GnRH-a induced changes in endometrial pinopodes

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Abstract
Our aim was to study the effect of GnRH-a on pinopodes during luteal phase support and explore the possible mechanism. Forty women with primary infertility due to male factors were enrolled for ART, and randomly divided into experimental and control groups. Seven days after ovulation, the experimental group received a subcutaneous injection of 0.1 mg GnRH-a, while the control group received a subcutaneous injection of 2 ml of 0.9% saline. Serum progesterone levels in the experimental group were significantly (P < 0.05) higher 3 days after compared with before treatment, and were higher (P < 0.05) compared with the control group. Protein expression levels of the progesterone receptor in the experimental group were significantly (P < 0.05) lower after compared with before treatment, and lower than the control group (P < 0.05). The number of pinopodes and the percentage of pinopode maturation were significantly higher in the experimental compared with control group (P < 0.05). The luteal support provided by GnRH-a may act through the corpus luteum, which may promote the secretion of progesterone, down-regulate progesterone receptor expression, increase the growth of pinopodes and improve endometrial receptivity, which ultimately increase the rates of clinical pregnancy, continuous pregnancy and live birth.

Introduction
Luteal phase support has been a key part of Assisted Reproductive Technology (ART). With the rapid development of ART, researchers have sought a simple, economical, safe and effective method of luteal phase support. The Chinese Medical Association of Reproductive Medicine Branch, Chinese Medical Association of Perinatal Medicine Branch and Chinese Medical Association Family Planning Branch jointly presented a consensus on luteal phase support and progesterone support in January 2015 (1). In addition to reviewing estrogen, progesterone, and human chorionic gonadotropin, the consensus mentioned a new approach for luteal phase support using gonadotropin-releasing hormone analogues (GnRH-a). Many studies showed that administration of GnRH-a in the luteal phase may improve ART outcomes in many countries, including China (2). Our previous study followed clinical pregnancy cases after the use of GnRH-a during the luteal phase, and found that luteal phase support by GnRH-a may be effective and safe in ART (3). However, the mechanism of GnRH-a action for luteal
phase support was unclear (4-6). It is likely that GnRH-a improved endometrial receptivity for embryo implantation.

Pinopodes are smooth, membranous protuberances appearing on the apical surface of uterine epithelium when viewed under the scanning electron microscope. Early studies suggested that the timing of the appearance of mature pinopodes corresponded to the period of best endometrial receptivity. Pinopodes may provide specific morphological markers for the window of endometrial receptivity (7-10), and clues for predicting the timing of embryo transfer (11). Therefore, we have studied changes to pinopodes after the use of GnRH-a during luteal phase support, and explored possible mechanisms of GnRH-a actions in ART.

Results

Baseline data for experimental and control groups

As shown in Table 1, the values of age, infertility duration, body mass index, and serum estradiol (E2) and progesterone (P) levels (at day 3–5 of menstruation) were not significantly different between the experimental and control groups (P > 0.05).

Serum estradiol and progesterone levels before and after treatment in the experimental and control groups

There were no significant differences in serum estradiol and progesterone levels between the two groups before treatment (P > 0.05, Table 2). But after GnRH-a treatment, serum progesterone levels of the experimental group were significantly higher than the control group (P < 0.05), while there were no significant differences in estradiol levels (Table 2).

Protein expression of endometrial estrogen and progesterone receptors in the experimental and control groups before and after treatment

We detected expression levels of estrogen (ER) and progesterone (PR) receptors in the experimental and control groups by western blot analysis (n = 4 per group) (Fig. 1). The expression of PR was significantly down-regulated in the experimental group after treatment, and was lower in the experimental compared with control group in the same period (P < 0.05, Fig. 1B, D). However, there was no significant difference in the expression of ER between the two groups before and after
treatment ($P < 0.05$, Fig. 1A, C).

**Relative numbers of endometrial pinopodes in the experimental and control groups before and after treatment**

The relative numbers of pinopodes in the two groups on day 3 (after GnRH-a) was higher than that on day 0 (before GnRH-a), and more mature pinopodes were present on day 3. Moreover, the relative number of pinopodes and the proportion of mature pinopodes in the experimental group were significantly higher than the control group at day 3 ($P < 0.05$, Fig. 2 and Tables 3, 4).

**Association between the expression of pinopodes (surface percentage) and serum hormone levels before and after treatment**

The calculated differences between serum estradiol and progesterone levels and the area of pinopodes before and after GnRH-a treatment showed bivariate normal distributions. As shown in the scatter diagram of Fig. 3, there was no significant correlation between the calculated differences of estradiol levels and the area of pinopodes in the experimental group before and after treatment ($r = -0.058$, $P = 0.809$). As shown in Fig. 4, there was significant linear correlation between the differences in progesterone levels and area of pinopodes in the experimental group before and after treatment ($r = 0.781$, $P < 0.001$). Thus, these findings indicated that there was a positive correlation between the change of pinopode area (richness) and progesterone levels in the experimental group after GnRH-a treatment.

**Statistical methods**

Statistical analysis of the data was performed using IBM SPSS Statistics 19.0. Statistical values were described as average ± standard deviation and enumerated data were described as a percentage. The t test was applied for statistical comparisons between two independent samples. Enumerated data were analyzed by the $\chi^2$ test (if two values were not listed for the Fisher’s exact test). Significant differences between the two groups were defined when $P < 0.05$.

**Discussion**

Recently, the administration of GnRH-a for luteal phase support during ART has been the focus of considerable research. Several research groups$^{(4,12)}$ showed that addition of GnRH-a for luteal phase
support could promote live birth and clinical pregnancy rates, and continuous pregnancy rates, using either agonist or antagonist programs. Recent studies showed that addition of GnRH-a in the luteal phase can also improve the implantation rate, clinical pregnancy rate and continuous pregnancy rate for frozen embryo transfers from natural cycles (13,14). Some studies reported that GnRH-a promoted hypothalamic-pituitary function and the secretion of LH, estrogen, and progesterone (similar to the natural cycle), and then promoted the development and implantation of embryos (4). One study reported that GnRH-a directly promoted the implanted early embryo to secrete human chorionic gonadotropin (15), which is conducive to pregnancy. However, the exact mechanism of GnRH-a luteal phase support remains unclear. This study aimed at exploring the possible mechanism of GnRH-a action in luteal phase support through investigating GnRH-a-induced changes to proposed endometrial receptivity markers – pinopodes.

Pinopodes were first observed on the rabbit endometrial epithelial cell surface by Psychoyos et al. in 1971 (3) using electron microscopy. Afterwards, a similar structure was found in human endometrium during the implantation stage (3). Under the scanning electron microscope, pinopodes are relatively large and smooth flower-like protuberances on the top of endometrial epithelial cells. Pinopodes usually appear during days 20–21 of the normal menstrual cycle (about 1 week after ovulation), and last less than 48 h (16). According to different stages of development, pinopodes are divided into three types; the developing pinopode, developed pinopode, and degrading pinopode. Pinopodes appear on the endometrial surface during the window of embryo implantation, and the appearance of mature pinopodes is a proposed hallmark of the optimal receptivity of endometrium (17). Early studies reported that the timing of the emergence of fully developed pinopodes and then pinopode degradation were consistent with the opening and closing times, respectively, of the window of embryo implantation. Fully developed pinopodes were proposed to be an important morphological feature for the establishment of uterine receptivity and opening of the implantation window (18,19), indicating that the endometrium was about to enter the sensitive implantation period. The more
abundant the pinopodes, the higher the pregnancy rate. Mikolajczyk et al. \(^{(17)}\) showed that either a reduction in the number or an inappropriate time of maturation of pinopodes were associated with pregnancy failure, suggesting that a lack of pinopodes was a feature of embryo implantation failure. During the formation of pinopodes, several studies have shown that progesterone mainly influenced the function of endometrial glandular epithelial and stromal cells, and the appearance of pinopodes was regulated by the level of serum progesterone and its receptor expression \(^{(20)}\). Moreover, studies found that the PR was expressed in both stromal and epithelial cells of endometrial glandular tissue, and the glandular secretory value reached a peak during the proliferative and early secretory phases \(^{(21)}\). As the level of progesterone increased, the expression level of PR in glandular epithelial cells was significantly decreased and was not detected or weakly expressed during the implantation window, while there was no obvious change in the expression level of ER in endometrial stromal and glandular epithelial cells during the implantation window. Thus, it is possible that down-regulated expression of PR in glandular epithelial cells and up-regulated progesterone levels in endometrial stromal cells lead to the development of endometrial pinopodes, which improve endometrial receptivity. Consistent with this proposal, our current results show that serum progesterone levels in the experimental group were significantly higher after GnRH-a treatment (day 3) compared with the control group, but found no difference in estradiol levels. Our previous data also showed that serum progesterone levels were significantly higher 14 days after GnRH-a treatment compared with no treatment \(^{(22)}\). Our current results also showed that the expression level of endometrial PR was down-regulated in the GnRH-a treated group, while the level of ER remained constant. Our electron microscopy analysis showed that more mature endometrial pinopodes were present after GnRH-a treatment. Nine and 15 specimens exhibited pinopodes in the control and experimental groups, respectively, with an expression of 75% in the experimental group significantly different compared with the control group. Using electron microscopy, we observed increased numbers of pinopodes in specimens from the experimental group after GrRH-a treatment, each with a consistent size, clear boundary, smooth surface, with or without short and small microvilli, and shaped like a mushroom. Fully developed pinopodes were morevisible
after treatment. We concluded that adding GnRH-a in the luteal phase promoted the secretion of LH and progesterone via the hypothalamic-pituitary and corpus luteum, respectively, and led to increased development of mature pinopodes. Moreover, the appropriate development and abundance of mature pinopodes was closely related to successful implantation. Additionally, GnRH-a treatment had no effect upon estradiol and ER levels. Consequently, it is possible that the appearance of pinopodes was mainly influenced by the level of progesterone. Recently, Haas et al. found that the addition of GnRH-a for luteal support during natural cycle frozen embryo transfer improved the outcome, but was not effective in the artificial cycle with no corpus luteum formation\(^{(23)}\). These findings provide further support that GnRH-a plays an important role in promoting progesterone secretion, down-regulating endometrial PR, promoting full development of pinopodes, improving endometrial receptivity, and eventually promoting increased clinical pregnancy, continuous pregnancy and live birth rates, via an action on the corpus luteum.

In summary, the luteal phase support provided by GnRH-a is attracting more research into understanding the mechanism of improving pregnancy outcomes, and the dependence on promoting pinopode maturation in improving endometrial receptivity. Our future research should expand the sample size, combined with relevant examination of other GnRH-a-regulated genes and proteins associated with altered endometrial receptivity and embryo development to identify the specific mechanism of GnRH-a actions.

**Materials And Methods**

**Subjects**

Forty women with primary infertility due to male factors were prepared for ART and enrolled during January 2018 to October 2018 at The Reproductive Medicine Center of First Hospital Affiliated to Suzhou University. Inclusion criteria included: i) a normal natural menstrual cycle with normal ovulation, ii) normal baseline endocrine levels, iii) no uterine fibroids or polyps, no endometrial tuberculosis, endometritis, endometriosis, hydrosalpinx and other gynecological diseases, iv) no polycystic ovarian syndrome and other endocrine diseases, v) no history of estrogen or progesterone medication or uterine surgery in the past 3 months. The women were 22
to 35 years old with an average age of 26.7 years. The average duration of infertility was 3.26 years. All patients provided signed informed consent, and the study was approved by the Medical Ethics Committee of the Institute of Reproductive Medicine Center. The female subjects were randomly divided into the experimental and control groups. There were no significant differences for the baseline parameters (age, duration of infertility, BMI, estradiol and progesterone levels) between the two groups ($P > 0.05$).

**Embryo implantation window model**

The selected women were prepared for ART treatment. During the ninth day of the menstrual cycle, follicle development was detected using a diagnostic ultrasound instrument (ALOCK-6). If the average follicle diameter had reached 14 mm, patients were then monitored daily. The ovulation day was defined when the follicle diameter reached 18 mm (a mature follicle) and then suddenly disappeared or was reduced more than 5 mm, and dark liquid areas appeared in the uterine-rectum nest. The implantation window was regarded as 7–10 days after ovulation.

**Experimental and control groups**

Subjects were successively divided into experimental and control groups according to their order of enrolment. On the seventh day after ovulation, the experimental group received a subcutaneous injection of 0.1 mg of GnRH-a (Diphereline, Ipsen Pty. Ltd.), while the control group received a subcutaneous placebo injection (2 ml of 0.9% saline).

**Collection of endometrial specimens**

Before injection of GnRH-a (day 0) and after 3 days (day 3), endometrial tissue was extracted from the uterine cavity by a single-use suction device (Ningbo TianyiMedical Instrument Co., Ltd.). Extracted endometrial tissues were divided into two parts, washed with 0.9% physiological saline and dried with absorbent paper. One part was quickly placed into 2.5% glutaraldehyde solution and stored at 4°C for subsequent electron microscope scanning. The second part was stored at −80°C prior to western blot analysis.

**Collection of serum samples**

Fasting peripheral venous blood (3 ml) was collected on the same day endometrial tissue was
extracted, and then centrifuged at 3500 rpm for 15 min. Serum samples were collected and estradiol and progesterone levels were measured by chemiluminescence immunoassays (Beckman Coulter Inc., USA).

**Assessment of scanning electron microscopy results**

The endometrium sample was dried by gradient ethanol dehydration and plated with metal film by a vacuum coating apparatus, then observed by scanning electron microscopy. According to previously described criteria (Aghajanova et al., 2003), during pinopode formation, the cell surface was smooth, microvilli were reduced and the thin endometrial surface was protruded and folded to a large degree, shaped like a mushroom. During the degeneration of pinopodes, protrusions were reduced and microvilli reappeared, projecting from the endometrial surface, and cell volume was markedly increased. Every specimen was taken from near the opening of each gland, five fields were counted, and the average count selected. According to the percentage of pinopodes estimated in the total uterine endometrium, the expression levels were divided into rich, moderate and micro (>50%, 20–50% and <20%, respectively).

**Western blot analysis**

Total protein was extracted from endometrial tissue samples in liquid nitrogen. During SDS-PAGE, 30 μg denatured protein was added per lane, and after electrophoresis the proteins were transferred to a PVDF membrane (at a constant flow of 300 mA) at room temperature. The membrane was blocked in 5% skim milk powder (Shanghai Biological Engineering) dissolved in Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 h. The primary antibodies (1: 20) were incubated overnight at 4°C, then rinsed three times with TBST for 10 min. The HRP-labeled secondary antibody was then incubated at room temperature for 1 h, then rinsed 3 times with TBST for 10 min. An ECL chemiluminescence imaging system was used to analyze results (Kit purchased from Santa Cruz Biotechnology).

**Conclusions**

We propose that during the luteal phase support, GnRH-a promoted the secretion of progesterone, down-regulated progesterone receptor expression, and improved the growth of pinopodes and
endometrial receptivity, which ultimately led to increased rates of clinical pregnancy, continuous pregnancy and live birth. These effects of GnRH-a may be mediated via corpus luteum function.

**Abbreviations**

GnRH-a: Gonadotropin releasing hormone analogues

**Declarations**

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**Author contribution statement**

W. Z., D. L. and Z. Z. wrote the manuscript. W. Z., D. L., Y. J. and H. T. prepared Figures 1 and 2, and Y. P and C. Z. prepared Tables 1, 2, 3 and 4. All authors reviewed the manuscript.

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**Declaration of conflicting interests**

The authors have no confliction or financial interests to declare.

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Tables
Table 1 The comparison of two groups of patients with general information
### Table 2
The comparison of serum estrogen and progesterone levels before and after treatment in two groups

| Variable               | Experimental group | Control group | t value |
|------------------------|--------------------|---------------|---------|
| D0-day E2 pg/ml       | 203.90±43.43       | 198.25±46.30  | 0.3     |
| D0-day P ng/ml        | 13.28±3.11         | 15.34±4.70    | 1.6     |
| D3-day E2 pg/ml       | 209.25±42.49       | 195.60±33.07  | 1.1     |
| D3-day P ng/ml        | 21.07±4.69         | 14.33±1.62    | 6.070   |

Table 3 The comparison of the expression levels of pinopodes in both groups before and after
| Groups            | Variable                  | Before treatment | After treatment | Z value |
|-------------------|---------------------------|------------------|-----------------|---------|
|                   |                           | Total            | 20              | 20      | -       |
|                   | Development%              | -                | -               | 2.052   |
|                   | Puberty                   | 10 50.0%         | 2 10.0%         |         |
|                   | Maturity                   | 7 35.0%          | 15 75.0%        |         |
|                   | Regression stage          | 3 15.0%          | 3 15.0%         |         |
|                   | Richness%                  | -                | -               | 2.484   |
|                   | Trace +20%                 | 3 15.0%          | 1 5.0%          |         |
|                   | Defined amount +20%-50%    | 12 60.0%         | 6 30.0%         |         |
|                   | Richness ++20%-50%         | 5 25.0%          | 13 65.0%        |         |
|                   |                           | Development%     | -               | -       |
|                   | Puberty                   | 11 55.0%         | 5 25.0%         | 1.868   |
|                   | Maturity                   | 6 30.0%          | 9 45.0%         |         |
|                   | Regression stage          | 3 15.0%          | 6 30.0%         |         |
|                   | Richness%                  | -                | -               | 0.262   |
|                   | Trace +20%                 | 3 15.0%          | 2 10.0%         |         |
|                   | Defined amount +20%-50%    | 13 65.0%         | 14 70.0%        |         |
|                   | Richness ++20%-50%         | 4 20.0%          | 4 20.0%         |         |

Table 4 Comparison of the expression levels of pinopodes in experimental group and control group before and after treatment
| Whether treatment | Variable          | Experimental group | Control group | Z value | P value |
|-------------------|-------------------|--------------------|---------------|---------|---------|
| Before treatment  | Total             | 20                 | 20            | -       | -       |
|                   | Development %     | -                  | -             | 0.254   | 0.799   |
|                   | Puberty           | 10 [50.0%]         | 11 [52.4%]    | 0.267   | 0.963   |
|                   | Maturity          | 7 [35.0%]          | 6 [30.0%]     | 0.267   | 0.007   |
|                   | Regression stage  | 3 [15.0%]          | 3 [15.0%]     | 0.267   | 0.007   |
|                   | Richness %        | -                  | -             | 0.267   | 0.007   |
|                   | Trace + 20%       | 3 [15.0%]          | 3 [15.0%]     | 0.267   | 0.007   |
|                   | Defined amount    | 12 [60.0%]         | 13 [65.0%]    | 0.267   | 0.007   |
|                   | Richness + 50%    | 5 [25.0%]          | 4 [20.0%]     | 0.267   | 0.007   |
| After treatment   | Development %     | -                  | -             | 0.046   | 0.963   |
|                   | Puberty           | 2 [10.0%]          | 5 [25.0%]     | 2.694   | 0.007   |
|                   | Maturity          | 15 [75.0%]         | 9 [45.0%]     | 2.694   | 0.007   |
|                   | Regression stage  | 3 [15.0%]          | 6 [30.0%]     | 2.694   | 0.007   |
|                   | Richness %        | -                  | -             | 2.694   | 0.007   |
|                   | Trace + 20%       | 1 [5.0%]           | 2 [10.0%]     | 2.694   | 0.007   |
|                   | Defined amount    | 6 [30.0%]          | 14 [70.0%]    | 2.694   | 0.007   |
|                   | Richness + 50%    | 13 [65.0%]         | 4 [20.0%]     | 2.694   | 0.007   |

**Figures**
A: Detection of estrogen receptor (ER) expression levels by western blotting in the experimental group (EG) and control group (CG). 1, 3, 5 and 7 are before treatment and 2, 4, 6 and 8 are after treatment. B: Detection of progesterone receptor (PR) expression levels by western blotting in the experimental group (EG) and control group (CG). 1, 3, 5 and 7 are before treatment and 2, 4, 6 and 8 are after treatment. C: Detection of estrogen receptor (ER) expression levels by western blotting. *P< 0.05. D: Detection of progesterone receptor (PR) expression levels by western blotting. *P < 0.05.
Figure 2

Pinopodes detected by electron microscopy. A, B and C: In the experimental group, maturing pinopodes in endometrial tissue were observed by electron microscopy at x1000, x3000, x10000. D, E and F: In the experimental group, fully mature pinopodes in endometrial tissue were observed by electron microscopy at x1000, x3000, x10000. G, H and I: Endometrial pinopodes of degeneration phase were observed using electron microscopy at x1000, x3000, x10000.
Figure 3

Scatter plots of the difference in serum estradiol (E2) and pinopodes area in the experimental group before and after GnRH-a treatment.
Figure 4

Scatter plots of the difference of serum progesterone (P) and pinopodes area in the experimental group before and after treatment.