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Endometrial Receptivity Markers in Mice Stimulated With Raloxifene Versus Clomiphene Citrate and Natural Cycles

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Abstract

Ovulation induction therapy with clomiphene citrate can suppress endometrial receptivity. Raloxifene may be an alternative therapeutic for women with ovulatory disorders. This study aimed to compare the expression of endometrial receptivity markers, including homeobox gene 10 (HOXA10), integrin β_3, and leukemia inhibitory factor (LIF), as well as pinopode production during the implantation window in mice stimulated with raloxifene and clomiphene citrate and natural cycles. Thirty-six 8-week-old female Kunming mice were randomly divided into 3 groups ($n = 12$) and administered daily raloxifene (22 mg/kg), clomiphene citrate (18 mg/kg), and normal saline (1 mL), respectively, by gavage. Two days later, mice were injected with 5 IU human chorionic gonadotropin and mated. Successfully mated female animals were identified with vaginal plugs designated gestation day 1. At day 4.5, pregnant donor mice were euthanized, and uterus samples were collected for immunohistochemistry, quantitative polymerase chain reaction, Western blot, and scanning electron microscopy analyses. Homeobox gene 10, integrin β_3, and LIF messenger RNA (mRNA) and protein levels were significantly higher in the raloxifene-treated animals compared with the clomiphene citrate group (all $P < .05$) but not significantly different from saline group values, except for LIF and integrin β_3 mRNA levels ($P < .05$). Pinopodes were abundant and well developed in the raloxifene and saline groups; however, in the clomiphene citrate-treated mice, fewer and poorly developed pinopodes were obtained. In mice, raloxifene had no effect on HOXA10, integrin β_3, and LIF expression as well as pinopode production, suggesting it has no adverse effects on endometrial receptivity. Raloxifene may provide a viable alternative oral ovulation induction agent to clomiphene citrate.

Keywords

raloxifene, homeobox A10, integrin β_3, leukemia inhibitory factor, endometrial receptivity

Introduction

An ovulation disorder is a common cause of female infertility affecting around a quarter of couples seeking fertility treatment.[1] Eighty-five percent of the affected women have hypothalamic pituitary dysfunction or are eugonadotropic, predominantly due to polycystic ovary syndrome (PCOS).[2] Ovulation induction therapy with clomiphene citrate is the main method for treating ovulation disorders.[1] Clomiphene citrate belongs to the class of selective estrogen receptor modulators (SERMs) which bind to estrogen receptors with both estrogen agonist and antagonist effects in different tissues.[3] Selective estrogen receptor modulators include clomiphene citrate, tamoxifen, and raloxifene and have a wide range of clinical applications from breast cancer treatment to osteoporosis prevention.[3] Although clomiphene citrate has been a first line of pharmacologic ovulation induction for over 40 years, side effects such as antiestrogenic action on the endometrium and cervical mucous are relatively common, and it can also suppress endometrial receptivity.[4] Tamoxifen shows similar ovulation rates and pregnancy rates to clomiphene[5] but may be superior in that it does not appear to have an adverse impact

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upon the endometrium.[6] However, the International Cancer Research Group has listed tamoxifen as a human endometrial carcinogen.[7]

Raloxifene, a second-generation SERM, has been approved for the prevention and treatment of postmenopausal osteoporosis as well as for the reduction in invasive breast cancer risk in women.[8] Raloxifene has also been approved for increasing follicular phase follicle-stimulating hormone and serum estradiol levels.[9] Recently, a prospective randomized study revealed similar ovulation rates between raloxifene- and clomiphene citrate-treated patients having PCOS,[10] with raloxifene showing fewer adverse effects on endometrial receptivity in an in vitro study.[11]

Embryo implantation represents a critical step during the reproductive process, and there is a specific time during which implantation is possible,[12] known as the "implantation window." In rodents and humans, the development of pinopodes closely coincides with the endometrial implantation window,[13-15] and fully developed pinopodes may be a morphological marker for the implantation window.[16,17] Women with decreased implantation show few or no pinopode.[18] Interestingly, pinopode number is reduced in the clomiphene citrate-treated cycles.[19] In mice, scanning electron microscopy (SEM) shows that pinopodes appear on day 4 of normal pregnancy, regressing within just 1 day.[20] The 3 stages of pinopode formation include development, full development, and degradation.[21]

Appearance of pinopodes is consistent with the expression of other markers of endometrial receptivity in humans. Homeobox gene 10 (HOXA10) regulates proliferation, differentiation, and decidualization of the endometrium, playing an important role in endometrial receptivity and embryo implantation. Expression of HOXA10 increases drastically in the midluteal phase of the menstrual cycle corresponding to the time of implantation.[22] In adult female HOXA10-deficient mice, implantation is severely compromised, and defective decidualization leads to recurrent pregnancy loss and infertility.[23] In patients with infertility due to implantation defects, such as endometriosis and PCOS, a defect has been found in the HOXA10 expression pattern.[24,25] Other important markers of endometrial function include leukemia inhibitory factor (LIF), a cytokine involved in the implantation cascade, and integrin β_3 involved in cell adhesion.[26] Leukemia inhibitory factor is involved in the regulation of multiple processes leading to and during implantation, including uterine transformation into a receptive state, decidualization, blastocyst growth and development, embryo–endometrial interaction, trophoblast invasion, and immune modulation.[27] Dissociation of integrin β_3 from focal adhesions is thought to allow embryo invasion, with high levels also assisting embryo attachment.[28] In mice, integrin β_3 expression peaks on day 5 of pregnancy, and if blocked, implantation is significantly inhibited.[29]

To the best of our knowledge, there is no report investigating the effect of raloxifene on endometrial receptivity in mice. The purpose of this study was to compare the effects of raloxifene and clomiphene citrate on the expression of HOXA10, integrin β_3, and LIF as well as pinopode production on uterine epithelium in mice undergoing ovulation induction and natural cycles.

Materials and Methods

Animals

Male and female Kunming mice were obtained from the Animal Experimental Center of Guangdong province (China). Thirty-six female and 12 male mice (20-25 g, 8 weeks old) were provided standard mouse chow and water ad libitum and housed in a temperature-controlled room at 25 $\pm$ 0.5°C, with a relative humidity of 60% to 80% and a 12-hour light–dark cycle. Vaginal smears were monitored daily, and only mice showing 2 consecutive estrous cycles were used in the experiment. The animal experiments were approved by the Animal Care Laboratory of Southern Medical University (Guangzhou, China).

Ovarian Stimulation Regimen

The 36 female mice were randomly divided into normal saline solution (SS, n = 12), raloxifene (RAL, n = 12), and clomiphene citrate (CC, n = 12) groups. The random number generator tool of the Excel software (Microsoft, Redmond, Washington) was used to assign numbers for randomization. Animals of the RAL and CC groups were dosed according to a previous report.[10] On the proestrus stage of the 5-day estrous cycle, mice were daily administered raloxifene (22 mg/kg; Lilly, SA, Spain), clomiphene citrate (18 mg/kg; Medochemie Ltd, Cyprus), and 1 mL of normal saline solution. Substances were administered by gavage for 2 days. At 17:00 two days later, each female mouse was injected with human chronic gonadotropin 5 IU (Lizhu Co, China) and mated with a sexually mature male Kunming mouse. Successfully mated female mice were identified by the presence of vaginal plugs. The day of vaginal plug presentation was designated gestation day 1.

Tissue Collection

The pregnant mice were euthanized with ether at day 4.5, the implantation window in mice[30] and uterus samples were collected. The endometrial samples were divided into 3 parts. The first portion was fixed in 2.5% glutaraldehyde for SEM analyses (pinopode development in the endometrium). The second one was fixed in 10% formalin and embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The remaining portion was frozen in liquid nitrogen and immediately stored at −80°C for RNA isolation and Western blot analysis.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA from endometrial tissue samples was prepared with TRIzol (Invitrogen, Carlsbad, USA). RNA purity was determined by absorbance at 260 and 280 nm (A260/280). Total RNA was reverse transcribed into complementary DNA in a

Table 1. Primer Sequences Used in Quantitative Real-Time RT-PCR Analysis.

Gene	Sequence (5′-3′)	Annealing Temperature (°C)	Product Size (bp)
HOXA10	Sense: GGCAGTTCCAAAGGCGAAAAT Antisense: GTCTGGTGCTTCGTGTAAGGG	55	86
Integrin β3	Sense: CCCCGATGTAACCTGAAGGAG Antisense: GAAGGGCAATCCTCTGAGGG	55	166
LIF	Sense: GGGCAGTGGCTGACTACAGA Antisense: TTCCAGGCCTTTCCTAAGCC	55	183
GAPDH	Sense: TGTGTCCGTCGTGGATCTGA Antisense: TTGCTGTTGAAGTCGCAGGAG	55	150

Abbreviations: bp, base pair; LIF, leukemia inhibitory factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HOXA10, homeobox gene 10; RT-PCR, reverse transcription–polymerase chain reaction.

reaction primed by an oligodeoxynucleotide $(dT)_{18}$ primer using reverse transcriptase M-MLV (Takara, Japan) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction primers for HOXA10, integrin β3, and LIF were synthesized by Shanghai Bioengineering Inc (Shanghai, China; Table 1). Amplification was performed on an ABI ViiA 7 Detection System (Carlsbad, USA) and revealed with SYBR Premix Ex Taq (Takara, Japan). The cycling conditions were 45 cycles of 95°C for 30 seconds, 95°C for 3 seconds, and 60°C for 34 seconds. Data were analyzed using the ViiA 7 software, and threshold cycle (C_t) values were determined. $2^{-\Delta\Delta Ct}$ was calculated to represent the relative messenger RNA (mRNA) expression of target genes.[31]

Western Blotting

Tissue samples were lysed with lysis buffer (#P1003; Beyotime Biotechnology, Jiangsu, China), containing a protease inhibitor cocktail (Merck, Germany), on ice for 15 minutes. After centrifugation at 12 000*g* for 5 minutes at 4°C, the resulting supernatant was collected for Western blot analysis. Protein concentrations were determined by the bicinchoninic acid protein assay (Thermo Fisher Scientific Inc, MA, USA). Then, 30 μg of total protein samples were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts). After blocking in 0.1% phosphate-buffered saline (PBS) and Tween 20 (PBST) containing 5% non-fat milk on a shaker for 1 hour, the membranes were incubated overnight at 4°C, with primary antibodies raised against HOXA10 (ab23392), LIF (ab113262), and integrin β_3 (ab75872; 1 μg/mL, 1:1000 dilution; Abcam, Cambridge, Britain) and rabbit anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AP0063; Bioworld Technology Inc, MN, USA) as an internal control. The blots were incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (BS12478; Bioworld Technology Inc, MN, USA) and washed 5 times with PBST. Bound antibodies were detected by electrochemiluminescence using SuperSignal West Dura substrates (Pierce Biotechnology Inc, MA, USA). Band intensity was quantified using the Image J software (NIH, USA). Relative protein expression was normalized to GAPDH.

Immunohistochemical Analysis

Tissue specimens were fixed for 4 hours in 10% formalin, embedded in paraffin, sectioned at 5 μm, and mounted on silane-coated slides. For immunohistochemistry, sections were dewaxed and rehydrated through descending grades of alcohol to distilled water, followed by incubation in 2% hydrogen peroxide. Subsequently, nonspecific binding was blocked with goat serum (Fuzhou Maixin Biotechnology, China) for 2 hours, followed by incubation overnight at 4°C with primary antibodies raised against HOXA10, integrin β_3, and LIF (Abcam, Cambridge, Britain; 1:400 dilution). Following 3 washes in PBS, the sections were incubated with HRP-conjugated goat anti-rabbit secondary antibody (Fuzhou Maixin Biotechnology, China) for 1 hour at room temperature. Immunoreactive sites were visualized with diaminobenzidine and mounted for bright-field microscopy (DMLB; Leica Microsystems, Germany). Negative control sections were incubated with 1% bovine serum albumin in place of the primary antibody. The reactivity in the endometrial glands and luminal surface epithelium of the uterine, stromal cells, and vessels were assessed. The intensity of staining of endometrial components was evaluated by 2 independent investigators blinded to grouping using a semiquantitative scoring system (0-3) as follows[4]: absent (0), weak or focal (+), moderate (++), and strong (+++).

Scanning Electron Microscopy

Glutaraldehyde-fixed (2.5%) samples were thoroughly washed with PBS buffer, dehydrated in a graded ethanol, placed in 2% isoamyl alcohol for 3 hours, and underwent critical point drying. The samples were attached to the sample stage for observation with the surface (endometrial cavity surface) up and painted with silver conductive plastic using a vacuum coating apparatus for coating metal samples. Then, the samples were observed under the JSM-6330F SEM (JEOL, Japan) and assessed by 2 independent SEM experts blinded to the grouping.

According to the literature,[32] scanning electron micrographs of pinopodes were graded semiquantitatively as follows: scanning photomicrographs were obtained from 12 randomly selected areas of each tissue specimen and saved as TIFF files

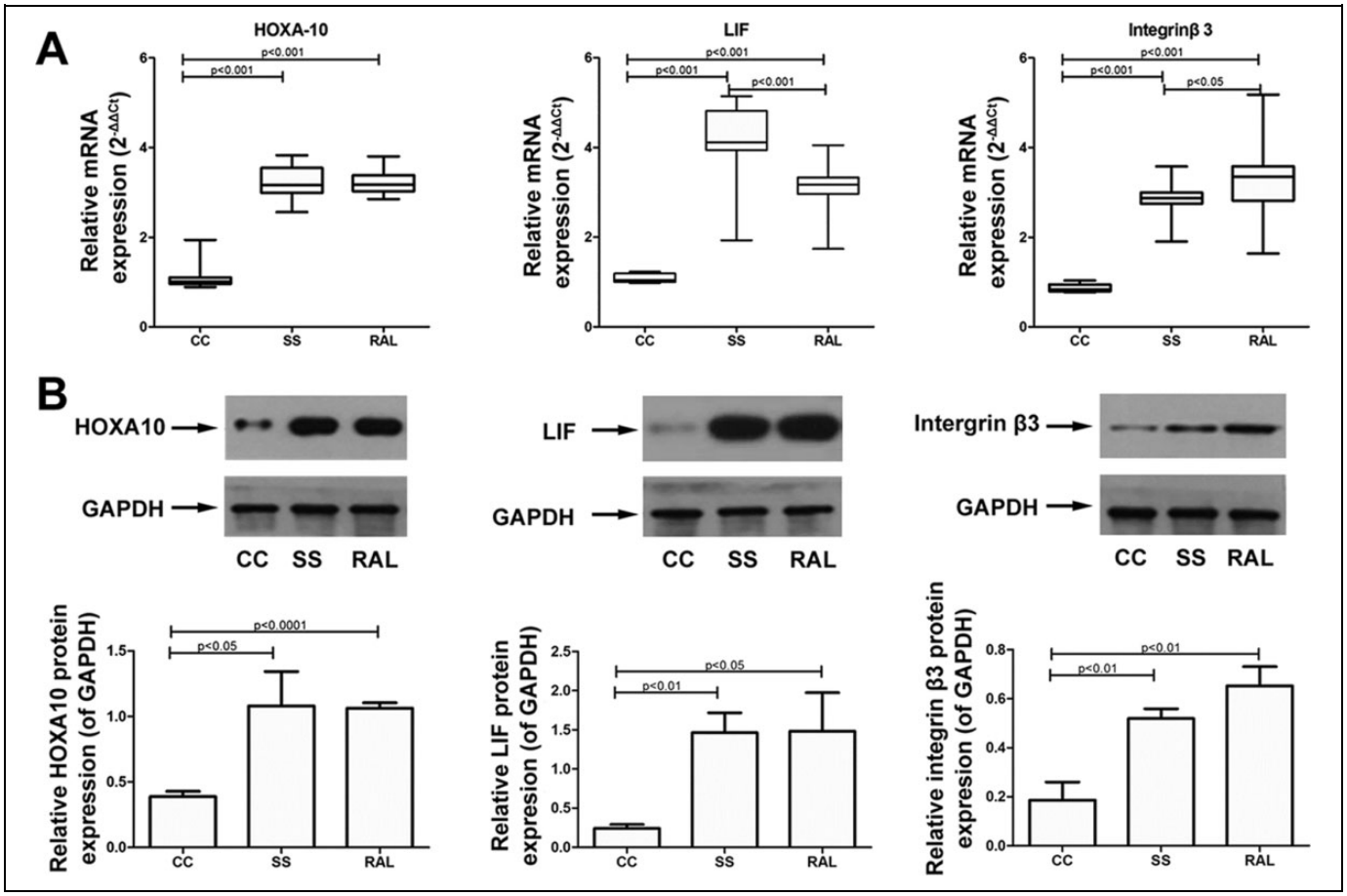

Figure 1. Homeobox gene 10 (HOXA10), integrin β_3, and leukemia inhibitory factor (LIF) messenger RNA (mRNA) and protein expression in uterine epithelial tissues from pregnant mice stimulated with raloxifene (RAL), clomiphene citrate (CC), and normal saline solution (SS). A, Homeobox gene 10, integrin β_3, and LIF mRNA amounts were evaluated by quantitative real-time reverse-transcriptase polymerase chain reaction. Data are median $\pm$ interquartile range (IQR; n = 12). B, Homeobox gene 10, integrin β_3, and LIF protein levels were evaluated by Western blot. Data are mean $\pm$ standard deviation (SD; n = 12 each group). Glyceraldehyde-3-phosphate dehydrogenase was used as an inner control.

for later viewing. Pinopodes were defined as smooth apical protrusions from the surface epithelium without microvilli. Pinopode production was scored as follows: 0 (absence of pinopodes), 1 (covering <25% of the surface epithelium), 2 (covering 25%-50% of the surface epithelium), or 3 (covering >50% of the surface epithelium). A single score was assigned to the most representative photomicrograph among the 12 images obtained for each tissue specimen, and mean scores for all specimens were calculated.

Statistical Analysis

Data were analyzed with the SPSS statistical software (version 19.0, IBM, New York, USA). Normally distributed data were expressed as mean $\pm$ standard deviation (SD) and compared using 1-way analysis of variance with least significant difference as the post hoc test. Nonnormally distributed data were expressed as median $\pm$ interquartile range and compared using the Kruskal-Wallis H test. Categorical data were frequencies and were analyzed using Fisher exact or χ^2 test, as appropriate. A 2-tailed $P < .05$ was considered statistically significant.

Results

Homeobox Gene 10, Integrin β_3, and LIF mRNA and Protein Expression in Uterine Epithelial Tissues

The median mRNA levels of HOXA10, integrin β_3, and LIF were all significantly lower in the CC group compared with the SS and RAL groups (1.10 $\pm$ 0.28, 0.86 $\pm$ 0.88, 1.08 $\pm$ 0.09 vs 3.21 $\pm$ 0.37, 2.86 $\pm$ 0.39, 4.10 $\pm$ 0.84 and 3.23 $\pm$ 0.26, 3.33 $\pm$ 0.85, 3.09 $\pm$ 0.53, respectively, $P < .001$; Figure 1A). No significant difference was found in the expression of HOXA10 mRNA between the SS and the RAL groups; however, significant differences in the expression levels of LIF and integrin β_3 were found between these groups ($P < .001$ and $P < .05$, respectively).

Similar results were obtained at the protein level, as demonstrated by Western blot. As shown in Figure 1B, protein expression levels of HOXA10, integrin β_3, and LIF were significantly higher in the SS and RAL groups compared with the CC-treated mice ($P < .05$), and no significant differences were found in HOXA10, integrin β_3, and LIF protein amounts between the SS and RAL groups.

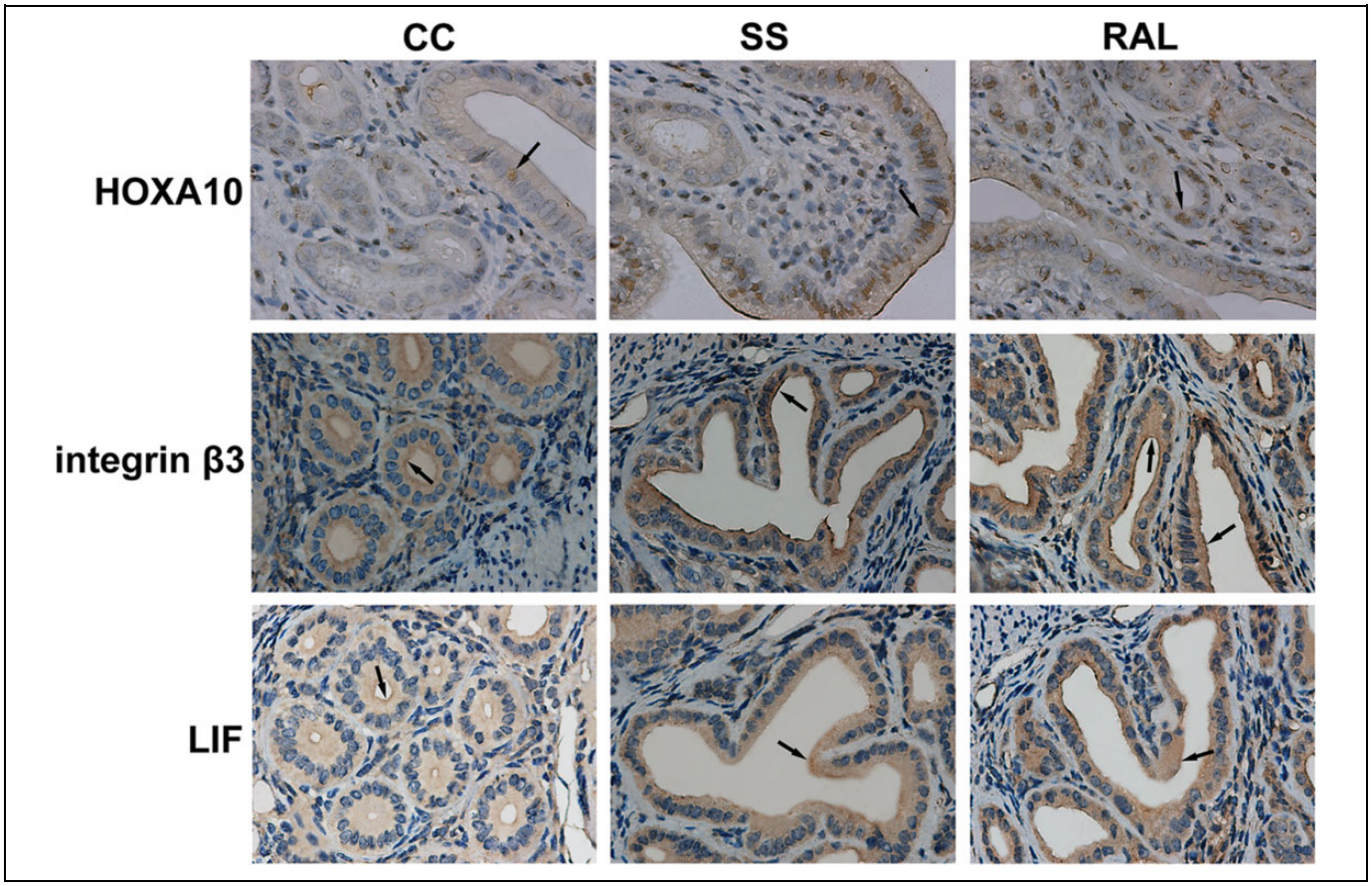

Figure 2. Homeobox gene 10 (HOXA10), integrin β_3, and leukemia inhibitory factor (LIF) protein expression in the uterine epithelial tissues in pregnant mice stimulated with raloxifene (RAL), clomiphene citrate (CC), and normal saline solution (SS). Homeobox gene 10, integrin β_3, and LIF protein expression levels were determined by immunohistochemistry (magnification ×400). Homeobox gene 10 was mainly expressed in the nucleus of endometrial luminal and glandular epithelial cells. Integrin β_3 and LIF were mainly found in the cytoplasm of both the endometrial luminal and glandular epithelial cells. Arrows: positive staining.

Immunohistochemistry of HOXA10, Integrin β_3, and LIF in Uterine Epithelial Specimens

Homeobox gene 10 was mainly expressed in the nucleus of endometrial luminal epithelial cells and glandular epithelial cells. Meanwhile, integrin β_3 and LIF were mainly expressed in the cytoplasm of both the endometrial luminal epithelial cells and the glandular epithelial cells (Figure 2). As shown in Table 2, the expression levels of HOXA10, integrin β_3, and LIF in the CC group were lower compared with the RAL and SS groups (all $P < .01$), and the RAL and SS groups showed similar expression levels (all $P > .05$).

Table 2. Expression Levels of HOXA10, Integrin β_3, and LIF Proteins in the Uterine Epithelium Estimated by Immunohistochemistry.[a]

	Moderate and Strong Positive Staining (++/+++), n (%)		
Group	HOXA10	Integrin β_3	LIF
CC (n = 12)	2 (16.7)	2 (16.7)	3 (8.3)
SS (n = 12)	12 (100.0)[b]	12 (100.0)[b]	12 (100.0)[b]
RAL (n = 12)	12 (100.0)[b]	12 (100.0)[b]	12 (100.0)[b]

Abbreviations: CC, clomiphene citrate; LIF, leukemia inhibitory factor; HOXA10, homeobox gene 10; RAL, raloxifene; SS, normal saline solution.
[a]The SS, RAL, and CC groups were evaluated.
[b]$P < .01$ versus CC group.

Histopathology of the Endometrial Samples

Hematoxylin and eosin staining showed mature endometrium in the RAL and SS groups, with loose interstitial space and large glandular cavity, rich in blood vessels and complex glands. Meanwhile, CC-treated mice showed an endometrium with a denser interstitial space, fewer blood vessels, and poorly developed glands, with smaller glandular cavity and less secretions in the glandular cavity (Figure 3).

Production of Pinopodes in Endometrial Tissues

Scanning electron micrographs of pinopodes were graded semiquantitatively (Figure 4) in tissue samples. The amounts of well-formed pinopodes were significantly higher in the RAL group compared with the CC group ($P < .001$). The SS group also had significantly more well-formed pinopodes compared

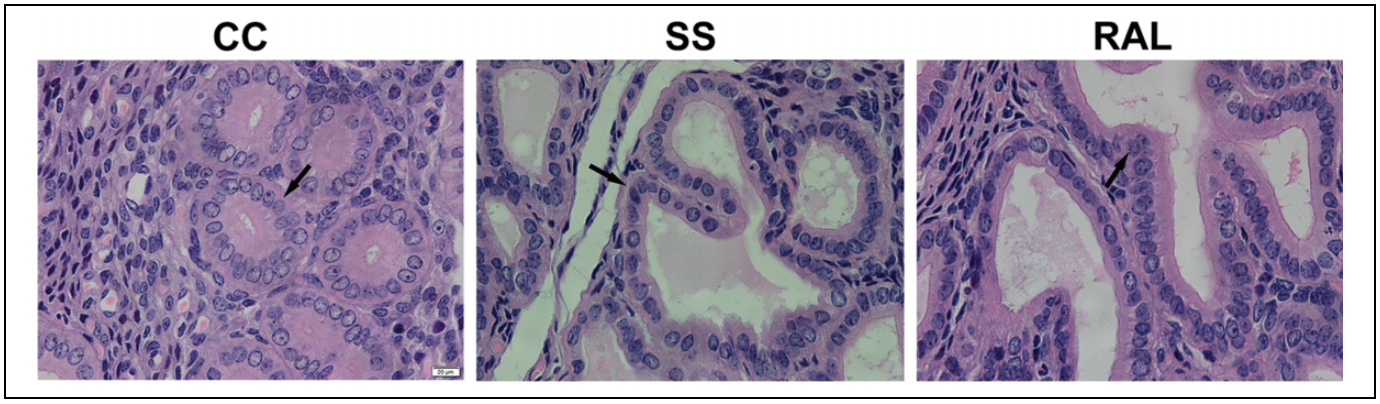

Figure 3. Histopathology of uterine epithelial tissues from pregnant mice stimulated with raloxifene (RAL), clomiphene citrate (CC), and normal saline solution (SS). Histopathology was determined by hematoxylin and eosin (H&E) staining (magnification ×400). Arrows: endometrial glands. Mature endometrium was found in the RAL and SS groups, with loose interstitial space and large glandular cavity, rich in blood vessels and complex glands, whereas CC-treated mice showed an endometrium with a denser interstitial space, fewer blood vessels, and poorly developed glands, with smaller glandular cavity and less secretions in the glandular cavity.

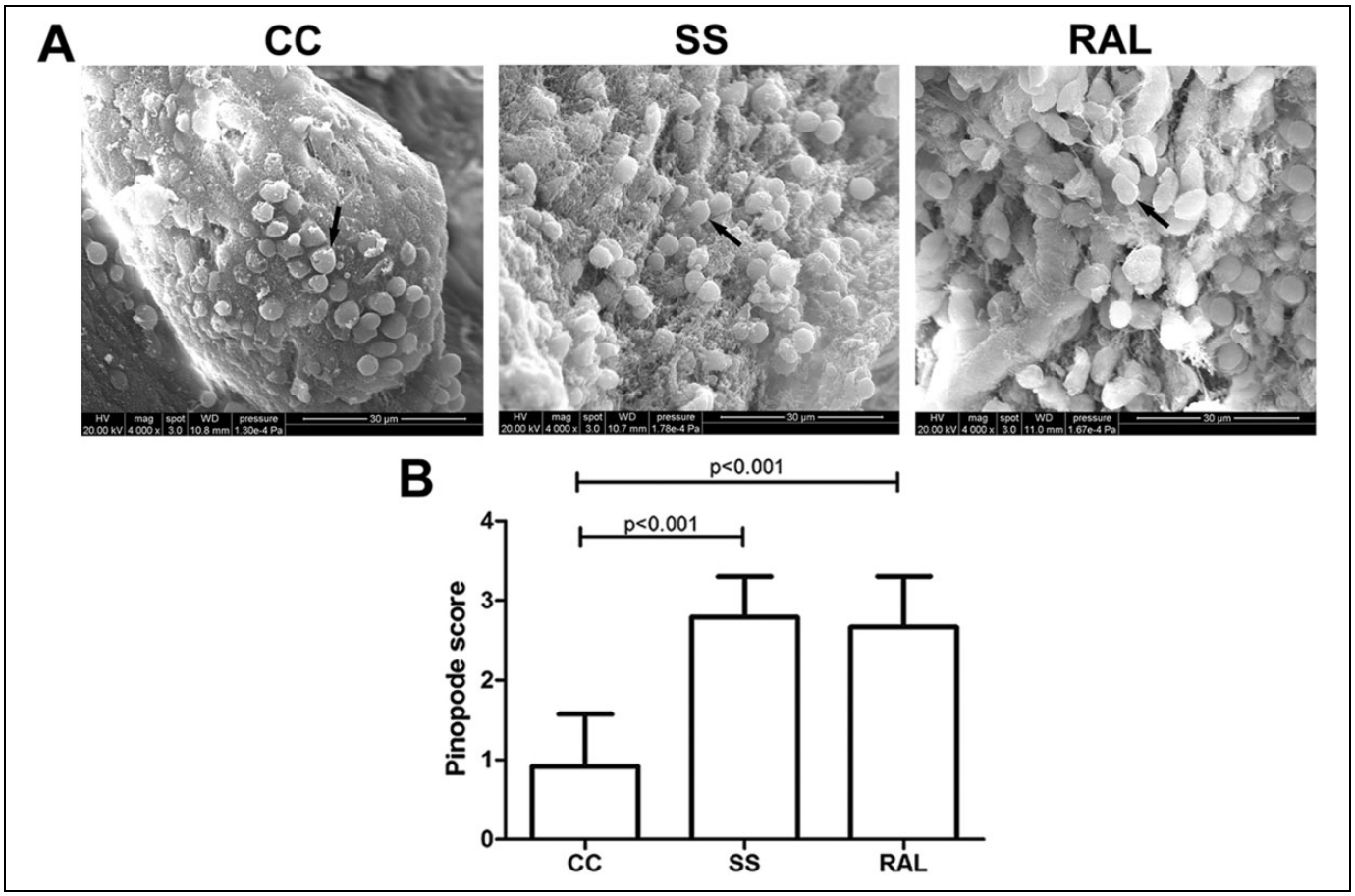

Figure 4. Pinopodes in uterine epithelial tissues from pregnant mice stimulated with raloxifene (RAL), clomiphene citrate (CC), and normal saline solution (SS). A, Pinopodes were determined by scanning electron microscopy (magnification ×4000). Arrows point to well-developed pinopodes. B, Scanning electron micrographs of pinopodes were graded semiquantitatively. Data are mean ± standard deviation (SD; n = 12).

with the CC group ($P < .001$). However, pinopode densities were comparable between the RAL and SS groups.

Discussion

Multiple studies, prospective and retrospective, have focused on clomiphene citrate and tamoxifen for ovulation induction. However, it was not until 2011 that the first prospective, randomized trial carried out a comparison between raloxifene and clomiphene citrate for ovulation induction.[10] To date, no study has reported the effects of raloxifene on makers of endometrial receptivity in mice, such as pinopodes, HOXA10, integrin β_3, and LIF. As clomiphene citrate may have some antiestrogenic effects in the endometrium,[4] this study aimed to compare the effects of raloxifene and clomiphene citrate, used to induce ovulation, on endometrial receptivity markers in mice during the implantation window, including HOXA10, integrin β_3, LIF, and pinopodes. We found that HOXA10, integrin β_3, LIF, and pinopodes were present in the endometrium of mature female mice, and compared with those mice undergoing natural cycles, clomiphene citrate significantly reduced the amounts of these 4 markers. However, raloxifene did not cause these adverse effects.

Our study shows for the first time pinopode production in the endometrium during the implantation window in mice after administration of raloxifene and clomiphene citrate. Interestingly, pinopodes were abundant and well developed in raloxifene- and saline solution-treated animals. However, in clomiphene citrate-treated cycles, the pinopodes were fewer and poorly developed. This finding indicates that raloxifene has no side effects on the endometrium.

We also demonstrated that HOXA10 expression was significantly higher in the RAL group compared to the CC-treated animals, with no statistically significant difference between the RAL and SS groups. This suggests that raloxifene is deprived of harmful effects on endometrial receptivity, while benefiting embryo implantation. These data corroborate another study that compared letrozole and clomiphene citrate for ovulation induction in rats and found that clomiphene citrate resulted in lower levels of HOXA10, suggestive of suppression of endometrium receptivity.[4]

We also examined integrin β_3 and LIF levels during the implantation window in mice. Consistent with the expression of HOXA10, integrin β_3 and LIF levels were significantly higher in the RAL and SS groups compared with the CC group, with no significant difference between the RAL and SS groups at the protein level, although a significant difference was observed at the gene level. This finding indicated that raloxifene may slightly influence integrin β_3 and LIF. These findings differ from a previous study reporting no adverse effect of clomiphene citrate on LIF expression in patients with unexplained infertility.[33] However, integrin β_3 results corroborate a previous report.[4]

Compared with clomiphene citrate, raloxifene had less effects on the amounts of endometrial receptivity markers, such as pinopodes, HOXA10, integrin β_3, and LIF. These findings might be explained by the different biological characteristics of raloxifene and clomiphene citrate. Raloxifene is an estrogen receptor antagonist or has a neutral effect on endometrial cells in vitro.[34,35] Both raloxifene and clomiphene citrate share a similar mechanism for ovulation induction and show similar estrogen receptor antagonist activity in the endometrium; however, the long half-life of clomiphene citrate (5 days),[36] in comparison with raloxifene (32.5 hours),[37] may play a role, since the latter would be more readily cleared. If raloxifene is administered for 5 days from days 3 to 7 of the menstrual period, raloxifene treatment for ovulation will hardly affect endometrium proliferation and show no adverse effects on endometrial receptivity.

Overall, in this experimental study comparing the effects of raloxifene and clomiphene citrate on markers of endometrial receptivity in mice, raloxifene was better than clomiphene citrate. These results suggest that raloxifene may be a promising oral ovulation induction drug, representing a viable alternative to clomiphene citrate, which can suppress endometrial receptivity. However, the molecular mechanisms underlying the effect of raloxifene on human endometrial receptivity and its safety on the embryo need to be further explored. These are the next steps of our research.

Declaration of Conflicting Interests

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References

1. Wilkes S, Murdoch A. Ovulation induction with clomifene: a primary care perspective. *J Fam Plann Reprod Health Care*. 2012; 38(1):48-52.
2. Brown J, Farquhar C, Beck J, Boothroyd C, Hughes E. Clomiphene and anti-oestrogens for ovulation induction in PCOS. *Cochrane Database Syst Rev*. 2009;(4):cd002249.
3. Goldstein SR, Siddhanti S, Ciaccia AV, Plouffe L Jr. A pharmacological review of selective oestrogen receptor modulators. *Hum Reprod Update*. 2000;6(3):212-224.
4. Bao SH, Sheng SL, Peng YF, Lin QD. Effects of letrozole and clomiphene citrate on the expression of HOXA10 and integrin alpha v beta 3 in uterine epithelium of rats. *Fertil Steril*. 2009; 91(1):244-248.
5. Boostanfar R, Jain JK, Mishell DR Jr, Paulson RJ. A prospective randomized trial comparing clomiphene citrate with tamoxifen citrate for ovulation induction. *Fertil Steril*. 2001;75(5): 1024-1026.
6. Deligdisch L. Effects of hormone therapy on the endometrium. *Mod Pathol*. 1993;6(1):94-106.

7. Ismail SM. Pathology of endometrium treated with tamoxifen. *J Clin Pathol*. 1994;47(9):827-833.
8. Silverman S, Christiansen C. Individualizing osteoporosis therapy. *Osteoporos Int*. 2012;23(3):797-809.
9. Baker VL, Draper M, Paul S, et al. Reproductive endocrine and endometrial effects of raloxifene hydrochloride, a selective estrogen receptor modulator, in women with regular menstrual cycles. *J Clin Endocrinol Metab*. 1998;83(1):6-13.
10. de Paula Guedes Neto E, Savaris RF, von Eye Corleta H, de Moraes GS, do Amaral Cristovam R, Lessey BA. Prospective, randomized comparison between raloxifene and clomiphene citrate for ovulation induction in polycystic ovary syndrome. *Fertil Steril*. 2011;96(3):769-773.
11. Lessey BA, Palomino WA, Apparao KB, Young SL, Lininger RA. Estrogen receptor-alpha (ER-alpha) and defects in uterine receptivity in women. *Reprod Biol Endocrinol*. 2006;4(suppl 1):s9.
12. Boron WF, Boulpaep EL. *Medical physiology. A cellular and molecular approach*. 2nd ed. Oxford, PA: Saunders/Elsevier; 2009.
13. Nikas G, Develioglu OH, Toner JP, Jones HW Jr. Endometrial pinopodes indicate a shift in the window of receptivity in IVF cycles. *Hum Reprod*. 1999;14(3):787-792.
14. Garcia-Velasco J, Simon C, Ardiles G, Neuspiller F, Remohi J, Nikas G. Assessment of endometrial response to HRT using the detection of pinopods in women with endometriosis undergoing oocyte donation. *Fertil Steril*. 1998;70:365.
15. Kolb BA, Najmabadi S, Paulson RJ. Ultrastructural characteristics of the luteal phase endometrium in patients undergoing controlled ovarian hyperstimulation. *Fertil Steril*. 1997;67(4): 625-630.
16. Quinn C, Ryan E, Claessens EA, et al. The presence of pinopodes in the human endometrium does not delineate the implantation window. *Fertil Steril*. 2007;87(5):1015-1021.
17. Pierson RA. Imaging the endometrium: are there predictors of uterine receptivity? *J Obstet Gynaecol Can*. 2003;25(5):360-368.
18. Bagot CN, Kliman HJ, Taylor HS. Maternal Hoxa10 is required for pinopod formation in the development of mouse uterine receptivity to embryo implantation. *Dev Dyn*. 2001;222(3):538-544.
19. Creus M, Ordi J, Fabregues F, et al. The effect of different hormone therapies on integrin expression and pinopode formation in the human endometrium: a controlled study. *Hum Reprod*. 2003;18(4):683-693.
20. Parr MB, Parr EL. Endocytosis in the uterine epithelium of the mouse. *J Reprod Fertil*. 1977;50(1):151-153.
21. Aghajanova L, Stavreus-Evers A, Nikas Y, Hovatta O, Landgren BM. Coexpression of pinopodes and leukemia inhibitory factor, as well as its receptor, in human endometrium. *Fertil Steril*. 2003;79(suppl 1):808-814.
22. Eun Kwon H, Taylor HS. The role of HOX genes in human implantation. *Ann N Y Acad Sci*. 2004;1034:1-18.
23. Benson GV, Lim H, Paria BC, Satokata I, Dey SK, Maas RL. Mechanisms of reduced fertility in Hoxa-10 mutant mice: uterine homeosis and loss of maternal Hoxa-10 expression. *Development*. 1996;122(9):2687-2696.
24. Taylor HS, Bagot C, Kardana A, Olive D, Arici A. HOX gene expression is altered in the endometrium of women with endometriosis. *Hum Reprod*. 1999;14(5):1328-1331.
25. Cermik D, Selam B, Taylor HS. Regulation of HOXA-10 expression by testosterone in vitro and in the endometrium of patients with polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2003;88(1):238-243.
26. Lindhard A, Bentin-Ley U, Ravn V, et al. Biochemical evaluation of endometrial function at the time of implantation. *Fertil Steril*. 2002;78(2):221-233.
27. Salleh N, Giribabu N. Leukemia inhibitory factor: roles in embryo implantation and in nonhormonal contraception. *ScientificWorldJournal*. 2014;2014:201514.
28. Kaneko Y, Lecce L, Day ML, Murphy CR. beta(1) and beta(3) integrins disassemble from basal focal adhesions and beta(3) integrin is later localised to the apical plasma membrane of rat uterine luminal epithelial cells at the time of implantation. *Reprod Fertil Dev*. 2011;23(3):481-495.
29. Liu N, Zhou C, Chen Y, Zhao J. The involvement of osteopontin and beta3 integrin in implantation and endometrial receptivity in an early mouse pregnancy model. *Eur J Obstet Gynecol Reprod Biol*. 2013;170(1):171-176.
30. Zorn TM, Soto-Suazo M, Pellegrini CR, Oliveira JG, Stumpf WE. Estradiol receptor binding to the epithelium of uterine lumen and glands: region- and time-related changes during preimplantation and periimplantation periods studied by autoradiography. *Histochem Cell Biol*. 2003;120(1):1-12.
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.
32. Usadi RS, Murray MJ, Bagnell RC, et al. Temporal and morphologic characteristics of pinopod expression across the secretory phase of the endometrial cycle in normally cycling women with proven fertility. *Fertil Steril*. 2003;79(4):970-974.
33. Kuscu NK, Koyuncu FM, Var A, Lacin S, Uyanik BS, Ceylan E. Clomiphene citrate does not adversely affect endometrial leukemia inhibitory factor levels. *Gynecol Endocrinol*. 2002;16(2): 151-154.
34. Punyadeera C, Kamps R, Defrere S, et al. Effects of selective oestrogen receptor modulators on proliferation in tissue cultures of pre- and postmenopausal human endometrium. *J Steroid Biochem Mol Biol*. 2008;112(1-3):102-109.
35. Blauer M, Heinonen PK, Rovio P, Ylikomi T. Effects of tamoxifen and raloxifene on normal human endometrial cells in an organotypic in vitro model. *Eur J Pharmacol*. 2008;592(1-3):13-18.
36. Szutu M, Morgan DJ, McLeish M, et al. Pharmacokinetics of intravenous clomiphene isomers. *Br J Clin Pharmacol*. 1989; 27(5):639-640.
37. Heringa M. Review on raloxifene: profile of a selective estrogen receptor modulator. *Int J Clin Pharmacol Ther*. 2003;41(8): 331-345.