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GnRH antagonists may affect endometrial receptivity

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Abstract

Study objective—HOXA10 is an essential regulator of endometrial receptivity. To determine the effect of gonadotropin releasing hormone (GnRH) antagonists on endometrial receptivity we assessed endometrial HOXA10 expression in GnRH antagonist, GnRH agonist, and natural cycles.

Design—Prospective case-control study

Setting—University academic medical center

Patients—Nineteen subjects were included: 12 subjects underwent controlled ovarian hyperstimulation (COH) with recombinant follicle stimulating hormone (rFSH) and used either a GnRH antagonist or a GnRH agonist; 7 control subjects underwent natural cycles.

Interventions—Pipelle endometrial biopsies were obtained 11 days after human chorionic gonadotropin (hCG) administration or spontaneous luteinizing hormone (LH) surge in untreated cycles, respectively. Immunohistochemistry was used to assess HOXA10 protein expression in endometrial glands and stroma.

Main outcome measure(s)—Endometrial HOXA10 protein expression

Results—HOXA10 expression was significantly decreased in endometrial stromal cells in GnRH antagonist treated cycles compared with GnRH agonist treated cycles or natural cycle controls. There was no significant difference in glandular cell HOXA10 expression among the three groups.

Conclusions—Use of GnRH antagonists may be associated with impaired HOXA10 expression in endometrial stromal cells, and thus may affect endometrial receptivity.

KEY WORDS

endometrial receptivity; GnRH antagonist; GnRH agonist; HOXA10; implantation

Introduction

Controlled ovarian hyperstimulation (COH) with recombinant follicle stimulating hormone (rFSH) is commonly used in the treatment of infertility. Gonadotropin releasing hormone (GnRH) analogs, both agonists and antagonists, are often employed to prevent a premature luteinizing hormone (LH) surge and subsequent ovulation. While a regimen of these medications can result in increased oocyte production, implantation rates remain relatively

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low; the majority of human embryos fail to implant (1,2). Endometrial receptivity is an essential part of implantation success, and it is crucial to determine the effect of these medications on the endometrium.

The effect of COH on implantation remains controversial (3–7). FSH and GnRH receptors have been identified in the endometrium, thus it is possible that gonadotropins and GnRH analogues may have a direct or indirect effect on the endometrium (8–10). Furthermore, high serum estradiol levels or other hormonal alterations that result from FSH stimulation may adversely affect the endometrium (5,7,11,12).

The effects of GnRH agonists and GnRH antagonists on endometrium and pregnancy rates have previously been investigated. Both medications are associated with advanced endometrial maturation of 2 to 4 days on the day of oocyte retrieval; no pregnancies occur when the advancement is greater than 3 days (13–15). However recently, endometrial development during cycles using GnRH antagonists were found to be histologically more similar to endometrium from natural cycles than endometrium exposed to GnRH agonists (16). Although most studies do not identify significant differences in clinical pregnancy rates with the use of GnRH antagonists (17,18), or with GnRH antagonists compared with GnRH agonists (19–21), several reviews and meta-analyses report an overall decrease in pregnancy rates with the use of GnRH antagonists compared with GnRH agonists (21–28).

Here we investigated the effect of GnRH antagonists on HOXA10, a well-characterized marker of endometrial receptivity (29,30). HOXA10 is a homeobox-containing transcription factor that regulates uterine development in the embryo as well as adult endometrial development during each menstrual cycle (29–33). HOXA10 expression is necessary for endometrial receptivity (30,33–36). Targeted mutation of HOXA10 renders mice infertile due to implantation failure: they produce viable embryos, and these embryos implant and develop normally in a wild-type surrogate, however wild-type embryos fail to implant in HOXA10 (–/–) mice (37). This phenotype is likely related to both the absence of HOXA10 during embryonic uterine development, and lack of adult maternal HOXA10 expression during cyclic endometrial development. Reduction of HOXA10 expression in mice using HOXA10 antisense results in diminished implantation proportional to the level of HOXA10 expression, indicating that altered levels of this protein regulates the degree of endometrial receptivity (35).

In the midluteal phase at the time of implantation, HOXA10 mRNA expression is up-regulated in both endometrial glandular and stromal cells in humans (30,38). HOXA10 has diverse effects on several aspects of adult endometrial development such as stromal decidualization, leukocyte infiltration, and pinopod development (34,39). Furthermore, HOXA10 regulates downstream target genes that are also involved in implantation such as β_3 integrin, EMX2, and IGFBP-1 (40–42). Defective endometrial HOXA10 expression has been described in association with endometriosis, polycystic ovary syndrome (PCOS), and hydrosalpinges, conditions associated with abnormal implantation (32,43,44). Thus the present study aimed to determine if GnRH antagonists affect HOXA10 expression, a well-characterized marker of endometrial receptivity and one of only a few genes known to be essential for implantation.

Materials and Methods

The study included 19 subjects: 12 subjects undergoing controlled ovulation stimulation with rFSH and a GnRH analogue, and 7 natural cycle controls. The study was approved by the Yale University School of Medicine Human Investigation Committee. Treated subjects were involved in an oocyte donation program. All subjects participated on a voluntary basis. Average age for the oocyte donors was 27 years (range 24–32 years), and average age for the control

subjects was 36 years (range 28 – 43 years). Subjects used no medications except for rFSH, GnRH antagonist, GnRH agonist, and human chorionic gonadotropin (hCG), and had no history of endometrial or other uterine disease.

All study subjects underwent ovarian stimulation with rFSH, and dosage was individualized as clinically indicated. Six subjects were treated with the GnRH agonist leuprolide acetate 0.5 mg daily starting on day 21 of the luteal phase, and once suppressed, COH commenced. The GnRH antagonist group started rFSH on cycle day 3, and once the lead follicle reached 13 mm diameter, 0.25 mg of GnRH antagonist was administered daily until hCG administration. For both COH groups, hCG was administered when the lead follicle reached 18 mm diameter. Pipelle endometrial biopsies were performed 11 days after hCG administration. Controls consisted of women in natural cycles, and endometrial biopsies were performed between cycle days 21 to 25 based on LH surge monitoring. Endometrial dating was confirmed based on the criteria of Noyes *et al.* (45). All endometrial biopsy samples were evaluated with immunohistochemistry (6 GnRH antagonist, 6 GnRH agonist, and 7 control samples) to assess HOXA10 protein expression.

Endometrial tissue was fixed in formalin, embedded in paraffin, cut into 5- μ m sections, and mounted onto slides. Slides were deparaffinized and dehydrated through a series of xylene and ethanol washes, followed by permeabilization in 95% cold ethanol. After a 5 minute rinse in distilled water, an antigen-presenting step was performed by steaming the slides in 0.01M sodium citrate buffer for 20 minutes, followed by cooling for 20 minutes. Slides were rinsed for 5 minutes in PBS with 0.1% Tween 20 (PBST), and sections were circumscribed with a hydrophobic pen. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 minutes followed by a 5 minute PBST wash. Nonspecific binding was blocked with 1.5% normal horse serum in PBST for 1 hour at room temperature. Slides were incubated in the primary antibody overnight at 4°C. HOXA10 antibody (sc-17159) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Normal goat IgG (Santa Cruz Biotechnology) was used as a negative control for HOXA10 antibody.

Biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). Horse α -goat secondary antibody was applied for 1 hour at 4°C. Slides were washed in PBST, incubated in ABC Elite (Vector Laboratories) for 15 minutes at room temperature, washed in PBST, and incubated for 5 minutes in diaminobenzidine (Vector Laboratories). A 20 second exposure to hematoxylin was used as a counterstain. Slides were rehydrated through 3 minute ethanol and xylene washes and mounted with Permount. Slides from the GnRH antagonist and GnRH agonist treated cycles were processed simultaneously.

The HOXA10 immunohistochemistry results were quantified by three evaluators blinded to treatment regimen. An H-SCORE was determined separately for the glandular cells and stromal cells from 4 high-powered fields on each slide. The H-SCORE was calculated with the following equation: $H\text{-SCORE} = \sum P_i (i + 1)$. Intensity (i) of HOXA10 nuclear staining is indicated by a value of 1, 2, or 3 (weak, moderate, or strong respectively), and P_i is the percentage of stained nuclei for each intensity, ranging from 0 – 100% (46,47). The three H-SCORE results for the glands from each slide were averaged, and the three H-SCORE results for the stroma were averaged. The GnRH antagonist, GnRH agonist, and control group glandular and stromal cell H-SCORES were compared using the Kruskal-Wallis One Way Analysis of Variance on Ranks test with Dunn's multiple-comparison procedure.

Results

The effect of controlled ovarian hyperstimulation with concomitant GnRH antagonist use on endometrial HOXA10 expression, a marker endometrial receptivity, was investigated. Subjects

consisted of 12 oocyte donors who underwent COH with concomitant use of a GnRH antagonist (n = 6) or GnRH agonist (n = 6), and 7 natural cycle controls. Peak serum estradiol levels were not significantly different between the 11 GnRH agonist and GnRH antagonist groups (2300 mIU/mL versus 2850 mIU/mL, p = 0.47). Similarly, progesterone levels did not differ between the two groups. Serum LH levels were significantly higher in the GnRH agonist group (1.6 mIU/mL) compared to the GnRH antagonist group (0.3 mIU/mL, p < 0.01).

Immunohistochemistry was employed to evaluate HOXA10 expression in the endometrial glands and stroma cell compartments. Representative immunohistochemistry results are shown in Figure I. HOXA10 expression in the endometrial glands and stroma was quantified, and H-SCORES were determined (Table I). There were no significant differences in HOXA10 expression in the glandular cells between any of the three groups (p = 0.213). However, a significant difference in HOXA10 expression was identified in stromal cells between the three groups (p = 0.003); there was significantly less HOXA10 expression in the stromal cells of subjects treated with GnRH antagonist (mean H-SCORE = 1.50 ± 0.18) compared with the stromal cells in the GnRH agonist group (mean H-SCORE = 2.51 ± 0.12) and with the untreated group (mean H-SCORE = 2.31 ± 0.07) (p < 0.05). Thus immunohistochemistry identified a significant decrease in HOXA10 protein expression in the endometrial stromal cells of women treated with GnRH antagonists.

Discussion

Controlled ovarian hyperstimulation is an effective means to produce multiple oocytes, and subsequently multiple high quality embryos, however the effect of the involved medications on endometrial receptivity remains controversial (3,7,14,26). GnRH agonists, and more recently GnRH antagonists, are often used during COH to prevent premature ovulation.

Women undergoing COH cycles using GnRH antagonists produce fewer follicles, thus fewer oocytes and embryos, and when a standardized number of embryos are transferred, lower implantation and pregnancy rates have been observed (25). One meta-analysis of 5 prospective randomized controlled trials (2 using cetrorelix and 3 using ganirelix) compared a fixed GnRH antagonist protocol to the long luteal GnRH agonist protocol, and identified significantly fewer clinical pregnancies per cycle with the GnRH antagonist protocol (OR 0.77, 95% CI 0.61, 0.96) (22). While none of the individual studies showed a significant difference in clinical pregnancy rates between the two protocols, each trial revealed a consistently lower pregnancy rate with the GnRH antagonist protocol. Similarly, Ludwig *et al.* performed a meta-analysis to evaluate pregnancy rates with GnRH antagonist compared with GnRH agonist and identified an overall lower pregnancy rate per cycle with the antagonist protocol (OR 0.82, 95% CI 0.68, 0.99) (27).

The effect of GnRH antagonists on implantation appears to be dose-responsive. High doses of ganirelix (0.5, 1, 2 mg) have been connected with low implantation rates (17,25). Implantation rates were highest in the 0.25 mg group (21.9%), and lowest in the 2 mg group (1.5%), and the higher doses were associated with higher miscarriage rates (17). In contrast, when embryos were cryopreserved after an ovulation stimulation cycle in which high-dose GnRH antagonists were used and later thawed and transferred, the implantation and pregnancy rates were unaffected by the use of GnRH antagonist during the initial stimulation cycle (20,25). These data suggest an effect of GnRH antagonists on the endometrium and hence, an effect on endometrial receptivity.

Little is known about the effect of GnRH antagonists on the endometrium, therefore we investigated the effect of GnRH antagonist use on endometrial receptivity by evaluating HOXA10 protein expression in endometrial glands and stroma. Endometrial stromal cell

HOXA10 protein expression was significantly decreased in cycles using GnRH antagonist compared with cycles using GnRH agonist or natural cycle controls. No difference was noted in glandular cell HOXA10 protein expression among the three groups. Our results identify a potential differential effect of GnRH antagonists on individual endometrial cellular components by demonstrating a negative impact on stromal cells, and no similar effect on glandular cells. This finding may explain the trend towards a lower rate of clinical pregnancies in cycles using GnRH antagonists compared with cycles using GnRH agonists.

In contrast to our results, one recent study demonstrated that endometrial development in oocyte donors treated with a GnRH antagonist was more similar to endometrium from natural cycles than the endometrium from GnRH agonist cycles (16). These investigators used a gene microarray to detect global changes in gene expression, however they reported 2-fold or greater differences in gene expression and would not have detected a small but significant difference in key regulatory genes such as HOXA10 (C. Simon and J. Horcajadas, personal communication).

GnRH antagonists may have a direct or indirect negative effect on the endometrium that could affect implantation (21,22,26). Currently there is no evidence that GnRH antagonists negatively impact oocyte quality, fertilization rates, or embryo quality (21,24,28). Recombinant FSH, GnRH agonist, and GnRH antagonist do not directly alter HOXA10 mRNA expression *in vitro*; this implies that the effect of GnRH antagonists is not directly on the endometrium, but may be due to altered hormone levels (36).

Despite the impact on endometrial receptivity, there are significant advantages to using GnRH antagonists instead of GnRH agonists. GnRH agonists are initiated in the luteal phase and cause an initial stimulatory effect before acting to down-regulate the GnRH receptors prior to gonadotropin stimulation, and are associated with hypoestrogenic side effects and greater gonadotropin stimulation requirements (23). In comparison, GnRH antagonists act by competitive blockade of pituitary GnRH receptors and cause rapid suppression of FSH and LH. The advantages of GnRH antagonists include a rapid dose-dependent effect, the lack of an initial stimulatory effect, shorter duration of treatment with fewer symptoms of estrogen deprivation, a decrease in gonadotropin requirement for stimulation, and lower risk of developing severe ovarian hyperstimulation syndrome (18,19,21–24,27,28).

The use of GnRH antagonists impaired HOXA10 expression in endometrial stromal cells, and thus may affect endometrial receptivity. However, GnRH antagonists have been shown to be effective, safe, and the therapeutic benefits may outweigh any negative impact, thus these medications should continue to have a role in controlled ovarian stimulation. The impact on endometrial receptivity is likely indirect, suggesting the potential to counter this effect by endocrine means. In conclusion, these results support a molecular basis for the lower pregnancy rates seen clinically with GnRH antagonist use. A greater understanding of GnRH antagonist effects on the endometrium, and further studies investigating other markers of implantation, will help to determine the optimal use of these medications.

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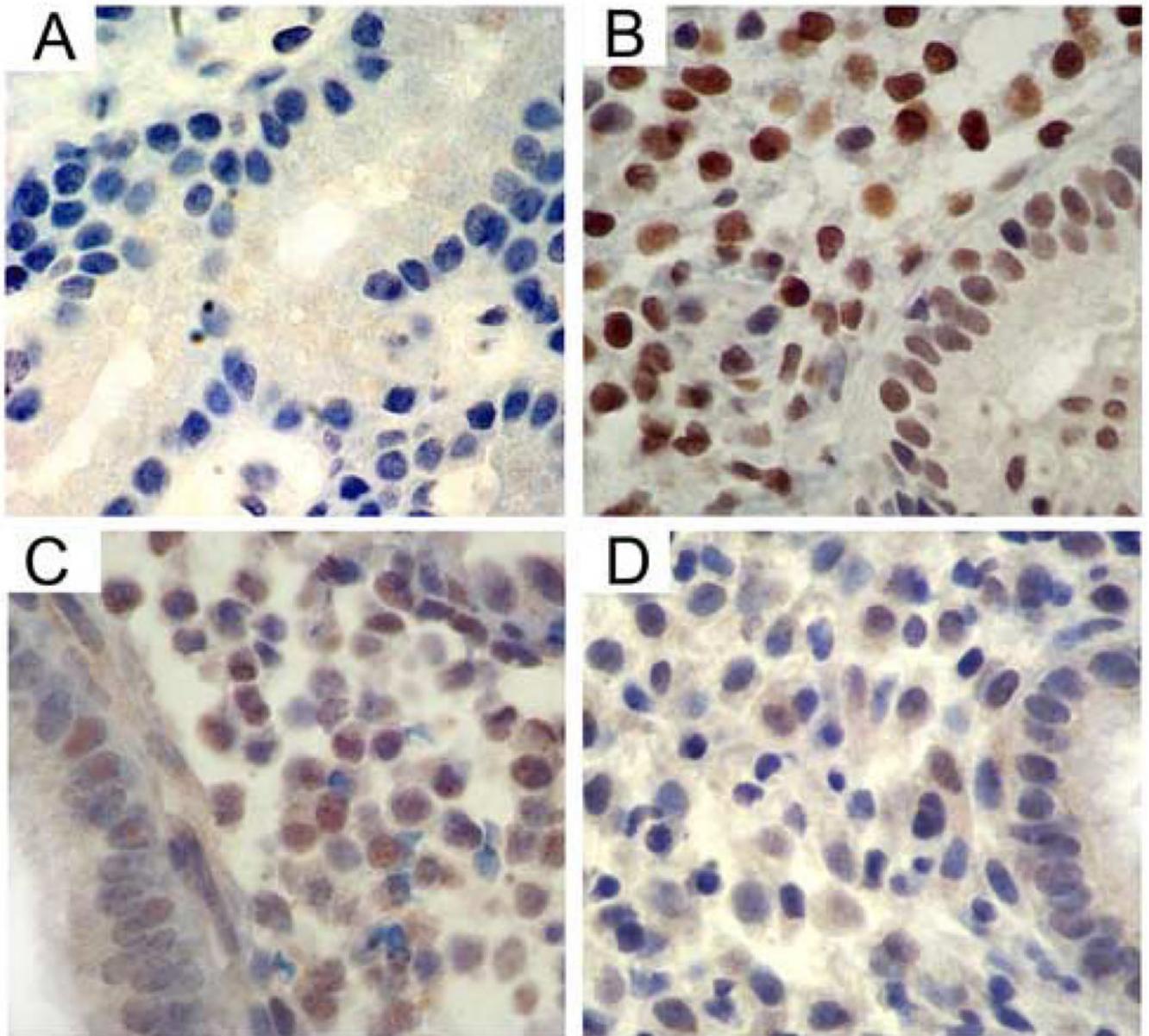


Figure I. Endometrial HOXA10 Immunohistochemistry

Immunohistochemistry identified HOXA10 protein expression in endometrial glands and stroma. H-SCOREs were determined separately for the glands and stroma. Shown are representative photomicrographs demonstrating HOXA10 expression in endometrium: (A) HOXA10 negative control omitting primary antibody, (B) Natural cycle control, (C) Subject treated with GnRH antagonist, (D) Subject treated with GnRH agonist.

Table 1HOXA10 immunohistochemistry mean H-SCOREs \pm standard error.

	Antagonist	Agonist	Control
Glands	0.59 \pm 0.10	0.67 \pm 0.19	0.84 \pm 0.09
Stroma	1.50 \pm 0.18 ^a	2.51 \pm 0.12	2.31 \pm 0.07

^aP < 0.05 for HOXA10 protein expression in GnRH antagonist treated cycles compared with GnRH agonist treated cycles or with controls.