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Endometrial HOXA10 mRNA Expression in PCOS Patients at High Risk for Ovarian Hyperstimulation Syndrome

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ABSTRACT

Background: *This study was designed to determine whether the risk of ovarian hyperstimulation syndrome (OHSS) contributes to the subfertility in PCOS. Endometrial HOXA-10 mRNA expression, a well-characterized gene essential to endometrial receptivity, was evaluated in PCOS patients whose embryos are planned to be frozen due to the risk of OHSSS.*

Methods: *Twent-five women with PCOS in high risk group for OHSS and age and BMI matched 25 non-PCOS infertile patients were included the study. Five fertile women were accepted as positive control. Following egg collection each group of subject underwent total embryo freezing. After the egg collection, endometrial sampling was performed with a pipella cannula from each gruop of participant and fertile control. Expression levels of HOXA-10 mRNA were determined by RT-PCR. Gene expression results are presented as Ct (cycle threshold), ΔCt, and ΔΔCt.*

Reults: *Average ΔCt value of HOXA-10 mRNA in PCOS, non-PCOS and fertile groups were found to 5.88, 6.77, and 7.79 respectively. Compared to endometrial HOXA-10 mRNA levels of fertile cases, the HOXA-10 mRNA levels of the patients in the PCOS group were found to be significantly lower (ΔCt 7.79 vs. ΔCt 5.88, $p<0.002$). Similarly, endometrial HOXA-10 mRNA levels in the non-PCOS control group were significantly lower than the HOXA-10 mRNA levels in the fertile group (ΔCt 6.77 vs. ΔCt 7.79, $p<0.001$). HOXA-10 mRNA levels in endometrial samples taken from patients in the PCOS group were found to be significantly lower than the HOXA-10 mRNA levels in non-PCOS control group.*

Conclusions: *HOXA-10 mRNA levels were found to be lower in PCOS patients with high risk for OHSS compared to both fertile and infertile patients without PCOS.*

OHSS risk in PCOS decreases endometrial HOXA-10 mRNA expression.

Keywords

OHSS, PCOS, HOXA-10 mRNA, Endometrium.

Introduction

PCOS is a metabolic disease spectrum that is the most common in reproductive biology and is associated with subfertility. Besides endocine problems, many PCOS patients suffer from infertility. In most of the cases, it may be necessary to resort to assisted reproductive techniques (ART) in order for couples to have a baby. However, unlike other infertile patient groups, there is a risk of developing ovarian hyperstimulation syndrome (OHSS) during ART methods to be used in PCOS patients [1,2]. OHSS is a serious clinical picture characterized by the accumulation of fluid in the pelvis and abdominal cavity as well as the formation of multiple

eggs due to hormonal drugs used to stimulate the ovaries during invitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) treatment. It is a condition that causes more frequent and serious clinical findings especially in polycystic ovaries syndrome (PCOS) patients [2-4]. Women with a priori risk for development of OHSS are those with PCOS or a high antral follicle count. Due to fluid leakage from enlarged ovaries to the abdominal cavity, in PCOS patients, OHHS may progress to abdominal bloating, decreased renal perfusion, increased risk of coagulation, and deterioration in liver function [2,3]. To prevent OHSS in PCOS patients, either the IVF/ICSI procedure is canceled or all embryos are frozen. Postponing embryo transfer may prevent OHSS or cause it to progress less severe.

Homeobox genes are the leading gene sequences responsible for endometrial receptivity. They are expressed in both luminal and glandular cells of the endometrium [5]. HOXA-10 is a gene of this family and plays a role in growth, differentiation, implantation, and decidualization in endometrial cells [6]. The expression of this gene becomes dominant in the midluteal phase [7]. Studies showed that endometrial biopsies, obtained from women with PCOS, demonstrated decreased HOXA10 mRNA expression [8,9]. Senturk et al. [10] reported an increase in endometrial HOXA mRNA expression after ovarian drilling in PCOS patients. However, the PCOS participants in these studies consisted of patient groups who did not undergo IVF/ICSI. To date, there is no study showing that OHSS has a direct effect on endometrial receptivity. On the other hand, OHSS may lead to indirect changes in endometrial receptivity by causing increased steroid synthesis in the ovaries [11]. HOXA-10 mRNA levels have not been studied in the endometrium of PCOS patients who underwent total embryo freezing due to the risk of OHSS during IVF/ICSI. This study was designed to determine whether the risk of OHSS as well as hormonal changes due to IVF/ICSI contributes to the subfertility seen in PCOS. For this purpose, endometrial HOXA-10, a well-characterized gene essential to endometrial receptivity, was evaluated in PCOS patients whose embryos are planned to be frozen due to the risk of OHSS.

Materials and Methods

A total of 55 patients, including 25 PCOS, 25 non-PCOS infertile cases and 5 fertile cases, were included in the study. The inclusion of patients in the PCOS group was done in two stages. The cases should have been diagnosed with PCOS first according to the Rotterdam criteria. Women carrying at least two of the three Rotterdam criteria (oligomenorrhea or amenorrhea, clinical hyperandrogenism and/or hyperandrogenemia, and polycystic ovaries) were accepted as PCOS. The initial antral follicle count of PCOS cases was ≥20.

In the second stage, among the patients who were diagnosed with PCOS and decided to undergo IVF/ICSI, those with a high risk of OHSS were included in the study. Those with 18 or more growing follicles (>11 mm) during the controlled ovarian stimulation follow-ups and those with estradiol levels above 2500 pg/mL on the day of ovulation trigger were considered in the high risk group for OHSS [12], and the PCOS group of was selected among these patients. Patients in the control group were selected from infertile patients who did not have clinical and laboratory findings of PCOS, had normal or low AFC, and had similar age and BMI to the PCOS group. Thus, because the OHSS risk is eliminated due to control group selection criteria we had the opportunity to investigate the effects of only IVF/ICSI-related hormonal changes on HOXA-10, while in the PCOS group, we had the opportunity to research the effects of OHSS risk on HOXA-10 in addition to IVF/ICSI. Five patients who had two or more children and required biopsy due to an endometrial pathology were accepted as positive control group. Thus, we were able to compare the expression patterns of HOXA-10 mRNA levels in fertile cases and in infertile cases.

Since all PCOS participants were anovulatory they were subjected to progesterone induced withdrawal bleeding to begin ovarian stimulation. All menstrual cycles studied in the non-PCOS group were ovulatory according to serum progesterone levels. Women in PCOS and control groups were treated according to a standard antagonist protocol with individually dosed recombinant FSH starting on day 2–3 of the menstrual cycle. Gonadotrophin-releasing hormone antagonist was started on the 5th or 6th day of stimulation. When at least three follicles reached 18-20 mm in diameter, maturation of follicles was induced with GnRHa (Gonapeptyl, Ferring, 0.1 mg/mL, Kiel, Germany). Gonapeptyl was administered as 1x2 dose (0.2 mg/mL, sc) to trigger ovulation. Egg collection was performed 36 hours after agonist trigger. Ovarian follicles were aspirated using a single-lumen, 17-gauge needle (Cook Medical, Bloomington, IN, USA) guided by trans-vaginal ultrasonography. It was decided to freeze all embryos in PCOS group due to risk of OHSS. In the control group, embryos were totally freezed for reasons other than OHSS. We can list the reasons for total freeze in non-PCOS controls as follows; The presence of high progesterone level on the day of ovulation trigger in five cases, insufficient endometrial thickness in six cases, the presence of fluid collection or polyps on the day of OPU in four cases, transfer cancellation due to other health reasons in three cases, three cases of egg freezing due to decreased ovarian reserve, planning transfer after myomectomy in two cases and in two cases freezing until PGD results are obtained. Following the egg collection, endometrial sampling was performed with a pipella cannula from patients in both PCOS and control groups. In the fertile group, endometrial samples were taken in the midluteal phase. The endometrial tissues were washed with a sterile saline solution to remove blood and transferred into RNA stabilization buffer and stored for future analysis. Expression of HOXA-10 mRNA was determined by RT-PCR. Presence of uterine septum, antiphospholipid syndrome, diabetes or recurrent spontaneous abortion, uni- or bilateral hydrosalpinx, endometrioma, endometrial hyperplasia, endometritis and uterine synechiae were excluded from the study. Patients who received hormonal medication for six months or more were excluded from the study.

In addition to demographics characteristics of women in

PCOS and control group age, body mass index (BMI) (kg/m^2), total testosterone, fasting glucose, insulin, serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were also measured. Serum glucose levels were measured in autoanalyzer by using hexokinase/G6PD method. The fasting serum insulin levels were also measured in autoanalyzer by using electrochemiluminescence immunoassay. Homeostatic model assessment [HOMA-IR] Formula was used for calculating insulin resistance. The study was approved by the local Research Committee of Memorial Kayseri Hospital and all patients signed an informed consent before the inclusion.

RT-PCR

Endometrial samples from PCOS and non-PCOS participants were transferred into RNA stabilization buffer (RNA Later; Qiagen) and then stored at -80 C until used. Total RNA was extracted from decidua with the Rneasy Mini Kits (QIAGEN). RNA quantity and purity was measured spectrophotometrically with the use of the Maestronano. Complementary DNA (cDNA) was obtained with the use of the Quantitect Reverse Transcription Kit (Qiagen). β-Actin gene (ACTB) was used as housekeeping gene. Realtime PCR reaction was performed with the use of Quantitect Probe PCR Kit and the RotorgeneQ (Qiagen) realtime PCR device. The primer sequences are the following: HOXA-10 forward: F 5'-GGT TTG TTC TGA CTT TTT GTT TCT-3', R 5'-TGA CAC TTA GGA CAA TAT CTA TCT CTA-3'; ACTB forward: F 5'-GCA AGC AGG AGT ATG ACG AGT-3', R -5' -CAA GAA AGG GTG TAA CGC AAC TAA-3'. Gene expression results are presented as Ct (cycle threshold), ΔCt, and ΔΔCt. For calculation of average Ct values, each endometrial sample was studied three times. The relative gene expression was determined by means of the $2^{-\Delta\Delta Ct}$ comparative method with the use of RT2 Profiler PCR Array Data Analysis version 5.5 (SA Biosciences). All data were normalized according to mRNA of β-actin. Comparative RNA expression analysis in all groups versus the fertile control group (calibrator) was performed as follows:

Fertile group: ΔCt= Ct (target gene)-ΔCt (housekeeping gene ACTB).

PCOS/or non-PCOS: ΔCt=Ct (target gene)-ΔCt (housekeeping gene ACTB).

ΔΔCt=ΔCt (PCOS group)-ΔCt (fertile group).

Statistical analysis

The normality distribution of data was tested with the Kolmogorov-Smirnoff test. The continuous variables were analyzed by means of analysis of variance test with post hoc Tukey procedure and Mann-Whitney U test. The categoric data were analyzed by means of the Pearson chi-square test. The relationship between the average ΔCt values of HOXA-10 mRNA and other demographic, hormonal and reproductive parameters was evaluated by Spearman's correlations analysis. Data are presented as the means ± SD. A p value of <.05 was considered statistically significant.

Results

The demographic and laboratory findings of each group of participants were shown in Table 1. The patients in the PCOS group and non-PCOS control group were found to be similar in terms of age and body mass index (kg/m2) values. Serum total testosterone and LH levels of patients in the PCOS group were significantly higher than the non-PCOS control group. FSH levels of both groups were similar. Serum insulin levels and HOMA-IR of the PCOS group were found to be significantly higher than the control group. The mean serum estradiol levels measured on the day of agonist trigger in the PCOS group were significantly higher than the control group and were consistent with the OHSS risk. There was a slight increase in progesterone levels measured on the day of agonist trigger in the PCOS group compared to the control group, but this increase was not statistically significant. The number of total oocytes, MII oocytes and 2PN embryos in the PCOS group were significantly higher than in the non-PCOS group.

Average ΔCt value of HOXA-10 mRNA in PCOS and non-PCOS control groups were found to 5.88 and 6.77 respectively. Average ΔCt value of HOXA-10 mRNA in fertile group was found to 7.79. When compared with the endometrial HOXA-mRNA levels of fertile cases, the HOXA-10 mRNA levels of the patients in the PCOS group were found to be significantly lower (ΔCt 7.79 vs. ΔCt 5.88, $p<0.002$). Similarly, endometrial HOXA-10 mRNA levels in the non-PCOS control group were significantly lower than the HOXA-10 mRNA levels in the fertile group (ΔCt 6.77 vs. ΔCt 7.79, $p<0.001$). HOXA-10 mRNA levels in endometrial samples taken from patients in the PCOS group were found to be significantly lower than the HOXA-10 mRNA levels in non-PCOS control group (ΔCt 5.88 vs. ΔCt 6.77, $p<0.03$). A negative but significant relationship was found between the average ΔCt values of HOXA-10 mRNAvalues and the MII oocyte count only ($r = -0.670$, $p <.002$). No significant correlation was found between the other evaluated parameters and ΔCt values.

Discussion

Our study is the first study investigating the endometrial HOXA-10 mRNA expression levels in PCOS patients who are in the high risk group for OHSS and whose total embyo freezing decision is made. Our study with this double control group showed that the endometrial HOXA-10 mRNA expression levels of the patients who underwent total embryo freezing were found to be significantly lower than the fertile group, regardless of whether the patients had PCOS or not. However, when PCOS and non-PCOS control groups are compared in terms of average ΔCt values of HOXA-10 mRNA, the decrease in gene expression of the patients in the PCOS group (ΔCt 5.88 vs. ΔCt 7.79) is more pronounced than in non-PCOS group (ΔCt 6.77 vs. ΔCt 7.79)

One of the possible reasons for this difference between the two groups may be greater number of follicle growth in PCOS patients and the negative effect of these follicles on endometrial HOXA-10 mRNA by increasing ovarian steroid synthesis. The results of Cermik et al. [8] strongly support this idea. These authors showed that high testosterone levels in vitro blocked HOXA-10

expression. In our study, high testosterone levels were detected in the PCOS group, which is a finding that supports the more severe decrease in HOXA-10 mRNA expression. However, I have to explain a situation that seems paradoxical among our findings. The expression levels of homeobox genes in healthy and fertile individuals begin to increase from the follicular phase and reach a maximum in the secretory phase [7,13]. Therefore, both estrogen and progesterone increase homeobox gene expression [13]. Despite the significant increase in estrogen levels measured on the day of hCG in the PCOS group compared to the control group, the decrease in HOXA-10 mRNA levels suggests that the positive effect of estrogen on this gene is blocked. We thought that there was no direct relationship between HOXA-10 reduction and progesterone levels, since progesterone levels were similar between the two groups. Increased androgen levels in PCOS patients may be one of the possible causes of inhibiting the positive effects of estrogen on HOXA-10. High testosterone levels have been reported to prevent estrogen and progetserone-induced HOXA-10 expression [8]. It has been noted that the same preventive effect is exerted by dihydrotestosterone. In our study, high testosterone levels in PCOS patients may have blocked the HOXA-10 inducing effect of estrogen. However, in the correlation analysis, we could not find a significant correlation between testosterone levels and the average ΔCt values of HOXA -10. High insulin levels and HOMA-IR values may also have led to a decrease in HOXA-10 expression in the PCOS group. However, we could not detect any correlation between these two metabolic parameters and HOXA-10 mRNA levels. Consistent with our results, Cermik et al. [8] reported that insulin had no effect on homeobox gene expression.

During the Spearman's correlations analysis, we found a negative but significant correlation only between the number of mature eggs collected and the average ΔCt values of HOXA-10 mRNA. We do not know why the increased follicle count has a negative effect on HOXA-10 mRNA. However, the increase in the number of follicle corresponds to the increase in estrogen, progesterone and androgen synthesis and release. Study by Cermik et al. [8] reported that the positive effect of estrogen and progesterone on HOXA-10 is blocked by testosterone. Since only physiological levels of estrogen increase HOXA-10 mRNA expression, supraphysiological estrogen levels secreted in PCOS patients in the OHSS risk group may not stimulate or block endometrial HOXA-10 mRNA expression. Blocking the expression of HOXA-10 mRNA by both increased androgens and increased estrogen levels may prevent the formation of pregnancy and block the development of the OHSS. In other words, the decreased HOXA-10 mRNA expression in PCOS cases in the risk group for OHSS may prevent pregnancy and provide a compelling mechanism against the worsening of the OHSS clinic. Since the clinical development of OHSS in our cases was prevented, we cannot directly attribute the decrease in HOXA-10 levels to the results of OHSS. However, subclinically, OHSS may have started in the ovaries and affected the endometrium. In order to make a definite comment on this issue, it is necessary to measure the expression of receptivity genes in PCOS cases with clinical OHSS.

As a result, HOXA-10 mRNA levels, one of the basic endometrial receptivity genes, were found to be lower in PCOS patients with high risk for OHSS compared to both fertile and infertile patients without PCOS. In addition to increased androgen levels, supraphysiological estrogen levels are among the possible causes of the decrease in HOXA-10 mRNA expression. No correlation was found between insulin levels, HOMA-IR and other parameters and HOXA-10 expression. The negative and significant correlation between the number of mature eggs collected and HOXA-10 expression suggests that the onset of a subclinical OHSS and related molecular changes in PCOS cases may adversely affect receptivity. This decrease in HOXA-10 mRNA expressions in PCOS cases may be a protective mechanism taken to prevent OHSS from becoming clinically apparent. We can make a definite judgment thanks to extensive studies, including larger case series and clinically clear OHSS cases.

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