Induction of a local pseudo-pregnancy via levonorgestrel-loaded microspheres for the treatment of endometriosis in a rabbit model

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BACKGROUND: Endometriosis is a chronic disease that responds to systemic pseudo-pregnancy therapy. However, side effects limit their long-term use, and recurrence often occurs after cessation of medication. Reducing side effects whereas improving therapeutic efficacy of pseudo-pregnancy therapy seems contradictory, but appealing. In order to address this dilemma, the efficacy and side effects of local pseudo-pregnancy therapy were investigated for the first time in an endometriosis animal model.

METHODS AND RESULTS: Levonorgestrel-loaded polylactic acid microspheres (LNG-microspheres) were prepared by using an oil-in-water emulsification–solvent evaporation method. Rabbits with experimental endometriosis were randomized to treatment with local pseudo-pregnancy therapy, local blank microspheres, systemic pseudo-pregnancy therapy, ovariectomy or the control. Local pseudo-pregnancy was induced by injection of LNG-microspheres directly into endometrial cysts. Compared with the systemic pseudo-pregnancy group, significantly higher intra-cystic drug levels were maintained for at least 6 months with much lower serum levels in the local pseudo-pregnancy group (P < 0.01). The high intra-cystic levonorgestrel simulated a state of potent pregnancy, which induced size reductions and endometrial atrophy comparable to those of ovariectomy. Moreover, major metabolic parameters and ovarian function were not disturbed by local pseudo-pregnancy therapy.

CONCLUSIONS: Induction of a local pseudo-pregnancy could achieve therapeutic efficacy comparable to that of ovariectomy without provoking any marked side effects in a rabbit endometriosis model. Thus it may be a preferable option for patients with endometriosis.

Key words: endometriosis / levonorgestrel / local pseudo-pregnancy / microsphere / rabbit

Introduction

The therapeutic effects of pregnancy on endometriosis are well-established. Severe decidualization and atrophy are commonly found in endometriotic lesions during pregnancy (Clement, 1977; Razzi et al., 2004; Schweitzer et al., 2006; Ueda et al., 2009). As Beecham stated, ‘Nature (since the beginning of time) has employed an efficient prophylactic and curative measure for endometriosis, i.e. pregnancy’ (Beecham, 1949). Thus, it is not surprising that pseudo-pregnancy therapy by systemic administration of progestogen to simulate pregnancy was introduced for the treatment of endometriosis. Pseudo-pregnancy therapy is effective in relieving the pain and controlling the progression of endometriosis for women who do not wish to have children in the short-term (Vercellini et al., 2003b).

However, the efficacy of common systemic pseudo-pregnancy therapy is significantly attenuated by poor compliance because of side effects, which include acne, nausea, weight gain, breast tenderness, abnormal uterine bleeding, elevated liver function, venous thrombosis and embolism. Furthermore, recurrence often occurs after cessation of therapy. Meanwhile, the efficacy of conventional systemic pseudo-pregnancy therapy in treating large ovarian endometrioma is also in doubt (Chapron et al., 2002).

Recently, there have been attempts to localize the pharmaceutical effects of progestogen in treating endometriosis. Levonorgestrel-releasing intrauterine system (LNG-IUS), which releases 20 µg of LNG per day, was reported to be beneficial in improving the symptoms and reducing recurrence following surgery (Fedele et al., 2001; Vercellini et al., 2003c; Lockhat et al., 2004). However, the level of
evidence in these studies is relatively low, and the efficacy of LNG-IUS for endometriosis still needs to be validated further (Abou-Setta et al., 2006; Anpalagan and Condous, 2008). A recent case report also casts doubt on its efficacy in treating ovarian and extragenital endometriosis (Hinterholzer et al., 2007). In this case, a patient with endometriosis was free of symptoms with depot progesterone injections, yet after switching to LNG-IUS she developed dysmenorrhea and rectal bleeding, which was diagnosed as ovarian and sigmoid endometriosis by laparotomy. Furthermore, studies on drug levels in LNG-IUS patients revealed that LNG in myometrial, fallopian tube and fat tissue were all of the same order with orally treated patients (1–5 ng/g) except for a much higher concentration in endometrium (808 ± 511 ng/g) (Nilsson et al., 1982). The low concentrations of LNG outside the genital tract may explain the unresponsiveness of ovarian and extragenital endometriosis to LNG-IUS.

Induction of a local pseudo-pregnancy by injection of progestogen-loaded biodegradable microspheres directly into endometriotic lesions may provide a more effective treatment with fewer side effects. Biodegradable microspheres, as a controlled drug delivery system, have the advantage of ease of administration, relatively constant rate of drug release and long duration of action. By local sustained delivery of progestogen via biodegradable microspheres, high levels of progestogen will be localized in the endometriotic lesions to generate a potent pseudo-pregnancy condition. Theoretically, this could result in superior effectiveness, limited adverse effects and better patient compliance during long-term treatment.

In this study, LNG was encapsulated into polyactic acid (PLA) microspheres for local sustained delivery. The efficacy and side effects of local pseudo-pregnancy therapy induced by LNG-microspheres were investigated for the first time in rabbits with experimental endometriosis.

Materials and Methods

Preparation of LNG-microspheres

An oil-in-water (o/w) emulsification–solvent evaporation method described by Beck was used with modest modification (Beck et al., 1983). Briefly, 500 mg of PLA (D, L-polyactic acid, Av. Mw 25000, Inherent visc. 0.31 dl/g, Shandong Institute of Medical Instruments, China) and 26.4 mg of LNG (Beijing Zizhu Pharmaceutical Co., China) were dissolved in 7.6 ml of dichloromethane (Tedia, USA). After the solution had been cooled to 0–4°C, it was emulsified by adding dropwise into 30 ml ice-cold 1% (w/w) PVA aqueous solution (PVA 4–88, Mw 31000, Fluka, Germany) and homogenizing simultaneously at 500 rpm for 10 min. The stable o/w emulsion was then added to 50 ml 1% (w/w) PVA aqueous solution and stirred with a magnetic stirrer at 250 rpm, 4°C, and overnight. The solidified microspheres were harvested by settlement separation three times in distilled water and lyophilized for 24 h.

Characteristics of LNG-microspheres

The morphology of the microspheres was examined by scanning electron microscope (SEM, S-3400N, Hitachi, Japan). Particle size distribution was described by analyzing the diameter of 100 microspheres in the SEM image. Injectability of the microspheres was tested using a syringe with needles from 18-gauge to 30-gauge. Drug loading efficiency was determined by dissolving 5 mg microspheres in 0.5 ml dichloromethane and vortex-mixing with 10 ml methanol, then collecting the supernatant after centrifugation. The concentration of LNG in the supernatant was measured by UV spectrophotometer at 244 nm (Beckman DU460, USA). The drug loading efficiency was calculated as (actual drug loading/theoretical drug loading) × 100%.

Experimental endometriosis

Experimental endometriosis was induced following a well-established method (Dunselman et al., 1988). Approval was obtained from Institutional Animal Care and Use Committee and the national guidelines for the care and use of laboratory animals were followed. In brief, 45 female New Zealand White rabbits were anesthetized by pentobarbital sodium and laparotomized. The left uterus horn was cut down and incised longitudinally, and the outer layer of myometrium was peeled away. Then 0.5 × 0.5 cm pieces taken by microscissors were implanted with 6–0 suture onto the abdominal peritoneum. Two months later, a second laparotomy was performed to identify the viability of the implants and measure them using a caliper. An ellipsoid volume formula (π/6 × length × width × height) was used for volume calculation.

Study interventions

At 2 months post-operatively, the implants transformed into endometrial cysts ~10 mm in maximum diameter. Four rabbits with unsuccessful induction were excluded. The remaining 41 rabbits were randomized to five groups (Fig. 1).

Contents of the endometrial cysts were aspirated before injection under sterile conditions. For the local pseudo-pregnancy group, suspension of 55 mg LNG-microspheres (containing 1 mg LNG) and 0.2 ml dispersed solution (containing 2% sodium carboxymethylcellulose and 1% Tween-80) was injected into the endometrial cysts. For the local blank microspheres group, suspension of 55 mg blank microspheres and 0.2 ml dispersed solution was injected into the endometrial cysts. For the systemic pseudo-pregnancy group, suspension of 1 mg LNG microcrystal and 0.2 ml dispersed solution was injected subcutaneously and the injection was repeated as soon as LNG was no longer detectable in plasma. The control group remained untreated, whereas the ovariotomy group underwent oophorectomy. Estrous cycles were evaluated by sequential vaginal smears. Plasma samples were collected at appropriate intervals and immediately stored at −80°C. Cyst fluid was collected monthly by fine needle (29-gauge) aspiration under the guide of ultrasound. LNG in samples was analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/MS/MS). The lower limit of quantification is 0.3125 ng/ml.

Specimen collection

At the end of the experiment, the volume of the endometrial cysts was remeasured by performing a third laparotomy. Blood samples were obtained by cardiac puncture and stored at 4°C for analysis of liver function, serum lipids and glucose. Finally, the implants, ovaries and the right uterus were removed and fixed in 10% buffered neutral formalin for histological examination. The specimen were then embedded in paraffin and cut into 6 mm sections. The sections were stained with hematoxylin and eosin (HE).

Statistical analysis

Data are expressed as mean ± SD. The Student’s paired t-test was used to compare the implant volume before and after treatment in each group. Differences among three or more groups were analyzed by Student–Newman–Keuls q-test. Values of P < 0.05 were considered significant.
Results

Characteristics of LNG-microspheres

The microspheres were approximately spherical with a relatively smooth, non-porous surface under SEM. Virtually no crystalline drug or fragment of polymer was found adhered to the surface. Under 1500× magnification, the microspheres appeared to be homogeneous and parenchymatous (Fig. 2). The particle size of LNG-microspheres was mainly distributed around 45 μm with the average value of 45.18 ± 8.96 μm, which is injectable using 18-gauge to 23-gauge needles, but not with needles finer than 26-gauge. The results demonstrated that the particle size of drug-loaded microspheres was uniform and appropriate for routine injection. The drug loading efficiency determined from three batches was estimated to be 37.5 ± 1.4%.

LNG level in plasma and cysts

The concentration-time profile after subcutaneous administration of LNG microcrystal is presented in Fig. 3A. The plasma concentration reached its peak within 6 h, and then dropped gradually in a relative short duration (40 days). So the administration was repeated continuously in order to simulate the conventional systemic pseudo-pregnancy therapy. As a result, LNG in plasma and cysts fluctuated greatly depending upon the time of administration and the time of sampling. The maximum concentration of LNG in plasma and cyst were 30.65 ± 5.63 and 20.30 ± 4.14 ng/ml, respectively.

In contrast, for rabbits treated with local pseudo-pregnancy therapy, the profile in plasma was rather stable and the concentration in plasma was much lower (P < 0.01) (Fig. 3B). The LNG concentration in plasma peaked (about 3 ng/ml) at 1 day after administration, and maintained at that level without much variation for 3 months. Then
the level started to decrease steadily and became undetected (below the limit of assay) by Day 188. The LNG concentrations in cysts were about 1300-fold higher than those in plasma. The profile of LNG in cysts paralleled that in plasma, which maintained the peak level (about 4000 ng/ml) in the first 3 months and then decreased gradually to 275 ng/ml 6 months later. Thus, unlike systemic pseudo-pregnancy group, significantly higher levels of LNG were maintained in the endometrial cysts for at least 6 months (P < 0.01).

Size changes of endometrial cysts
There were no differences among the five groups when considering the mean volume of the cysts at the time of the second laparotomy; the groups were homogeneous prior treatment. Six months after the second laparotomy, the endometrial cysts treated with local blank microspheres and the control showed a significant increase (P < 0.01) in size. Rabbits treated with systemic pseudo-pregnancy therapy showed a slight non-significant increase in the cyst volume with respect to the initial value, however, the cysts were still significantly smaller than in the control group (P < 0.01). The volume of endometrial cysts in animals treated with local pseudo-pregnancy therapy and ovariectomy reduced significantly compared with both the pretreatment value and the control (P < 0.01). The impact of local pseudo-pregnancy therapy upon the size of endometrial cyst was comparable to the effect of ovariectomy, which decreased the cyst volume to approximately 30% of the initial value at 6 months after treatment. However, the differences of the volume of endometrial cysts among local pseudo-pregnancy, ovariectomy and systemic pseudo-pregnancy groups at third laparotomy did not reach significance level (Fig. 4).

Morphologic changes of endometrial cysts
Consistent with earlier reports (Dunselan et al., 1988), the endometrial implants protruded from the abdominal peritoneum and became vascularized cysts by 2 months. Eight of the forty-one endometrial cysts developed adhesions to intestine and/or bladder. Six months after treatment, endometrial cysts developed progressively into much larger cysts in the control and local blank microspheres group, endometrial cysts treated with local pseudo-pregnancy therapy and ovariectomy withered noticeably, whereas those treated by systemic pseudo-pregnancy therapy did not show any remarkable changes. However, adhesions did not regress along with the atrophy of the endometrial cysts in the local pseudo-pregnancy therapy group (n = 2) or ovariectomy group (n = 2), nor did they improve in systemic pseudo-pregnancy group (n = 1), local blank microspheres group (n = 2) and the control group (n = 1). No new adhesion formation was observed at third laparotomy.

The histological appearance of endometrial cysts resembled those of human endometrioma with a layer of cuboidal epithelium, glands and stromal tissue. Treatment with local pseudo-pregnancy therapy induced highly atrophic changes in the endometrial epithelium and stroma, whereas local blank microspheres did not induce any marked differences. In both groups, the majority of the microspheres remained in the cyst, although a small portion can be seen outside the cyst wall allowing some microsphere leakage. The microspheres outside the cyst generated focal minor foreign body reactions. For rabbits treated with systemic pseudo-pregnancy therapy, secretory changes with intracellular vacuoles can be seen in the epithelium and gland, however, typical decidualization was not observed in the stroma. The endometrial cysts in ovariectomy group were highly atrophic. Only pyknotic nuclei can be seen in the epithelium and stroma (Fig. 5).

Evaluation of major metabolic parameters
The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (CHO), triglycerides (TG), plasma glucose (GLU) and weight gain were significantly (P < 0.01) elevated in the
systemic pseudo-pregnancy group. In contrast, no statistically significant differences were observed between the local pseudo-pregnancy group and the control, although the mean values of cholesterol and glucose did show slight increase. Treatment with local blank microsphere had no measurable influence on these major metabolic parameters, which remained at the control level. The level of cholesterol in rabbits treated with ovariectomy exhibited a slight non-significant elevation (Table I).

Estrous cycle, uterine weight and ovarian function
Except for systemic pseudo-pregnancy and ovariectomy treatment, which induced arrest of estrous cycle, no cycle disturbances were noted. The weight of right uterus in the local pseudo-pregnancy group (2.68 ± 0.64 g), local blank microspheres group (2.46 ± 0.69 g) and the systemic pseudo-pregnancy group (2.73 ± 0.57 g) were comparable to that of the control (2.41 ± 0.66 g), whereas the right uterus in the ovariectomized group was significantly decreased (0.69 ± 0.18 g, \( P < 0.01 \)). Numbers of large follicles (≥1 mm) in the ovary were counted in each group. There was no difference among local pseudo-pregnancy, local blank microsphere and control groups (11.1 ± 2.5, 10.1 ± 2.2, 9.5 ± 2.1, respectively), although no large follicle was found in the systemic pseudo-pregnancy group.

Discussion
By intra-cystic injection of LNG-microspheres, high levels of LNG were localized in the endometrial cysts for at least 6 months. The local high level of LNG simulated a state of potent pregnancy, which induced significant size reduction and endometrial atrophy upon the endometrial cysts. Since ovarian function was not suppressed in the local pseudo-pregnancy group, the predominant mechanisms for endometrial atrophy are probably progesterone receptor-mediated effects of local high concentration of LNG (Critchley et al., 1998), or other signaling pathways as suggested by Salmi et al. (1998).

Microspheres, prepared by the solvent evaporation method, usually have a tri-phasic drug release pattern. An initial burst is followed by a slow drug release phase and a final rapid drug release (Luan and Bodmeier, 2006b). However, in this study, no initial burst or final rapid drug release was observed. Absence of initial burst may be attributed to the smooth, non-porous surface of the microspheres and the hydrophobic property of LNG and PLA (Herrmann and Bodmeier, 1995; Wang et al., 2002; Luan and Bodmeier, 2006a; Allison, 2008). Final rapid drug release is generally caused by the erosion of the microspheres (Luan and Bodmeier, 2006b). In this study, the erosion of the microspheres had no apparent effect on the LNG release profile because the microspheres did not biodegrade much before the majority of the drug had been released. LNG was released from the microspheres mainly by the diffusion and final rapid drug release was accordingly absent. As a result, a stable and continuous release profile was maintained with this delivery system and toxicity problems associated with initial burst and final rapid drug release were avoided.

Compared with conventional systemic pseudo-pregnancy, major metabolic parameters and ovarian function were not disturbed in rabbits treated with local pseudo-pregnancy therapy, indicating that the effective dose of local pseudo-pregnancy therapy is well tolerated. Nevertheless, the statistically insignificant elevation of cholesterol and glucose in plasma may be indicative of elevated risk of cardiovascular disease and diabetes. Further studies are needed to determine the effective radius without any adverse metabolic side effects. Further, microsphere-encapsulated third-generation progestogens, for example...
gestodene and dienogest, that have fewer androgenic and glucocorticoid effects, might be more valuable (Sitruk-Ware, 2004).

Six months after injection, the serum level was below the limit of assay (0.3125 ng/ml) in the local pseudo-pregnancy group. However, according to previous studies, it takes ~1 year for PLA microspheres to biodegrade completely (Beck et al., 1979). This is a concern because the PLA microspheres will accumulate following repeated injections. Theoretically, this drawback could be avoided by substituting PLA

Figure 5 Representative gross morphology and histological appearance (HE stain. original magnification: \( \times 100 \) and \( \times 400 \), respectively) of endometrial cysts for each group. Local pseudo-pregnancy (Local Preg): the ovoid open spaces outside the cyst indicate where most of the microspheres have not been biodegraded. Decreased cytoplasm and condensed nuclei of the endometrial epithelial cells indicate atrophic changes. Stromal tissue is rather loose with scattered pyknotic nuclei. Local blank microspheres (Blank ms). Systemic pseudo-pregnancy (Systemic Preg): note the vacuolated cells in epithelium and glands. Ovariectomy (OVX) and the Control.
with another polymer that can release LNG in a similar kinetics but biodegrade faster, for example, poly(lactic-co-glycolic acid).

In this study, we observed an interesting phenomenon: at the end of the study period, in the local pseudo-pregnancy group, the LNG serum level fell below the limit of assay, yet the intra-cystic level remained high (275 ng/ml). Thus the release of LNG from PLA microspheres is still ongoing after 6 months, indicating that endometrial cysts remain in local high level of LNG. In order to study the long-term efficacy of LNG-microspheres, further research in a rat model is underway.

Ovarian endometrioma is probably the most applicable type but may not be the only one for local pseudo-pregnancy therapy. LNG-microspheres can also be injected into the cul-de-sac, or the rectovaginal septum to treat peritoneal lesions and deep-infiltrating endometriosis. We intend to investigate LNG-microsphere treatment efficacy on peritoneal endometriosis by intrapelvic injection in a baboon model described by D’Hooghe et al. (1995).

Although laparoscopic cystectomy is now considered to be the ‘gold standard’ for the management of ovarian endometrioma (Chapron et al., 2002), recurrence has been observed in 7–30% of patients within 3 years after laparoscopic surgery and 40–50% after 5 years (Seracchili et al., 2009). Moreover, normal ovarian tissue is frequently stripped away along with the endometriotic capsule during cystectomy, which inevitably impairs the follicular reserve. Coagulation or laser vaporization without excision of the capsule is associated with a significantly higher risk of cyst recurrence (Beretta et al., 1998; Vercellini et al., 2003a). As the Italian Senate’s Investigative Committee’s findings summarizes the challenge of endometriosis: there is no cure, treatments are often ‘hit-and-miss’ (Bianconi et al., 2003a). Thus medical treatment will still play an important role in the therapeutic strategy provided it can be administered over a prolonged period of time (Vercellini et al., 2003b).

In summary, the present study has showed the efficacy and side effects of local pseudo-pregnancy in treating experimental endometriosis. By sustained delivery of LNG directly into endometrial cysts via biodegradable microspheres, exceptional high concentrations of LNG were maintained in the lesions for at least 6 months with much lower levels in plasma. The high concentrations of LNG in the lesions are responsible for the superior effectiveness whereas the low levels in plasma contribute to minor adverse effects. Therefore, for patients with endometriosis, induction of a local pseudo-pregnancy via progestogen-loaded microsphere might be a preferable treatment of choice and a potential alternative to surgery. However, the restrictions of extrapolating its efficacy in rabbit model to the human must be borne in mind. Further, investigation of local pseudo-pregnancy therapy and its drug delivery system will be required to prove its clinical feasibility.

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