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Research Article

Laparoscopic Ovarian Drilling Regulates Endometrial LIF and Integrin Expression in PCOS

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Abstract

The study was planned to investigate whether laparoscopic ovarian drilling (LOD) alters the expression levels of leukemia inhibitory factor (LIF) and integrin beta 3 (ITGB3) in the endometrium of infertile women with clomiphene-resistant PCOS. Expression of LIF and integrins mRNA in the endometrium obtained before and after LOD during the implantation window were measured. Expression of each gene was evaluated using real-time reverse transcriptase polymerase chain reaction (RT-PCR). Expression levels of endometrial LIF and integrin were lower in PCOS subjects before LOD compared to healthy fertile controls. Compared with fertile subjects LOD up-regulated endometrial LIF and integrin expression. Fold changes of LIF and integrin following LOD were found to be 3.12 and 3.88 respectively. Fold change increase in LIF and integrin were noted as statistically significant ($P < .03$, $P < .01$). LOD increses endometrial LIF and integrin expression and improves receptivity in clomiphene-resistant PCOS women.

Keywords: *Laparoscopic Ovarian Drilling (LOD); Leukemia Inhibitory Factor (LIF); Integrin Beta 3 (ITGB3); Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)*

Introduction

Although the receptivity of endometrium of patients having PCOS might not be altered during the window of implantation the underlying mechanisms of PCOS associated infertility remain elusive [7]. Despite a clear ovulation defect is a main cause of subfertility in PCOS, recent findings showed that failed receptivity may also contribute to PCOS-related subfertility [7,12]. Moreover, increased miscarriage rates in PCOS further support the possibility of receptivity defect [12]. In line with this failed expression of some receptivity molecules in the endometrium can contribute to subfertility have been linked to PCOS [7,12]. Decline in the expressions of some receptivity genes have been reported in PCOS [7].

In order to treat ovulation defect many drugs including aromatase inhibitors, insulin sensitising drugs and gonadotropins have been developed. Nevertheless, many PCOS women who do not respond to increasing doses of clomiphene and fail to ovulate. This clinical condition is defined as clomiphene resistant [10]. Laparoscopic ovarian drilling (LOD) is a surgical method to treat hormonal and ovulatory defects in PCOS. LOD is an effective treatment for ovulation defects in women with clomiphene resistant [11]. It has been reported that medical treatment and LOD were equally effective in inducing ovulation in clomiphene-resistant PCOS [8,9].

Integrins are integral membrane proteins consist of alpha and beta chains. A gene encodes the αv protein has been shown to heterodimerize with β chain. Heterodimer of αv and β3 (ITGB3) is accepted as a receptor for vitronectin [16]. By inducing endometrial angiogen-

esis ITGB3 induce embryo attachment [16]. Leukemia inhibitory factor (LIF) is a member of the cytokine family. It has a fundamental role in endometrial receptivity [17]. It has criticial role in many cellular events such as proliferation, differentiation, and cell survival [17]. Although different studies have have investigated the endometrial expression of integrins and LIF in some infertile women [14,15] there is no study investigating the effect of LOD on receptivity molecules. We therefore, investigated whether laparoscopic drilling of PCOS ovaries alters the expression levels of LIF and integrins in the endometrium of clomiphene-resistant PCOS.

Materials and Methods

The study consists of fifteen women with PCOS, six women with non-endometriotic ovarian cyst, and six fertile control subjects who were undergoing tubal sterilization. PCOS was defined according to revised Rotterdam criteria, which require two of the following three manifestations: 1) oligo- and/or anovulation, 2) clinical and/or biochemical hyperandrogenism, and 3) polycystic ovaries determined by ultrasonography [1]. Women in control group had no evidence of hyperandrogenic skin manifestations or polycystic ovaries on ultrasound. PCOS subjects **were selected from clomiphene resitant patients. O**vulation failure after administration of 150 mg clomiphene daily for 5 days starting on the third day of the cycle is defined as clomiphene resistant. **Failure to achieve ovulation with clomiphene, followed by letrazole, led to the decision of laparoscopy.** Venous blood ofsamples were taken from study subjects for hormonal assays, lipid profile, glucose and insulin analysis. The blood of the women was sampled during the early follicular phase of progesterone induced withdrawal bleeding. Insulin resistance was calculated using the homeostasis model assessment insulin resistance index (HOMA-IR)[2]. Women taking antiandrogens, antidiabetics and lipid lowering drugs were excluded.

LOD method

Laparoscopic ovarian drilling with thermal dose adjusted according to ovarian volume (60 J/cm^3 of ovarian tissue) in the midluteal phase. Three to five (450 - 750 J) punctures per ovary were peformed [3]. Laparoscopic cystectomy was performed in control participants. Fertile control women underwent endometrial sampling. Endometrial biopsies were taken with a Pipelle from PCOS and controls before surgery. The endometrial tissue was divided into two sections, one of which was fixed in formalin, and the other washed three times with a sterile saline solution to remove blood and transferred into RNA stabilization buffer, and stored at -80°C until analysis. The ones with PCOS or ovarian cyst underwent a second endometrial biopsy 3 months after the LOD during midluteal phase.

RT-PCR

Sample preparation

Endometrial samples within the RNA stabilization buffer (RNALater; Qiagen) were homogenizatedwith Tissuelyser (Qiagen).

RNA isolation

Homogenized endometrial samples were used for total RNA isolation. RNeasy Mini Kit (Qiagen) was used for isolation. Both quantity and purity of RNA were detected spectrophotometrically with the help of the Maestro-nano(Maestrogen).

cDNA synthesis

Quantitect Reverse Transcription Kit (Qiagen) was used for obtaining complementary DNA (cDNA). Following reverse transcription (RT) genomic DNA was eliminated by incubating each RNA sample in Wipeout buffer at 42°C. RT mixture (1 mL Quantiscript Reverse Transcriptase, 4 mL Quantiscript RT Buffer, 1 mL RT Primer Mix) and target RNA at 1 mg concentration in 20 mL total volume were prepared on ice. The reaction mixture was incubated at 42°C for 15 minutes and then was kept at 95°C for 3 minutes to inactivate RT. The Quantiscript Reverse Transcriptase used in that system was optimized for RNA at a range of 10 pg^{-1} mg concentrations [6].

Measurement of LIF and ITGB3 mRNA

Primers and standard positive controls of LIF and ITGB3 were synthesized with the use of Primerdesign. Beta-actin (ACTB) was accepted as housekeeping gene. The mRNA levels of endometrial samples were normalized according to the beta-actin mRNA level. RT-PCR

reaction was performed with the use of Quantitect Probe PCR Kit (Qiagen) and the Rotorgene Q (Qiagen) realtime PCR device. RT-PCR results were expressed as Ct (cycle threshold), ΔCt, and ΔΔCt. Endometrial samples were studied three times and average Ct values were calculated. Sequences of all primers designed to be used as forward and reverse primers for RT-PCR were: ACTB: F 5'-GCA AGC AGG AGT ATG ACG AGT-3',R -5' -CAA GAA AGG GTG TAA CGC AAC TAA-3'; LIF: F 5'-GGA GGT CAC TTG GCATTC AG-3', R 5'-GG AAG AGA ACG AAG AAC CTA CC-3'; ITGB3: F 5'-ACC ATC TCT TTA CCT CCT AATTCC-3', R 5'-CTG GCT CTA CAA TAG CAC TCT C-3'. The relative LIF and ITGB3 expression were determined by means of the $2^{-\Delta\Delta Ct}$ comparative method. Fold change ($2^{-\Delta\Delta Ct}$) is the normalized gene expression in the PCOS group divided the normalized gene expression in the fertile control group.

Statistical analysis

Kolmogorov-Smirnoff test was used to analysis of data. Continuous variables were analyzed with Mann-Whitney U test. The categoric variables were analyzed by means of the Pearson chi-square test. A *P* value of < .05 was accepted as significant. The results are expressed as mean±SD. Fold increases were considered to be positive when the mRNA level was ≥ 3-fold higher than that of initial expression and negative if < 2-fold [6].

Results

Demography and hormone profiles of each group of subject was shown in table 1. The fertile group had at least two children. The age of PCOS and the ovarian cyst group were similar. BMI of PCOS and fertile groups were higher than the ovarian cyst group. Fertile women had no evidence of hyperandrogenic skin manifestations or polycystic ovaries on ultrasound. Fasting insulin, glucose levels, and HOMA-IR were significantly higher in PCOS group compared to the control groups.

	PCOS	Control	*P value
Age	25.8 ± 2.21	26.0 ± 1.12	0.11
Body mass index (kg/m^2)	26.9 ± 3.51	23.2 ± 0.14	0.02
Total testosterone (ng/dl)	70.4 ± 4.61	33.2 ± 3.44	0.01
HOMA-IR	3.89 ± 3.12	2.11 ± 1.13	0.01
Fasting insulin (mU/ml)	16.3 ± 1.13	8.2 ± 1.83	0.001
Fasting glucose (mg/dl)	94.1 ± 7.16	70.1 ± 2.11	0.001

Table 1: *Demographic characteristics of PCOS and control groups.*
*Data are presented as mean ± SD, *p < 0.05 is accepted statistically significant.*

Expression levels of LIF and ITGB3 were lower in the endometrium of PCOS women before LOD compared with fertile women. Compared with fertile subjects LOD of PCOS ovaries up-regulated endometrial LIF and ITGB3 mRNA expression. Fold changes of LIF and ITGB3 mRNA after LOD were found to be 3.12 and 3.88 respectively. Fold change increase in LIf and ITGB3 mRNA were found to be statistically significant (P < .03, P < .01). Expressions of LIF and ITGB3 mRNA in the endometrial samples did not change significantly after surgical removal of benign ovarian cysts. Although ovarian cystectomy were not associated with a significant change in LIF and ITGB3 mRNA, a trend toward increased endometrial LIF and ITGB3 mRNA expression were noted after surgery compared with the preoperative values. Fold change increase in LIf and ITGB3 were noted 2.11 and 2.33 respectively after ovarian cystectomy in control group. This positive regulation was noted as insignificant (P < .43 and P < .11).

Discussion and Conclusion

Although endometrial receptivity of some patients having PCOS might not be altered during the window of implantation the underlying mechanism of PCOS related subfertility remain elusive. Defects in receptivity molecules have been linked to a wide range of gyneco-

logical disorders including PCOS [19]. Decline in the expressions of some receptivity molecules such as integrin and homeobox genes have been reported in PCOS women [18]. Failed expression of above receptivity molecules might further complicate achieving pregnancy in PCOS women.

There is a few study showing the possible effect of PCOS on receptivity genes. This study was the first to investigate the expression levels of endometrial LIF and ITGB3 before and after LOD in PCOS patients. Our study showed that expression levels of endometrial LIF and ITGB3 mRNA were lower in patients with PCOS before LOD compared with healthy fertile controls. Significantly increased LIF and ITGB3 mRNA expressions were deteceted in endometrium obtained three months after LOD. We found a 3.1-fold increase in endometrial LIF mRNA and a 3.8-fold increase in endometrial ITGB3 mRNA following LOD. Fold changes in LIF and integrin after LOD were significant. One possible mechanism underlying the increased LIF and ITGB3 expression following LOD may be progesterone resistance [4]. Defective progesterone receptor expression in PCOS may cause decreased expression of progesterone-responsive LIF and ITGB3 mRNA. Hence, LOD of PCOS ovaries may restore progesterone resistance and can lead to increased LIF and integrin expression.

Increased serum androgen levels may be the main culprits of decreased expression of LIF and ITGB3. In ood agreement with this, PCOS women with high levels of androgen showed low receptivity genes expression suggesting androgens may lead to receptivity defect [13]. Possibly, androgens can antagonize the expression of estrogen induced receptivity genes that might lead to decline in LIF or ITGB3. After LOD, decline in androgen production may explain improve in LIF and ITGB3 mRNA expression. The presence of AMH receptors in endometrium and the reported inhibitory effect of AMH on human endometrial cell proliferation [5] enable us to think that high levels of AMH may inhibit the expression of LIF and ITGB3 in PCOS. Following LOD decline in AMH levels may block the inhibitory effect of AMH on integrins and LIF.

The results of current study showed that there is a receptivity failure in the endometrium of women with PCOS. This receptivity defect may affect implanation process regardless of other causes of infertility, such as defective ovulation and hyperandrogenism. LOD increses endometrial LIF and ITGB3 mRNA expression and improves receptivity in patients with clomiphene-resistant PCOS. We concluded that subfertility in PCOS is not only due to defective folliculogenesis but also the result of defective expression of endometrial integrins and LIF.

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