Hormonal profile and endometrial morphology in letrozole-controlled ovarian hyperstimulation in ovulatory infertile patients

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Objective: To evaluate the clinical response and endometrial morphology during the implantation window on ovarian hyperstimulation with the aromatase inhibitor letrozole in infertile ovulatory women.
Design: Prospective trial in infertile patients.
Setting: Tertiary care hospital.
Patient(s): Eight ovulatory infertile patient candidates for ovarian superovulation.
Intervention(s): Subjects were monitored in one control cycle. In the next cycle, they received letrozole 5.0 mg daily on days 3 through 7 after menses.
Main Outcome Measure(s): Number of ovulatory follicles; dominant follicle diameter; endometrial thickness; hormonal profile of FSH, LH, E_2, A, T, and P; endometrial histological dating; and pinopode formation assessed by scanning electron microscopy.
Result(s): Cycles stimulated with letrozole resulted in more ovulatory follicles than did natural cycles (mean ± SD 2.0 ± 0.9 vs. 1.0 ± 0.0), which attained a greater preovulatory diameter (mean ± SD 23.8 ± 2.7 vs. 19.3 ± 2.1 mm), with similar endometrial thickness at midcycle compared with spontaneous cycles. Endocrine profile of medicated cycles was characterized on day 7 by increased levels of LH (5.9 ± 0.8 vs. 3.5 ± 0.4 IU/mL), reduced E_2 (98.4 ± 11.4 vs. 161.5 ± 14.7 pmol/L), and elevated androgens. Preovulatory and midsecretory E_2 were similar to spontaneous cycle, and P levels during midluteal phase were significantly elevated (44.2 ± 4.6 vs. 27.7 ± 4.6 pmol/L). Endometrial morphology during the implantation window in letrozole-stimulated cycles was characterized by in-phase histological dating and pinopode expression on scanning electron microscopy.
Conclusion(s): Letrozole induces moderate ovarian hyperstimulation in ovulatory infertile patients with E_2 levels similar to spontaneous cycles and higher midluteal P, leading to both a normal endometrial histology and development of pinopodes, considered to be relevant markers of endometrial receptivity.

Key Words: Aromatase inhibitors, letrozole, infertility, ovulation, controlled ovarian stimulation, endometrium

In the management of the infertile couple, controlled ovarian stimulation is one of the most common practices to increase women's fecundity. This intervention is frequently associated with IUI improving the chance of pregnancy.

In anovulatory women, the use of clomiphene citrate is widely accepted as first-line therapy because of its low cost, easy administration, and high security. Its use is associated with an ovulation rate of 60%–80% but with a much lower pregnancy rate of 50% (1, 2). The use of clomiphene citrate in ovulatory women has no clear advantage. In fact, meta-analysis studies show a significant but modest effect in this group (3), and some studies have observed a reduced pregnancy chance compared with placebo (4).

Recently, Mitwally and Casper (5–7) published some data showing the effect of the aromatase inhibitor letrozole over the ovarian cycle in different conditions. They reported that letrozole induces a moderate ovarian stimulation on ovulatory and anovulatory women and improves the ovarian response to FSH in unexplained infertility and poor responders.

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Aromatase is a cytochrome P-450 enzyme complex that catalyses the conversion of androgen (A) and T into estrogens. There are two types of aromatase inhibitors: steroidal (type I) and nonsteroidal (type II) inhibitors. The triazole (antifungal) derivative letrozole (Femara; Novartis, East Hanover, NJ) is a nonsteroidal reversible, competitive aromatase inhibitor that is highly potent and selective. Letrozole is completely absorbed after oral administration, with a half-life of 2–4 days, and attains maximal estrogen suppression in 2–3 days (8).

The proposed mechanisms of ovarian stimulation by letrozole are a central effect on releasing the pituitary–hypothalamic axis from estrogen negative feedback and a local ovarian effect blocking androgen conversion to estrogen, with the concomitant accumulation of androgens inside the ovary, augmenting the follicular FSH receptor expression, and promoting folliculogenesis. Testosterone was found to augment follicular androgen and FSH receptor expression in primates, which support a probably stimulatory role of androgen in early follicular growth (9).

The dose of letrozole used in previous trials was 2.5 mg/d given on days 3–7 of the menstrual cycle, although Biljan et al. (10) obtained more mature follicles with a higher dose (5 mg/d).

Until the beginning of the trial there was no published information about the effect of the drug over the endometrium.

Considering that ovulatory infertile patients require new options for ovarian superovulation, we proposed to evaluate the clinical effect of a 5-mg/d letrozole dose on a group of those patients and also to study the endometrial morphology during the period of the implantation window.

MATERIALS AND METHODS

Eight patients between the ages of 25 and 35 years were recruited through the infertility clinic at San Borja-Arriarán Clinical Hospital (Santiago, Chile) to use the aromatase inhibitor letrozole for ovarian stimulation. This pilot study was a nonrandomized prospective study that included ovulatory women with unexplained infertility. Unexplained infertility was diagnosed by exclusion of known factors of infertility. Ovulation was confirmed by follicular monitoring with transvaginal sonography during a natural (no-treatment) cycle and/or by midluteal P of >15 nmol/L, associated with regular menstrual cycle. Tubal patency was confirmed by hysterosalpingography and/or pelvic laparoscopy, and male factor infertility was excluded by semen parameters meeting the 1999 World Health Organization criteria (11).

All the study couples had >1 year of infertility. Participants were excluded if they had preexisting ovarian cysts or if they used oral contraceptives or any hormonal medication within 3 months before enrollment. Patients were counseled regarding the novel use of aromatase inhibitors to enhance ovarian function. Before their enrollment in the study, informed written consent was obtained from all subjects. During participation in the study, patients used condoms or sexual abstinence to prevent pregnancy.

The present study was approved by the Research Ethics Board of the San Borja-Arriarán Clinical Hospital and was conducted in the Infertility Unit of San Borja-Arriarán Hospital, Department of Obstetrics and Gynecology, and the Institute of Maternal and Child Research, University of Chile, from October 2001 to March 2002.

After enrollment, each volunteer was followed through one natural cycle to obtain baseline hormonal parameters and to confirm the presence of spontaneous ovulation.

Monitoring in the natural cycle included transvaginal ultrasound commencing on day 3 to document the absence of preexisting ovarian cysts, followed by daily transvaginal ultrasonographic evaluation of the follicular growth with a 5-MHz vaginal transducer attached to a Medison scanner (Model Sonoace 8800 Digital GAIA MT; Medison Co., Seoul, Korea). The maximum follicular diameter was measured in all patients. Both ovaries were identified, and the largest diameter was measured in both the longitudinal and transverse dimensions in all follicles. The day of ovulation was designated as the day of maximum follicular enlargement, which was followed the next day by sudden disappearance or filling of this follicle, showing loss of clear demarcation of its walls and intrafollicular echoes (12, 13).

The endometrial thickness was measured at the greatest diameter perpendicular to the midsagittal plane in the fundal region, including both layers of the endometrial cavity. We recorded the endometrial thickness and the echo pattern on each evaluation.

Blood samples for FSH, LH, T, A, and E_2 were then drawn on cycle day 3 and 7 and daily when a follicle larger than 18 mm was seen, until sonographic ovulation. Preovulatory samples were determined retrospectively as the day before follicular rupture as seen on transvaginal sonography. Samples for E_2 and P were obtained on day +7 after follicular rupture. Additionally, on postovulatory day +7, endometrial biopsies were taken from the uterine fundus by using Pipelle (Laboratoire CCD, Paris, France). Endometrial sampling was always performed on ovulation day +7 as the best correspondence to the window of implantation (14).

In the subsequent cycle, subjects received letrozole (Femara; Novartis; 5.0 mg/d) from day 3 to day 7 of the menstrual cycle according to the protocol. Participants were followed in the medicated cycle by using hormone blood levels, transvaginal ultrasound, and endometrial samples, as in the natural cycle.

Endometrial samples were divided in two portions, one for light-microscopy study and the other for scanning electron microscopy investigation, considering a recent study (15) concluded that scanning electron microscopy, but not light

TABLE 1

Sonographic characteristics of natural and letrozole cycles.

Variable	Mean value for natural cycle (± SD)	Mean value for letrozole cycle (± SD)	*P* value
Ovulatory follicles	1.0 ± 0.0	2.0 ± 0.9	.02
Greatest follicle diameter (mm)	19.3 ± 2.1	23.8 ± 2.7	.01
Greatest endometrial thickness (mm)	12.1 ± 1.7	12.3 ± 2.3	.815
Ovulatory day	13.9 ± 2.7	14.0 ± 1.4	.199

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microscopy, is the appropriate tool for the evaluation of the stage of pinopod formation.

Endometrial Dating

Endometria were fixed in 10% formalin and embedded in paraffin; 6-μm sections were stained routinely with hematoxylin and eosin for light microscopy. The histologic evaluation of each sample was performed by three experienced pathologists (blinded to the patient's condition) according to the histopathological criteria of Noyes et al. (16). An out-of-phase biopsy was defined as at least a 3-day lag between the chronological and the histological day determined. Chronological day was determined by counting forward from the ovulation day as detected by ultrasonographic scans. Interobserver variability for endometrial dating was estimated in 1 day.

Scanning Electron Microscopy

Endometrial samples were fixed in 2.5% (wt/vol) glutaraldehyde solution in a sodium cacodylate buffer (0.15 M, pH 7.3) and postfixed in a solution of 1% (wt/vol) osmium tetroxide in a sodium cacodylate buffer (0.15 M, pH 7.3). The tissue samples were then dehydrated in acetone series, dried in a critical-point by using carbon dioxide, mounted on the specimen holder, and coated with gold palladium. For each biopsy, four to six fragments were evaluated. The scanning electronic microscopy studies were performed by three pathologists who were blinded with regard to patient identification and the ultrasonographically detected ovulatory day.

Hormone Assays

Blood samples were processed by centrifuge, and the serum was stored at −20°C until used. Luteinizing hormone and FSH concentrations were measured in duplicate by a commercial immunoradiometric assay from Diagnostic Products Co (Los Angeles, CA). The sensitivity of these assays are 0.05 mIU/mL for LH and 0.06 mIU/mL for FSH; the intra-assay coefficients of variation (CVs) are 6.5% and 3.6%, and the interassay CVs are 7.6% and 6.2% for LH and FSH, respectively. Samples from the control and stimulated cycle of each subject were always assayed in the same LH and FSH assay. The serum levels of E_2 and T were measured with a commercial RIA from Diagnostic System Laboratories (Webster, TX). The sensitivity of this assay was 5 pg/mL and 0.01 ng/mL, respectively, for E_2 and T. The intra-assay CVs are 4.1% and 5.1%, and the interassay CVs are 6.7% and 6.4%, respectively. Progesterone and A concentrations were measured with a commercial RIA from Diagnostic System Laboratories. The sensitivity of those assays were 0.02 ng/mL and 0.01 ng/mL, respectively; the intra-assay CVs were 3.2% and 4.8%, and the interassay CVs were 6.1 and 7.2, respectively.

Statistics

Results are expressed as mean ± SD. When variables showed normal distribution, paired *t* test was used. Categorical data were compared by Fisher's exact test. $P<.05$ was considered statistically significant. All statistics were run on SPSS 10.0 for Windows (Release 10.0, SPSS Inc., Chicago, IL).

RESULTS

Eight participants were enrolled in this study. All women completed both study cycles. In two women, it was not possible to obtain an endometrial sample because of cervical stenosis.

Sonographic features of natural and letrozole cycles are depicted in Table 1.

The number of ovulatory follicles was doubled in medicated cycles compared with in natural cycles.

The maximal follicular diameter attained before ovulation was consistently greater in letrozole cycles than in spontaneous cycles ($P=.01$, paired *t* test). Besides, spontaneous ovulation was documented in all natural and medicated cycles. Ovulation day (day of maximum follicular enlargement, which was followed the next day by disappearance of this follicle) was similar in natural and letrozole cycles.

Endometrial thickness at midcycle was comparable between natural and treatment cycles, and all natural and

TABLE 2

Endocrine profile of spontaneous and letrozole cycles.

Variable	Mean value for natural cycle (± SD)	Mean value for letrozole cycle (± SD)	*P* value
Day 7 FSH (IU/mL)	4.6 ± 0.7	4.9 ± 0.6	.63
Day 7 LH (IU/mL)	3.5 ± 0.4	5.9 ± 0.8	.003
Day 7 E_2 (pmol/L)	161.5 ± 14.7	98.4 ± 11.4	.002
Preovulatory E_2 (pmol/L)	427.2 ± 40.7	430.8 ± 48.8	.93
Preovulatory E_2/mature follicle (pmol/L)	427.2 ± 40.7	252.5 ± 49.9	.018
Day 7 A (nmol/L)	7.36 ± 1.36	8.58 ± 1.5	.016
Day 7 T (nmol/L)	1.28 ± 0.17	1.63 ± 0.21	.006
Postovulatory day + 7 Progesterone (pmol/L)	27.7 ± 4.6	44.2 ± 4.6	.008

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medicated cycles had a trilaminar endometrial pattern at midcycle.

Hormonal levels in the treatment cycles were similar to natural cycles on day 3 before letrozole administration.

The endocrine profile of spontaneous and letrozole cycles is depicted in Table 2.

On cycle day 7 we observed a significant increase of LH, A, and T in letrozole-treated cycles, whereas FSH levels were not different between natural and medicated cycles.

Preovulatory E_2 levels were not different between natural and letrozole cycles; however, when we expressed the results in terms of E_2 concentration per mature follicle, we observed a significant reduction (50%) in medicated cycles (P=.018, paired t test). Progesterone serum levels in postovulatory day +7 (midluteal phase) were almost double in the letrozole cycles compared with in the natural cycles, consistent with the number of corpora lutea formed.

The histological endometrial dating of natural and letrozole cycles was not different among them, but when the biopsies were classified as in phase or out of phase, the samples of treated cycles were all in phase, whereas in natural cycles, 50% of the samples show a delayed growth.

When examined by scanning electron microscopy, the endometrial apical surface was predominantly covered in all instances by ciliated and microvillous cells. Pinopodes were observed in 100% of endometrial biopsies of natural and letrozole cycles, as shown in Figure 1 and Table 3. Interestingly, in both cycles, pinopodes characteristics were coincident with the histological dating of the endometria.

DISCUSSION

Clomiphene citrate has been widely used, in ovulation induction of anovulatory patients (mostly in polycystic ovary syndrome) and in ovarian stimulation associated with IUI, in ovulatory infertile patients who have unexplained infertility, minimal or mild endometriosis, and male factor. This drug is the first-line therapy because of its low cost and security, but it is well known that this antiestrogen has many undesirable effects on cervical mucus, endometrium, and uterine blood flow (17).

The long-lasting action on the estrogen receptor is the cause of the drug's undesirable effects on endometrium and cervical mucus, and many studies report reduction in glandular density and diameter and other glandular and stromal alterations of the endometrium (18, 19).

The second-line therapy in ovarian stimulation is injectable gonadotropins, but there are some limitations, such as multiple gestation, ovarian hyperstimulation syndrome, and high costs.

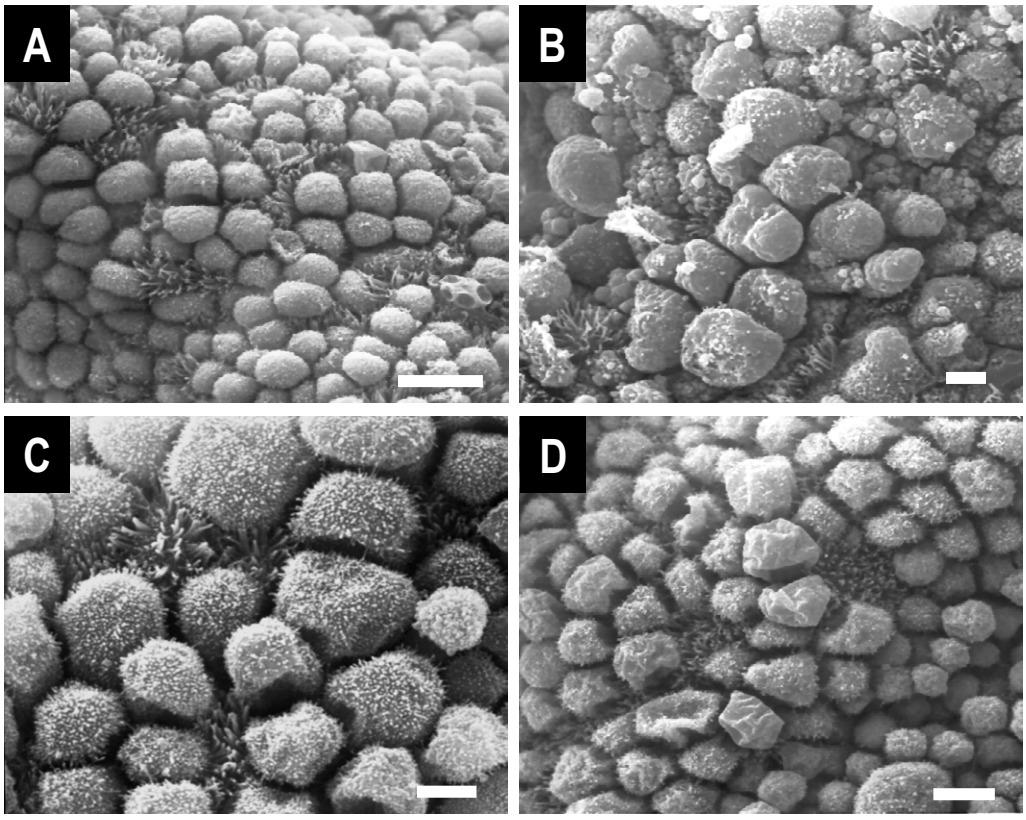
The report of the efficacy of letrozole in ovarian stimulation opens a new possibility in the management of infertile patients. Its relatively short half-life (45 hours) and excellent bioavailability make letrozole a good candidate as a first-line therapy.

Fisher et al. (20) compared the effect of clomiphene citrate and letrozole on normal ovulatory women, showing no difference on follicular recruitment, endometrial sonographic appearance, and gonadotropin levels; however, E_2 level was more than two times higher in clomiphene-treated cycles. In another study, letrozole was reported to improve endometrial thickness and pregnancy rates in a limited group of ovulatory and anovulatory patients who were treated in previous cycles with clomiphene citrate (5).

On the basis of the fact that letrozole had not been previously evaluated with respect to its effects on endometrial morphology, the present investigation addressed this matter in this pilot study, before its use in a clinical trial. Our results are in accord with previous reports in the efficacy of letrozole to induce multiple ovulatory response.

FIGURE 1

Pinopode formation in endometria of control and letrozole-treated cycles evaluated by scanning electron microscopy. (**A**) Control cycle endometrium (original magnification, ×1,500; bar = 10μm). (**B**) Letrozole-treated cycle endometrium of the same patient (original magnification, ×2,500; bar= 5 μm). (**C**) Control cycle endometrium (original magnification, ×3,000, bar = 5 μm). (**D**) Letrozole-treated cycle endometrium of the same patient (original magnification, ×1,500; bar = 10 μm).



Cortinez. Characterization of letrozole-stimulated cycle. Fertil Steril 2005.

The hormonal changes induced by the aromatase inhibitor are similar to those in the previous studies, although with the employed dose in the present investigation (5 mg for 5 days), we observed a significant increase in the levels of LH, A, and T during the follicular phase. In contrast, the levels of E_2 were significantly reduced dur-

TABLE 3

Endometrial dating and pinopode formation in natural and letrozole cycles.

Variable	Natural cycles (n = 6)	Letrozole cycles (n = 6)	*P* value
Histological dating (mean ± SD)	18.6 ± 0.6	19.6 ± 0.6	0.506[a]
In-phase biopsies (%)	3 (50)	6 (100)	0.182[b]
Out-of-phase biopsies (%)	3 (50)	0 (0)	NS
Pinopode formation (SEM), positive samples (%)	6 (100)	6 (100)	—

Note: NS = not statistically significant.
[a]Paired *t* test.
[b]Fisher's exact test.

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ing the follicular phase, when we considered E_2 concentration per mature follicle.

As expected, P levels were significantly augmented in the luteal phase. This was not unexpected given the presence of multiple corpora lutea and the fact that letrozole does not affect P synthesis.

The effect of letrozole in the serum level of E_2 and P leads to a change in the E_2/P ratio during the luteal phase, suggesting an action over the endometrial maturation improving receptivity. The advantage of this therapy over clomiphene or gonadotropins may be the increase in the number of follicles with E_2 levels, similar to spontaneous cycles, and a lower E_2/P ratio.

For the first time we report the effect of ovarian stimulation with letrozole over the endometrial morphology and the expression of pinopodes, one of the most widely accepted markers of endometrial receptivity. Our study shows a normal morphology of the endometrium and full expression of pinopodes during the window of implantation in all biopsies of letrozole-stimulated cycles.

Despite the high variability from cycle to cycle of endometrial markers reported by Ordi et al. (21), the only parameter with a higher agreement in the same study was histological dating. Using the same prospective ultrasonographic assignment as the day of endometrial sample, we found a small but consistent advancement in endometrial maturity in letrozole cycles, and we think this may be explained by the E/P ratio, which favors P in medicated cycles.

The presence of developed pinopodes during the implantation window is considered a marker of endometrial receptivity, validated by basic and clinical studies (22); in the present report, we confirmed its presence in all the biopsies of letrozole-stimulated cycles.

In summary, the results of this preliminary study suggest that the aromatase inhibitor letrozole may be an alternative as a first-line drug for ovarian superovulation in ovulatory infertile patients, considering its moderate stimulatory effect over the ovary and its favorable effect on endometrial morphology.

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