Effects of Two Different Doses of Raloxifene for Ovulation Induction on Pinopode Expression in Mouse Peri-implantation Endometrium: Postprint

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Abstract

Objective: To investigate the effect of two different doses of raloxifene (RAL) for ovulation induction on pinopode expression in the peri-implantation endometrium of mice. Methods: Healthy Kunming strain female mice, 6–8 weeks old, with two consecutive normal estrous cycles and no prior mating, were randomly divided into 4 groups: normal saline (SS) group, CC group, RAL 180 mg group, and RAL 240 mg group, with 12 mice per group. In the proestrus of the third estrous cycle, gavage was administered (SS group: 1 mL normal saline, once daily for 2 days; CC group: 1 mL, CC 18 mg/kg, once daily for 2 days; RAL 180 mg group: 1 mL, RAL 33 mg/kg, once daily for 2 days; RAL 240 mg group: 1 mL, RAL 44 mg/kg, once daily for 2 days). At 5:00 PM two days later (after two administrations), each female mouse was intraperitoneally injected with 5 IU hCG and then co-caged with normal male mice at a 1:1 ratio. The next morning, vaginal plugs were checked, and mice with plugs were considered to be on day 1 of pregnancy (D1). On D4.5, female mice in each group were sacrificed by ether inhalation anesthesia, endometrial tissues were harvested, and scanning electron microscopy was used to observe endometrial pinopode development. Results: Under electron microscopy, pinopodes in the endometrium of mice in the RAL 180 mg group, RAL 240 mg group, and SS group were mature and abundantly expressed, with no significant differences among these three groups in pairwise comparisons, whereas pinopodes in the CC group were immature and scarce. The semi-quantitative expression of pinopodes showed no significant differences among the RAL 180 mg group, RAL 240 mg group, and SS group, but was significantly higher than that in the CC group. Conclusion: Both doses of RAL did not affect pinopode expression in the endometrial epithelium of mice during the peri-implantation period, suggesting that ovulation

induction with either dose of RAL does not affect endometrial receptivity in mice.

Full Text

Effects of Raloxifene at Two Different Doses for Ovulation Induction on Endometrial Pinopodes in Mice During the Implantation Window

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Abstract

Objective To compare the expression of pinopodes, the marker of endometrial receptivity, during the implantation window in Kunming mice stimulated with two different doses of raloxifene (RAL). **Methods** Forty-eight 8-week-old female Kunming mice were randomly divided into 4 groups (n=12), namely saline group, clomiphene citrate (CC, 18 mg/kg) group, RAL (33 mg/kg) group and RAL (44 mg/kg group). In each group, the mice received intragastric administration of 1 mL of normal saline containing CC or RAL at the specified doses or saline only as indicated for ovulation induction, once daily for 2 days. The mice received then injection with 5 IU human chorionic gonadotropin (HCG) and mated and on day 4.5 of gestation, the pregnant mice were sacrificed for examination of the uterus with scanning electron microscopy. **Results** Abundant and well developed pinopodes were observed in the endometrium of the mice in the 2 RAL groups and in the saline control group. The mice in CC group showed obviously reduced endometrial pinopodes with poor development. **Conclusions** RAL at two different doses does not obviously affect the expression of pinopodes in the uterine epithelium of mice, suggesting the safety of RAL at these two doses for ovulation induction without causing adverse effects on endometrial receptivity.

Keywords: raloxifene; clomiphene citrate; endometrial receptivity; pinopode

Introduction

For patients with infertility caused by ovulatory disorders, ovulation induction is an effective treatment modality. However, in clinical practice, the clinical pregnancy rate following ovulation induction therapy is often substantially lower than the ovulation rate, particularly with clomiphene citrate (CC) [1]. Although CC has been used as a first-line ovulation induction drug for over 50 years, its

side effects as a selective estrogen receptor modulator (SERM), including anti-estrogenic effects on cervical mucus and the endometrium [2], remain quite common. Moreover, CC exerts inhibitory effects on endometrial receptivity [3], resulting in relatively low clinical pregnancy rates after ovulation induction.

Raloxifene (RAL) belongs to the second generation of SERMs and has been used to treat postmenopausal osteoporosis while also reducing the risk of invasive breast cancer [4]. Previous studies have demonstrated that RAL can elevate serum follicle-stimulating hormone (FSH) levels during the follicular phase in premenopausal women [5]. A prospective randomized controlled trial in 2011 confirmed the ovulation-inducing effect of RAL in patients with polycystic ovary syndrome (PCOS), with ovulation rates similar to those achieved with CC [6]. Meanwhile, in vitro research models have shown that RAL has less impact on endometrial receptivity compared to CC [7].

Morphologically, the most representative ultrastructural change in the endometrium during the implantation window is the formation of pinopodes. Scanning electron microscopy reveals that the appearance of pinopodes maintains strict consistency with the endometrial implantation window [8], serving as a reliable indicator of favorable endometrial receptivity [9]. Similar to humans, pinopode formation in rodents follows a strict temporal pattern and can be distinguished into three stages: developing, fully developed, and degenerating [10]. Given that pinopodes are widely recognized and commonly used markers of endometrial receptivity, our research group established RAL and CC ovulation induction mouse models in 2016 to observe the effects of these two ovulation-inducing drugs on the expression of pinopodes and other peri-implantation endometrial receptivity markers [11].

Our previous results showed that RAL at 22 mg/(kg · d) (equivalent to 120 mg/d in adult humans) had no adverse effects on pinopode expression in mouse endometrium [11]. However, it remains unclear whether the effects of RAL on endometrial receptivity markers exhibit dose-dependent characteristics. Since literature reports indicate that RAL at 120 mg/d yields relatively low ovulation rates in PCOS patients [7], we hypothesized that 120 mg/d may not be the optimal ovulation induction dose. Therefore, in this experiment, we increased the RAL ovulation induction dose in mice to 33 mg/(kg · d) (equivalent to 180 mg/d in humans) and 44 mg/(kg · d) (equivalent to 240 mg/d in humans), while simultaneously comparing these regimens with a CC ovulation induction mouse model. We observed the effects of these two different RAL doses on peri-implantation pinopode expression to investigate dose-dependent effects on endometrial receptivity, aiming to provide further experimental evidence for clinical RAL ovulation induction therapy.

Methods

Experimental Animals

We used 60 clean-grade, healthy Kunming white mice (48 females and 12 males), aged 6-8 weeks and weighing 20-25 g, that had never been mated. The animals were obtained from the Guangdong Experimental Animal Center, randomly assigned to cages, and numbered. They were maintained under conventional housing conditions at 25±2°C and 60-80% relative humidity, with controlled lighting (12-hour light period from 8:00 to 20:00 daily) and free access to standard pellet food and water. Female mice were group-housed (4-6 per cage), while male mice were individually caged.

Experimental Design and Procedures

Animal Grouping Female mice with normal estrous cycles for two consecutive cycles (cycle length 5-6 days) were selected for the experiment. The study was conducted during the proestrus phase of the third estrous cycle. Using the random number generator in Excel analysis tools, the female mice were randomly assigned to four groups (n=12 each): saline (SS) group, CC group, RAL 180 mg group, and RAL 240 mg group.

Drug Administration Qualified mice received intragastric administration during the proestrus phase of the third estrous cycle. Dosing calculations followed the novel method described by Huang Jihan et al. [12], which incorporates animal body shape coefficients and standard body weights for dose conversion between humans and experimental animals. We estimated equivalent doses for mice based on human clinical regimens: CC (100 mg/dose, once daily for 2 days) and RAL (180 mg or 240 mg/dose, once daily for 2 days) for a 60 kg adult. Each mouse received 1 mL of gavage solution: CC 100 mg group (CC 18 mg/kg), RAL 180 mg group (RAL 33 mg/kg), RAL 240 mg group (RAL 44 mg/kg), and SS group (1 mL normal saline). Specifically, gavage was performed daily between 8:30-9:00 AM for two consecutive days. At 5:00 PM on the second day (after two administrations), each female mouse received an intraperitoneal injection of 5 IU human chorionic gonadotropin (HCG) to induce ovulation and was then cohabited with a normal male mouse at a 1:1 ratio. The presence of a vaginal plug the following morning at 7:00 was designated as pregnancy day 1 (D1).

Specimen Collection On day 4.5 of gestation (D4.5), 12 female mice from each group were sacrificed by ether inhalation anesthesia. The uterus was rapidly exposed and endometrial tissue was collected. Each sample was divided into two portions: one was fixed in 2.5% glutaraldehyde and stored at 4°C for SEM specimen preparation (ensuring a smooth uterine cavity surface), while the other was fixed in 4% paraformaldehyde for 18 hours, routinely dehydrated, paraffin-embedded, and sectioned continuously (5 μm thickness) for hematoxylin-eosin (HE) staining.

Scanning Electron Microscopy For SEM specimen preparation, the 2.5% glutaraldehyde-fixed samples were thoroughly washed with PBS buffer, dehydrated through a graded ethanol series, immersed in 2% isoamyl acetate for 3 hours, and critical-point dried. The samples were mounted on specimen stages with the uterine cavity surface facing upward, coated with silver conductive adhesive, and metal-coated using a vacuum coater for SEM observation.

SEM observation was performed by two electron microscopy experts at ×4000 magnification. According to reference [13], pinopodes were semi-quantitatively scored as follows: each specimen was randomly scanned in 12 fields, microphotographed, and saved in TIF format for subsequent scoring. Pinopodes were defined as smooth, round protrusions of the endometrial epithelium lacking microvilli surface structures. The scoring system was: 0 (no pinopode expression), 1 (pinopode coverage <25%), 2 (coverage 25-50%), or 3 (coverage >50%).

Statistical Analysis Experimental data were analyzed using SPSS 21.0 software. Measurement data are expressed as mean ± standard deviation. For multiple group comparisons, one-way ANOVA was used if variances were homogeneous, with LSD test for multiple comparisons. If variances were heterogeneous, Welch' s correction was applied with Tamhane' s T2 test for multiple comparisons and correction. $P<0.05$ was considered statistically significant.

Results

Histological Morphology of Endometrial Tissues

Under HE staining, the endometrium of mice in the SS, RAL180, and RAL240 groups showed mature development with loose stroma, abundant vasculature, rich and complex glands with large lumens. In contrast, the CC group exhibited poorly developed endometrium with dense stroma, fewer blood vessels, poorly developed simple glands with small lumens and scant secretions (Figure 1 [Figure 1: see original paper]).

Pinopode Expression in Endometrium

Scanning electron microscopy revealed abundant, well-developed pinopodes in the endometrium of mice in the SS, RAL180, and RAL240 groups. The CC group, however, showed poorly developed pinopodes with reduced numbers. Semi-quantitative scoring demonstrated that the CC group score (1.08±0.56) was significantly lower than those of the SS group (2.68±0.62), RAL180 group (2.57±0.70), and RAL240 group (2.45±0.75) ($P<0.01$). No significant differences were observed among the SS, RAL180, and RAL240 groups ($P>0.05$, Figure 2 [Figure 2: see original paper], Figure 3 [Figure 3: see original paper]).

Discussion

The endometrium permits embryo implantation only during an extremely brief period. In humans, this window typically occurs during the mid-luteal phase of a normal menstrual cycle, 7-13 days after the LH surge [14] or 6-10 days after ovulation [15]. In rodents such as rats and mice, the implantation window occurs on day 4 after fertilization and lasts approximately 24 hours [16-17]. During this period, endometrial receptivity reaches its peak, constituting the "implantation window."

Our previous research [11] demonstrated that RAL at 22 mg/(kg·d) had no adverse effects on the histological morphology of mouse endometrium during the implantation window. In the current study, the RAL180 and RAL240 groups received increased RAL doses of 33 mg/(kg·d) and 44 mg/(kg·d), respectively. The results showed that both RAL groups, along with the SS group, exhibited well-developed secretory endometrium with loose stroma, abundant vasculature, and rich, complex glands with large lumens. Conversely, the CC group displayed poorly developed endometrium with dense stroma, fewer vessels, and simple glands with small lumens. These findings are consistent with our previous study, suggesting that both RAL doses do not impair the secretory histological morphology of mouse endometrium during the implantation window compared to CC.

Furthermore, SEM examination revealed abundant, well-developed pinopodes in the implantation window endometrium of the RAL180, RAL240, and SS groups, with no significant differences in pinopode expression among these three groups. In contrast, the CC group showed fewer, poorly developed pinopodes, significantly lower than both RAL dose groups and the SS group. These results indicate that both RAL doses do not affect pinopode expression in mouse implantation window endometrium, suggesting that RAL has less impact on endometrial receptivity than CC.

In 2006, Lessey et al. [7] demonstrated in Ishikawa cell cultures that RAL could promote the expression of integrin 3, a molecular marker of endometrial receptivity, through competitive binding to ER- , whereas CC lacked this promoting effect. Our study investigated the effects of two different RAL doses and CC on pinopodes as markers of endometrial receptivity in mice. The results align with Lessey' s in vitro findings, suggesting that competitive binding to ER- may be one mechanism through which RAL does not adversely affect pinopode expression in mouse endometrium.

Numerous studies have confirmed that CC exerts estrogen receptor antagonistic effects on the endometrium [18-20]. In CC-stimulated ovulation cycles, endometrial thickness is significantly suppressed [21], and supplementation with estrogen can substantially improve endometrial thickness [22]. Sequential estrogen-progesterone supplementation can improve endometrial receptivity and increase pregnancy rates [23]. In contrast, recent in vitro cell culture experiments have demonstrated that RAL exhibits estrogen antagonistic or neutral effects on the

endometrium [24-25]. Although RAL may also display estrogen receptor antagonism in the endometrium, this effect shows individual variation [22]. Additionally, CC' s long half-life of 5 days leads to prolonged occupation of estrogen receptors and subsequent receptor depletion, whereas RAL has a shorter half-life of only 32.5 hours, resulting in faster clearance from the body. Consequently, RAL ovulation induction has less impact on implantation window endometrium than CC, though the specific molecular mechanisms require further investigation.

Conclusion

This study represents the first prospective, randomized, controlled animal experiment in China comparing the effects of two different RAL doses and CC on peri-implantation endometrial pinopodes. We found that both RAL doses had no obvious adverse effects on pinopodes, a marker of endometrial receptivity, and were significantly superior to CC, with no apparent dose-dependency. These findings suggest that RAL may be a promising new oral ovulation induction drug and a potential alternative to the current first-line agent CC.

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