maintenance treatment, although clinical pilot studies are needed to confirm its efficacy.

Ovarian endometrioma is the most applicable case to apply such treatments via ultrasound-guided aspiration and intra-cystic injection of LNG microspheres. Although histological examination is unavailable to exclude malignancy, this procedure could be employed as a treatment option in treating endometrial cysts for selected patients. The pouch of Douglas may be another ideal place to administer LNG microspheres for two reasons. First, most peritoneal and deep infiltrating endometriosis lesions are confined in the pelvis, especially in the pouch of Douglas. Second, since women have an upright posture, LNG microspheres will accumulate mainly in the pouch of Douglas. We would like to test its efficacy in our further research.

Without further evidence it is inappropriate to claim that local application of LNG microspheres will be effective for human endometriosis. The major symptoms of human endometriosis are recurring pelvic pain and infertility and we did not investigate the effects of local administration of LNG microspheres on pain or fertility in this study. Moreover, animal models differ from human disease in many ways. For example, unlike human endometriosis, the ER/PR ratio is similar in both the endometriotic and the endometrial tissues in surgically induced rat endometriosis model (Ortega-Moreno, 1994). In addition, ~9% of patients with endometriosis do not respond to treatment with progestins (Wu et al., 2006). The mechanism of progesterone resistance in endometriosis may be related to an overall reduction of PR and the lack of the PR isoform B (PR-B; Attia et al., 2000), which may result from promoter methylation of PR-B (Wu et al., 2006). In this case, the efficacy of local application of LNG microspheres may be attenuated. Therefore, the most imminent work at present is to conduct a pilot clinical study to assess the efficacy, the pharmacokinetic profiles, effective radius, possible side effects and complications of local application of LNG microspheres in human patients.

In summary, the present study showed that in a rat endometriosis model the suppressive effect of a single intra-cystic injection of LNG microspheres was comparable to that of GnRHs, and was sustained for 1 year. Moreover, the local effective dose of LNG microspheres had no deleterious influence on metabolic parameters or the estrous cycle. Thus, local application of LNG microspheres can be done repeatedly over a long time period and is a potential therapeutic strategy for endometriosis.
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Authors’ roles

P.Y. conceived the idea, initiated the project, designed the research, performed the experiments, analyzed the results and drafted the manuscript. B.C. designed the research and analyzed the results. Y.H. and X.X. designed the research, analyzed the results and revised the manuscript.

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Conflict of interest

None declared.

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