Effect of GnRH antagonist therapy on the expression of MUC-1 and heparin binding growth factor expression in the endometrium of hyperstimulated rats

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Summary

Purpose: To determine the effect of GnRH-antagonist therapy on the expression of heparin binding-epidermal growth factor (HB-EGF) and MUC-1 glycoprotein in hyperstimulated rat ovaries. *Methods:* 30 female Wistar rats were divided into three groups (control, FSH and FSH+cetrorelix). Control rats were given 0.2 ml oil/saline mixture for four days beginning from the day of estrus. In the second group, 30 IU/ml purified hFSH was injected SC for four days beginning from the day of estrus. The rats of the third group were injected 30 IU FSH for four days and 10 IU cetrorelix SC for three or four days. The rats were sacrificed and the staining intensity of HB-EGF and MUC-1 of the epithelial cells and stromal cells of the endometrium of the rats was calculated by H-score. *Results:* Slight MUC-1 immunoreactivity was seen in the epithelial and decidual cells of the control and FSH groups. In the FSH+cetrorelix group, moderate MUC-1 immunostaining appeared in the epithelial and desidual cells. In rats in the control and FSH+cetrorelix groups, HB-EGF immunoreactivity in the epithelial cells and decidual cells was moderate. Strong immunoreactivity was seen in the FSH group. When the MUC-1 H-score values were compared statistically with the control and other groups, FSH+cetrorelix immunoreactivity in epithelial and decidual cells were significantly different from control and FSH groups. HB-EGF immunoreactivity of the epithelium and decidua was similar in the control and FSH+cetrorelix groups, but epithelial and decidual immunoreactivity of the FSH group was different from the other two groups. *Conclusion*: Our findings suggest that GnRH antagonists exert direct effects on the expression of HB-EGF and MUC-1 expression in the rat endometrium.

Key words: GnRH-antagonist; MUC-1; HB-EGF; Rat endometrium.

Introduction

Implantation is a very complex sequence of events that leads to an intimate association of the blastocyst with the endometrium. To date, some bilogical markers such as cytokines, growth factors, pinopodes, calcitonin, and HOX genes are found to serve as the link in the regulation of molecules that provide the physical contact between embryo and uterus. Because impaired uterine receptivity is one of the major reasons for the failure of assisted reproductive techniques (ART) and the phenomena of implantation and trophoblast invasion is currently considered as the major limiting factor for the establishment of pregnancy, the identification of biological markers of endometrial receptivity may have a prominent clinical significance by improving implantation rates in natural and ART cycles.

The crucial role of steroid hormones to prepare and drive the endometrium for successful embryonic implantation is beyond any doubt. Over the last two decades, gonadotropin-releasing hormone (GnRH) agonists have been used in ovarian stimulation protocols in ART in combination with gonadotrophins to prevent a premature LH surge. GnRH agonists induce an initial rise of gonadotropins (flare-up) before they achieve supression through desensitization. Recently, GnRH antagonists have been introduced into ovarian stimulation protocols. However, there are conflicting results reported in implantation and pregnancy rates of the patients of who were stimulated with GnRH-antagonists as compared with patients who were stimulated with agonists [1]. Some reported lower implantation and pregnancy rates in the antagonist groups as compared with the agonist [2-7] while some others found no difference [8].

It has been postulated that cytokines, growth factors and cell adhesion molecules produced by the uterine mucosa may play a role in maternal-embryonic interaction, enhancing endometrial receptivity by controlling the expression of adhesion and anti-adhesion proteins [9].

In this study, we determined the effect of GnRH-antagonist therapy on the expression of heparin-binding epidermal growth factor (HB-EGF) and MUC-1 glycoprotein which were among the main growth factors and cell adhesion molecules in hyperstimulated rat ovaries.

Materials and Methods

Animals

Thirty intact adult female Wistar rats (200-250 g) were included in the study, which presented at least four regular 4-day estrous cycles as determined by daily vaginal smears. Animals were maintained under a 12:12 h light:dark cycle with food and water available ad libitum. The study was approved by the local ethical committee of Celal Bayar University Hospital, Manisa, Turkey, and carried out at the Animal Research Laboratory of

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the University Hospital. All procedures were in accordance with the recommendations of the Declaration of Helsinki on care and use of animals. The rats were randomly divided into three equal groups (control, FSH and FSH+cetrorelix). Control rats were given 0.2 ml oil/saline mixture four days beginning from the day of estrus. In the second group, purified FSH (Gonal-f; Serono, Aubonne, Switzerland) was dissolved at a concentration of 30 IU/ml and injected SC on the fourth day beginning from the day of estrus.

The rats of the third group, FSH and cetrorelix (Cetrotide, Serono, Aubonne, Switzerland) were injected SC with 30 IU FSH for four days and 10 IU cetrorelix for only three or four days. The rats were caged with the males overnight and examined for a vaginal plug the next morning. The presence of the plug determined the day (0.5) of pregnancy. The rats were sacrificed on gestation day 5.5. Then the abdomen was carefully opened and access to the uterus was gained by pushing intestinal tissue to the side. The uterus was then removed by surgical cuts at the cervix and ovaries. Uteri were dissected and immediately fixed with 10% formaldehyde for 24-48 h. Tissues were dehydrated through a series of increasing ethanol concentrations and finally cleared with xylene. Tissues were then embedded in paraffin serial tissue sections (5 micron thick) and cut and mounted on poly-l-lysine coated slides.

Immunohistochemistry

After deparaffination at 60°C overnight, sections were held in xylene for one hour. After washing with serial concentrations of ethanol (95%, 80%, 70%, and 60% for 2 min each), sections were washed with distilled water and phosphate-buffered saline (PBS) for 10 min. They were held in 2% trypsin in Tris buffer at 37°C for 15 min, and then washed in PBS (3-5 min). The limits of sections were drawn with a Dako pen (S-2002; Dako, Carpinteria, CA) and incubated in 3% hydrogen peroxide for 15 min to inhibit the endogenous peroxidase activity. The uterine tissues were then given a 3-5 min wash in PBS. The primary antibodies, polyclonal anti-MUC-1 in a 1/100 dilution (sc-6827; Santa Cruz, CA) and anti-HB-EGF in a 1/100 dilution (sc-1414; Santa Cruz, CA, USA) were incubated for 18 hours. They were then given an additional 3-5 min wash in PBS, followed by incubation with biotinylated anti-rabbit immunoglobulin G (IgG) and administration of streptavidin-peroxidase (Histostain Plus kit Zymed 87-9999; Zymed, San Francisco, CA). After washing the secondary antibody with PBS three times for 5 min, the sections were washed for 5 min in Dako DAB substrate system containing diaminobenzidine to detect the immunoreactivity, and then stained with Mayer's hematoxylin. They were covered with a mounting medium (catalog no. 1012; Signet Laboratories, Dedham, MA) and observed with light microscopy (Olympus BX-40). Control samples were processed in an identical manner, but in the absence of the primary antibody. Two observers, blind to the clinical information of the samples, independently evaluated the staining scores. Staining intensity was assigned according to a semiquantitative immunohistochemical scoring system as follows: absent (-), weak (+), moderate (++), and strong (+++).

Measurement of H-score

The staining intensity of the epithelial cells and stromal cells was calculated by H-score using the following equation: H-score = $\sum$Pi(i + 1), where i = intensity of staining with a value of 1, 2, or 3, (weak, moderate, or strong, respectively) and Pi is the percentage of stained alveolar cells for each intensity, varying from 0% to 100%.

Statistical analyses

Data were expressed as the mean ± SD. Statistical analysis was done with the ANOVA test using SPSS for Windows release 10.0; p values less than .05 were considered significant.

Results

Slight MUC-1 immunoreactivity was seen in the epithelial and decidual cell of the control group (Figure 1a) and FSH group (Figure 1b). In the FSH+cetrorelix group, moderate MUC-1 immunostaining appeared in the epithelial and decidual cells (Figure 1c). In rats belonging to the control group (Figure 2a) and FSH+cetrorelix (Figure 2c), HB-EGF immunoreactivity in the epithelial cell and decidual cells was moderate. Strong immunoreactivity was seen in the FSH group (Figure 2b).

When the MUC-1 H-score values were compared statistically with the control and other groups, FSH+cetrorelix immunoreactivity in epithelial (14.400 ± 2.591) and decidual (13.000 ± 3.559) cells were significantly different from the control (epithelium 5.600 ± 1.713; decidua 7.800 ± 2.486) and FSH groups (epithelium 5.556 ± 1.236; decidua 6.083 ± 1.443) ($p < 0.01$). HB-EGF immunoreactivity of the epithelium and decidua was similar in the control group (14.300 ± 2.058; 14.200 ± 1.874, respectively) and FSH+cetrorelix group (13.200 ± 2.150; 14.100 ± 1.912, respectively), but epithelial (24.400 ± 2.066) and decidual (23.200 ± 3.293) immunoreactivity of the FSH group were different from the other two groups ($p < 0.01$) (Table 1).

Table 1. — *Mean H-SCORE for MUC-1 and HB-EGF in control, FSH and FSH+cetrorelix groups*.

	MUC-1		HB-EGF	
	Epithelium	Decidua	Epithelium	Decidua
Control	5.600 ± 1.713	7.800 ± 2.486	14.300 ± 2.058	14.200 ± 1.874
FSH	5.556 ± 1.236	6.083 ± 1.443	24.400 ± 2.066**	23.200 ± 3.293**
FSH+ cetrorelix	14.400 ± 2.591**	13.000 ± 3.559**	13.200 ± 2.150	14.100 ± 1.912

Anova statistical test was used to compare the staining intensities.
Statistical data are shown as mean ± SEM. **= $p < 0.001$.

Discussion

In some clinical studies, lower implantation and pregnancy rates were recorded in the antagonist groups as compared with the agonist in ovulation induction cycles [2-6]. Recently, two meta-analyses evaluating the efficacy of GnRH antagonists in comparison with the long agonist protocol were published. In the first Ludwig *et al*. [6] did not show any significant difference in pregnancy rates between the two groups, whereas in a more recent meta-analysis, Al-Inany and Aboulghar [7] found lower pregnancy rates in the antagonist compared with the agonist group. Since cetrorelix was not detectable in serum and follicular fluid during oocyte retrival and embryo transfer when small doses (0.1 and 0.5 mg) were used [8], Tarlatsis *et al*. [1] revealed that in IVF treatment, the risk of embryo exposure to the antagonist is minimal. Morever, the implantation and pregnancy rates after the

Fig. 1a

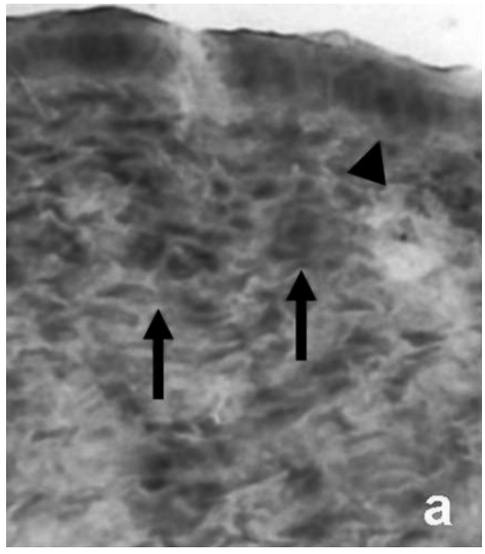

Fig. 1b

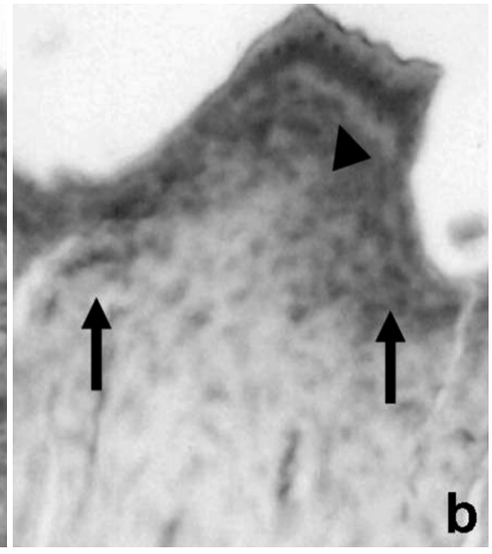

Fig. 1c

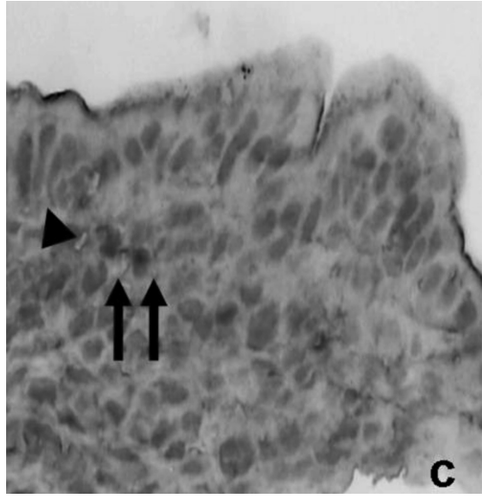

Figure 1. — Immunohistochemical staining of MUC-1 in the epithelial cells (arrowhead) and decidual cell (arrows) endometrium of both the control (a) and FSH groups (b) show slight staining. The FSH+cetrorelix group (c) shows moderate staining intensity of epithelial cells (arrowhead) and decidual cells (arrows) (original magnification x 400).

transfer of frozen-thawed pronuclear oocytes did not differ between the agonist and antagonist group [10]. Kolibianakis *et al*. [11] searched for a possible explanation for the lower pregnancy rate, of which GnRH antagonists could have had an adverse effect on endometrium quality. They found that the endometrial maturation at oocyte retrival was more advanced by around 2.5 days after the use of GnRH antagonists, as compared with the expected chronological date. The endometrial maturation was more advanced with higher luteinizing hormone levels at the beginning of stimulation and increased duration of recombinant follicle stimulating hormone (rFSH) stimulation before the antagonist initiation.

Here, we examined the effect of GnRH-antagonist therapy on the expression of implantation markers, HB-EGF and MUC-1 glycoprotein, in the hyperstimulated rat ovary. HB-EGF belongs to the epidermal growth factor family, which interacts with the EGF receptor [12]. Tamada *et al*. [13] examined the effects of the possible involvement of HB-EGF in the initiation of implantation in rats. They intraluminally injected anti-HB-EGF antiserum into uterine horns and found that the number of implantation sites per rat was lower in the anti-HB-EGF-treated group than in controls. Furthermore, HB-EGF induced implantation in the delayed implanting rats. HB-EGF was also found to be expressed in human endometrium at the time of implantation [14, 15]. These results suggest that HB-EGF participates in the implantation process in rats. In this study, we found that HB-EGF immunoreactivity of the epithelium and decidua was similar in the control group and FSH+cetrorelix group, but epithelial and decidual immunoreactivity of the FSH group was stronger than the two other groups. Lessey *et al*. [15] demonstrated that 17-β estradiol and progesterone, alone or in combination, stimulate the expression of HB-EGF in stromal cells. This report could explain the reason for higher HB-EGF staining scores in the FSH only group in our study.

Fig. 2a

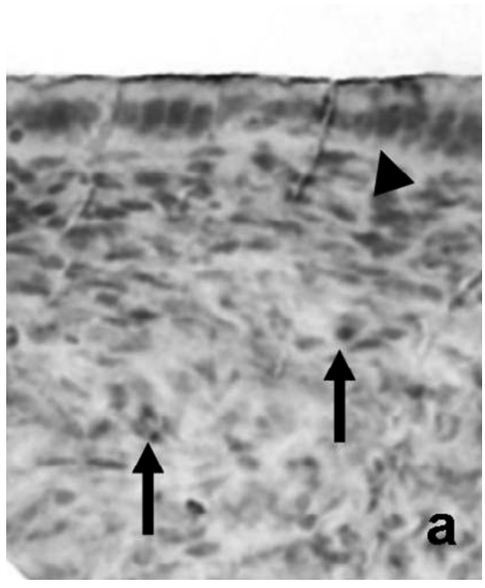

Fig. 2b

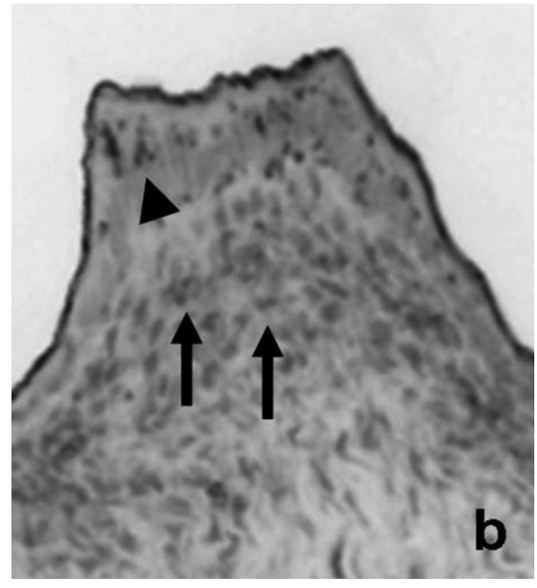

Fig. 2c

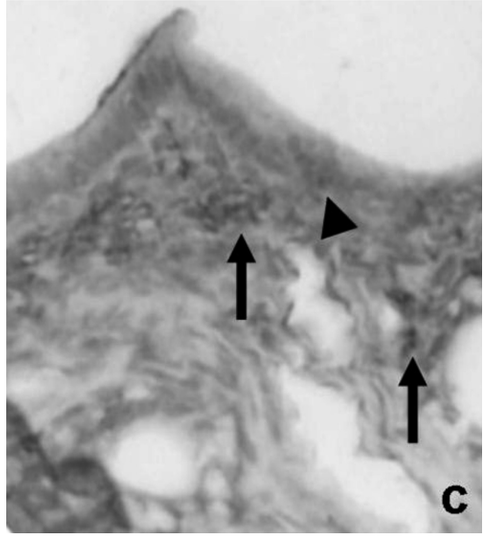

Figure 2: Immunohistochemical staining of HB-EGF in the endometrium of both the control (a) and FSH+cetrorelix group (c) show moderate epithelial cell (arrowhead) and decidual cell (arrows) staining. The FSH group (b) shows strong staining intensity of epithelial cells (arrowhead) and decidual cells (arrows) (original magnification x 400).

During the implantation window period, the human endometrium and the embryo express adhesion molecules which contribute to blastocyst attachment into the uterine mucosa [16]. Among these molecules, there are mucins and integrins [17]. Some authors studied these in mice and revealed that MUC-1 possibly acts as an anti-adhesion molecule during embryo attachment since it disappears on days 4-5, corresponding to the attachment phase of implantation [18, 19]. In humans, Bergh and Navot [20] reported that MUC-1 expression is high one week after ovulation, the moment that implantation would be expected. Meseguer *et al*. [21] showed that the expression of MUC-1 in the endometrium varies during the menstrual cycle. Cavagna *et al*. [22] revealed that MUC-1 possibly acts as a barrier to implantation, which must be removed to allow the blastocyst to interact with the endometrium, but the actual role of MUC-1 in human implantation remains to be determined. In this study, immunohistochemical staining of MUC-1 in the epithelial and desidual cells of endometrium of both the control and FSH groups showed slight staining while the FSH+cetrorelix group showed moderate staining intensity of epithelial and decidual cells of the endometrium.

The results of the current study do not give any certain explanation as to whether GnRH antagonists – which have recently been introduced in clinical practice – cause a lower pregnancy rate by affecting endometrium quality adversely. However, our findings suggest that GnRH antagonists exert direct effects on the expression of HB-EGF and MUC-1 expression in the endometrium, which have been shown to have significant roles during the implantation window and endometrium receptivity. Further investigations are needed to resolve this controversial phenomenon.

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