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The Effect of Altered *Mucin1*, *FGF2*, and *HBEGF* Gene Expression at The Ectopic Implantation Site and Endometrial Tissues in The Tubal Pregnancy Pathogenesis: A Case-Control Study

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Abstract

Background: Ectopic pregnancy (EP) is defined as implantation and development of an embryo outside of the uterine tissue. Women undergoing assisted reproductive technologies (ART), particularly frozen embryo transfer (FET), are in high-risk populations for EP. *Mucin1* (*MUC1*), fibroblast growth factor-2 (*FGF2*), and Heparin-binding epidermal growth factor (*HBEGF*) genes are involved in the endometrial receptivity pathway, leading to normal eutopic implantation; Although, their relevance in the tubal pregnancy after FET is unknown. We aimed evaluation of *Mucin1*, *FGF2*, and *HBEGF* expression fold as endometrial receptive markers in the EP patients following the FET cycle.

Materials and Methods: A case-control study was conducted on ten patients (five EP patients and five women in the pseudo-pregnancy group, as the control samples). Pseudo-pregnancy group was established in women who were candidates for hysterectomy for benign diseases. Fallopian tube biopsies and corresponding endometrial tissues from these patients were taken during the hysterectomy. However, the fallopian tube and endometrial tissues of EP patients were obtained during salpingectomy. The mRNA expressions of *MUC1*, *FGF2*, and *HBEGF* genes in the fallopian tube and endometrial tissues were measured by real-time polymerase chain reaction (PCR) assay.

Results: *MUC1* mRNA expression level in the endometrium of the case group was higher than in the control group (P=0.04); however, its mRNA expression in the fallopian samples of the case group in comparison with the control group was significantly decreased (P=0.001). The *HBEGF* mRNA expression level was not significantly different between the case and control endometrium, whereas its expression was significantly increased in the case fallopian samples compared with the control ones (P=0.001). The same pattern was observed for *FGF2* mRNA expression level in the fallopian samples of the case group but was significantly reduced in the endometrial samples in comparison with the control samples (P=0.03).

Conclusion: *MUC1*, *FGF2*, and *HBEGF* gene mRNA expression changes may explain the embryo rejection from the uterus and the establishment of a receptive phenotype in fallopian cells.

Keywords: Ectopic Pregnancy, *FGF2*, Frozen Embryo Transfer, *HBEGF*, *Mucin1*

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Introduction

An embryo moving in the fallopian tube to the uterus implantation site and also a suitable milieu for the embryo during this short lifetime of development in the tubal environment, is necessary to achieve a successful embryo implantation (1). The interactions and paracrine signaling network among the tubal epithelium, smooth muscle, and immune cells coordinate pivotal functions for successful later embryo implantation (1, 2). Any alterations in these interactions create a different environment that may influence an embryo tubular implantation (3).

A tubal pregnancy is defined as an embryo implantation within the fallopian tube, which is more prevalent, more than 90 %, type of the ectopic pregnancy (EP) (4). On the other hand, women undergoing assisted reproductive technologies (ART), particularly frozen embryo transfer (FET) procedures, are in high-risk populations for EP, in such a way that in an ART pregnancy, the increasing

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rate of an EP incidence, more than 8.6%, was reported in comparison with the actual non-ART pregnancy (natural conception) (5).

To prepare for embryo implantation, a considerable changes occur in the composition of the apical plasma membrane of the uterine epithelium. Among these changes, the dynamic expression of the genes involved in the endometrial receptivity pathway such as Mucin 1 (*MUC1*), fibroblast growth factor 2 (*FGF2*), and Heparin-binding epidermal growth factor (*HBEGF*) are previously proved (6). While the MUC1 gene plays an anti-binding role during implantation *FGF2* and *HBEGF* genes mediate blastocyst adhesion to the endometrial cells (7, 8).

Furthermore, substantial studies have demonstrated that a molecular basis that includes abnormal gene and protein expression, as well as the aberrant function and structure of the endometrial and fallopian tissues, are among the primary causes of EP (9, 10). Although, the functions of the *MUC1*, *HBEGF* and *FGF2* genes are well-established in the normal eutopic pregnancy, their relevance in the tubal pregnancy after FET is unknown. In the case of a tubal pregnancy, it has been suggested that signals emerging from the fallopian cells can compete with the uterus signals and attract the embryo toward misplaced implantation (11). A greater understanding of the functions and structure of fallopian tube epithelial cells, can lead to knowledge about protective mechanisms against the ectopic implantation that made our aim of the present study.

Materials and Methods

This case-control study was approved by the Research Ethics Committee of the Royan Institute, Tehran, Iran (IR.ACECR.ROYAN.REC.1399.091) and the Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RETECH.REC.1398.490) and informed written consent was obtained prior to the collection of tissue samples from each patient.

Study design and participants

Ten patients who had referred to the Royan Infertility Clinic, Tehran, Iran, from April 2021 to February 2022 were invited to participate in the present study. Volunteers who included in the study met the following criteria:

Inclusion criteria for all participants were aged between 30-40 years, normal body mass index (BMI) according to World Health Organization (WHO) categories ($18.5 \leq BMI < 25$ kg/m^2) (12, 13), and having the history of the regular menstrual cycle. Exclusion criteria include a history of pelvic inflammatory disease (PID) such as Gonorrhea, Chlamydia and/or salpingitis, fallopian tube problems such as adhesion or hydrosalpinx, history of tubal ligation, heterotopic pregnancy, receiving methotrexate, the record of using intrauterine devices such as intrauterine device (IUD), a history of the EP, a history of endometriosis, uterine abnormalities, myoma, hyperplasia, polycystic ovary syndrome (PCOS) and thyroid disease.

We categorized our participants in two equal case and control groups (n=5).

Ectopic pregnancy group

Five fallopian tube tissues were collected from women in the case group diagnosed with an ectopic embryo implantation in the ampulla of the fallopian tube during salpingectomy. All of them were in the 6 to 8 weeks of their gestational age. All participants in this group conceived after embryo transfer after freeze embryo transfer cycle and were taking exogenous progesterone (P4, Progestin®, Aburaihan Pharmaceutical Co, Tehran, Iran) and estradiol (E2) (Aburaihan Pharmaceutical Co, Tehran, Iran) for endometrial preparation and continued both hormones as luteal phase support (14). Each fallopian tube sample containing an embryo and a gestational sac that is defined as a tubal pregnancy was removed from the ampoule, about 10 mm distance from the gestational sac was dissected from the specimen during surgery.

Pseudo-pregnancy group (as the control group)

Ethical constraints prohibited the use of the fallopian tissue from women bearing a viable embryo. Therefore, patients who were candidates for hysterectomy for benign diseases were requested to receive human chorionic gonadotrophin (hCG, 5000 IU, Pregnyl; Organon, Norway), in the mid-luteal phase. This method makes hormonal conditions that are identical to those found in normal pregnancies. This protocol known as a pseudo-pregnancy that has previously been used by other research groups (7, 15). Fallopian tube biopsies from the ampulla and corresponding endometrial tissues of these patients were taken during hysterectomy.

Tissue samples processing, RNA extraction and cDNA synthesis

Biopsy specimens of the ampulla and endometrium tissues were directly placed in the RNAlater solution (AM7020, Ambion, Austin, TX, US), then were freezed by the snap freeze method (samples were rapidly frozen by placing tissues in liquid nitrogen for ten seconds) and finally, stored at -80°C until RNA extraction.

The extraction of RNA was carried out according to the manufacturer's protocol. (Trizol, Cat No: 15596026, Invitrogen, USA). Using DNase I (Fermentas, Cat No: E00381, Sanktleon-rot, Germany), probable genomic DNA contamination, was eliminated of the extracted RNA. The complementary DNA (cDNA) was produced using the first strand cDNA synthesis kit (Cat No: k1632, Thermo Scientific™ RevertAid™, Lithuania) as directed by manufacturer.

Relative expression levels of each target gene were normalized by the beta-actin gene (*β-actin*, as the housekeeping or internal control gene) (Table 1).

Table 1: Primer sequences used for real-time polymerase analysis

Gene	Forward primer (5´-3´)	Annealing temperature (°C)	Product size (bp)
MUC1	F: CAGCCTCTCTTACACAAACCCA R: AGAACCTGAGTGGAGTGGAATG	60	122
HBEGF	F: CATCCCCACAATCTGGCTTAGT R: ACCCCTACATCCTGACCATACA	60	157
FGF2	F: CTGTACATTTTTGGGGTCAGCTG R: CCAGCATTTCGGTGTTGAAGAA	60	167
β-actin	F: CAAGATCATTGCTCCTCCTG R: ATCCACATCTGCTGGAAGG	60	90

In a final reaction volume of 20 µL, each Real time polymerase chain reaction (PCR) reaction sample contained 5 µL of SYBR Green PCR Master Mix (Cat No: RR820L, Takara, China,), 11 µL of dH_2O (Cat No: W4502, Sigma-aldrich®,. Life Technologies TM), 1 µL of each forward and reverse primer (Metabion, Martinsried, Germany), and 2 µL of single-strand cDNA. Real time PCR conditions are described as following: initial denaturation (one cycle at 95°C for 10 minutes), followed by 40 cycles of denaturation (95°C for 10 seconds), annealing (60°C for 60 seconds, depends on the primer) and extension (72°C for 30 seconds), and a final extension (one cycle at 72°C for 10 minutes).

The relative standard curve and $2^{-\Delta\Delta Ct}$ techniques were used to determine the expression level of each target gene (16).

Statistical analysis

The IBM SPSS statistics 21 program was used to perform the statistical computations (IBM Corp., Armonk, NY). The normal distribution of the values was analyzed by the Student's t test with a two-tailed distribution. The non-normal distributions were examined using the Mann-Whitney non-parametric test (P≤0.05). The Levene's test for equality of variances were performed. The level of significance was set at P≤0.05.

Results

Demographic information

There were no statistically significant differences between the patients with the EP group and the control group in terms of patients' age (37.00 ± 2.34 vs. 38.80 ± 0.83) and BMI (23.90 ± 0.95 vs. 24.32 ± 0.44), respectively. The other demographic information is summarized in Table 2.

Table 2: Demographic information of patients in the control and ectopic pregnancy groups

Variable	Control group	EP group	P value
Age (Y)	37.00 ± 2.34	38.80 ± 0.83	0.14
BMI (kg/m^2)	23.90 ± 0.95	24.32 ± 0.44	0.39
hCG dose (IU)	3.8 ± 0.83	-	NA
Gestational age (Y)	-	6.2 ± 0.83	NA
Number of transferred embryos	-	2.0 ± 0.7	NA

Values are reported as means ± standard deviations (SD). BMI; Body mass index, hCG; Human chorionic gonadotrophin, EP; Ectopic pregnancy, and NA; Comparison not applicable.

Genes expression at the level of mRNA in endometrium and fallopian samples of patients with ectopic pregnancy and control groups

The RNA expression level of *MUC1* in the endometrium of the case group (0.33 ± 0.06) was significantly higher than the control group (0.05 ± 0.002, P=0.04); in contrast, its expression in fallopian samples was significantly decreased (0.068 ± 0.01 vs. 3.04 ± 0.8, for case and control group, respectively) (P=0.001, Fig.1).

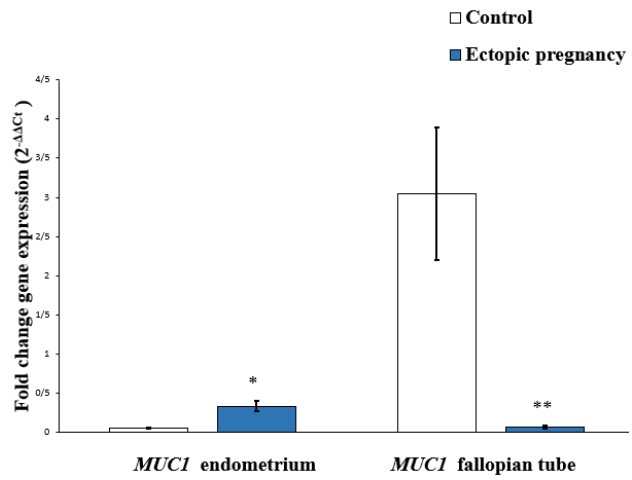

Fig.1: *MUC1* mRNA expression in endometrium and fallopian samples. *; P<0.05 and **; P<0.01.

As shown in Figure 2, the level of mRNA expression of *HBEGF* in the endometrium of our case group was not significantly different from the control group (0.33 ± 0.03 vs. 0.30 ± 0.03, respectively) (P=0.6), whereas its expression was significantly increased in the fallopian samples of the case group in comparison with the control group (1.46 ± 0.28 vs. 0. 61 ± 0.06, respectively) (P=0.001). The same pattern was observed for *FGF2* mRNA expression level in the fallopian samples of case and control groups, (3.68 ± 0.77 vs. 2.01 ± 0.75 respectively) (P=0.04, Fig.3); however, its expression was significantly decreased in the EP endometrial samples of the case group (1.24 ± 0.05) in comparison with the control group (1.87 ± 0.04, P=0.03).

Protein-protein interaction analysis using the STRING database showed that *MUC1*, *FGF2*, and *HBEGF* have a related signaling pathway (Fig.4).

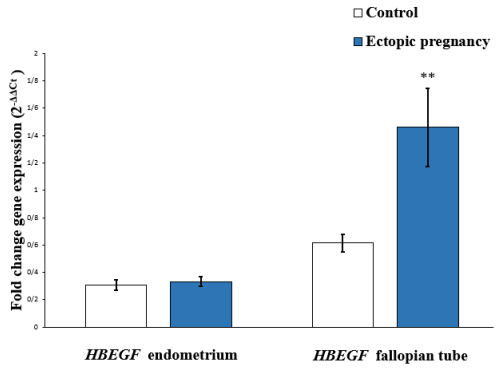

Fig.2: *HBEGF* mRNA expression in endometrium and fallopian. **; P<0.01.

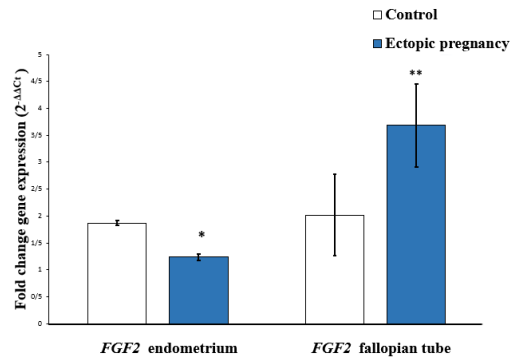

Fig.3: *FGF2* mRNA expression in endometrium and fallopian samples. *; P<0.05 and **; P<0.01.

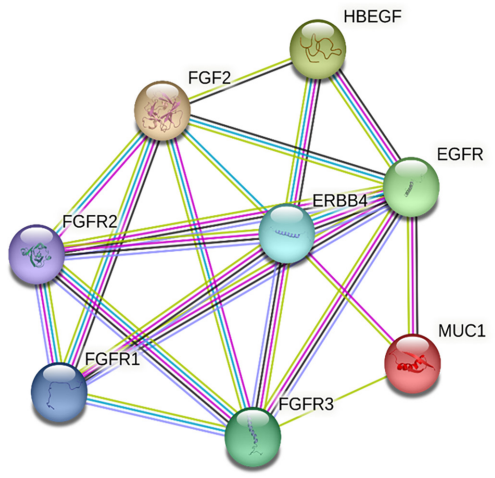

Fig. 4: Molecular interactions of three analyzed factors: FGF2, HBEGF, and MUC1. STRING database protein-protein interaction analysis.

Discussion

Different mRNA expression levels of *MUC1*, *HBEGF* and *FGF2* genes were observed in the fallopian and endometrium samples of our case group in comparison with our control group.

There are different molecular factors that lead to a tubal pregnancy, although it may directly occur due to an impaired embryo transfer in the uterine environment (17). The risk factors of an ectopic pregnancy have remained uncertain, and the etiology and molecular mechanisms behind its higher incidence in fresh/FET cycles following assisted reproductive technology are unspecified as well.

An increase mRNA expression level of *MUC1* in the fallopian tubes in comparison with the endometrium provides a gradient signal to prevent implantation in an ectopic site in normal pregnancy (18), it may be because of its anti-adhesive action. Therefore, the down-regulation of *MUC1* mRNA expression in the endometrial luminal epithelium and during the window of implantation has a role in establishing normal eutopic implantation. The same mechanism occurs in the epithelium of the fallopian tube, in which the reduction in the *MUC1* mRNA expression level facilitates an embryo attachment. In the current study, *MUC1* mRNA expression level is downregulated in the fallopian tissues, consistent with other research (7, 18); however, there was an increased mRNA expression level in the endometrial samples. It seems this alteration in the endometrial *MUC1* mRNA expression level contributes to the suboptimal embryo-endometrial dialogue and also, leads to the rejection of the embryo from the uterus. Interestingly, *in vitro* study suggested that the human embryo promotes the reduction in the protein expression of *MUC1* to facilitate endometrial cell attachment (19). It is possible that an alter capability of some embryos after a freeze-thawing procedure leads to the decrease of *MUC1* mRNA expression in the endometrium and guides the embryo to the other direction (e.g., fallopian tube).

On the other hand, both *FGF2* and *HBEGF* genes are important factors for the endometrial remodeling and trophoblast adhesion improvement (20). The *HBEGF gene*, that is expressed in the glandular and luminal epithelium of the endometrium and fallopian tube, plays a role in the decidualization process, consequently during the embryo implantation process (21). Interestingly, FGF2 and HBEGF protein expressed at the surface of endometrial epithelial cells mediates the process of blastocyst binding to the endometrium (22).

The FGF2 protein plays an important role in the regulation of cell survival, cell division, angiogenesis, and cell differentiation (23). The FGF2 protein is produced by human endometrial epithelial cells, while its in vitro protein expression level is regulated by the recombinant hCG hormone supplementation, also provides the endometrial receptivity enhancement (24).

The human blastocysts with high implantation potential regulate the expression level of genes that are involved in the endometrial receptivity through the secretion of regulatory molecules (22).

Endometrial selectivity and receptivity are two words that describe the endometrium's function as a biosensor of embryo quality. Selectivity is an endometrium-programmed feature that recognizes and rejects embryos with inadequate developmental ability. On the other hand, the receptive phenotype allows the endometrium to provide an ideal milieu for the embryo growth (25). A poor-quality embryo may be rejected by the endometrium, however, the fallopian tube's epithelium is unable to accomplish the same that results in an ectopic implantation. Changes in the expression patterns of genes

involved in the endometrial reception or in the biology of the fallopian tube have a potential role in underlying causes of EP (26, 27).

Some elements, including tubal damages, the type of embryo transfer technique, multiple embryos in an ET cycle, and a high volume of the transfer medium, could have an adverse effect on an IVF-ET procedure success (28). Although, these risk factors do not explain how and why the embryo passes through the uterus and enters and implants in the fallopian tube. Several studies on the ectopic implantation of the embryo have found that leukemia inhibitory factor (LIF), *HOXA10*, and *MUC1* gene expression were significantly changed at the site of EP (29). Our investigation found changes in both the endometrium and the fallopian tissues, which is consistent with previous research (20, 29). However, our investigations are unable to distinguish between cause and consequence.

Surprisingly, *MUC1*, *FGF2*, and *HBEGF* genes are targets of the hormones, P4 and E2, and also, their expressions are affected by these hormones (8, 19, 30-34). Both P4 and E2 are master regulators of uterine receptivity. During the frozen embryo transfer cycle, that endometrial preparation with exogenous steroids, P4 and E2, and the embryo is developed and the luteal phase is supported. The optimal doses for luteal phase support are essential to provide a suitable physiological hormone level. It is shown that elevated E2 and P4 levels are risk factors for EP following FET cycles. Because of its impact on uterine contractions and tubal movements, the extra-oral administration of E2 between two phases, ovarian puncture and embryo transfer, may increase the EP occurrence rate (35). On the other side, the P4 hormone may lead to an EP occurrence which may cause a malfunction in the fallopian tube ciliary (36, 37). Therefore, defective responses to hormonal therapy may be an EP index model that suggests further studies.

There were some drawbacks in our study. The gestational age in patients with EP lasted longer than the time we were able to create a condition of pseudo-pregnancy with repeated hCG injections. We considered that prolonging the pseudo-pregnant condition and, as a result, delaying elective surgery for a longer period would be unethical. Because truly ethical disqualification decisions that prohibit the fallopian tissue biopsy from those women having viable embryos, we used a pseudo-pregnancy model which could potentially influence our results. There are only limited reports of this model (7, 15, 38), we consider future research also to be needed to improve our understanding.

Conclusion

It seems, an altered expression of *MUC1*, *FGF2*, and *HBEGF* genes may underpin an embryo rejection from the uterus and induce a receptive phenotype in the fallopian epithelial cells. Understanding the molecular mechanisms of an embryo implantation site, ectopic and eutopic is essential. Further study of high doses of P4 and E2 for luteal phase support in FET cycles, to develop effective prevention strategies against ectopic implantation.

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Authors' Contributions

N.N.; Writing manuscript. F.Gh., F.K., R.A; Provided concept, Method of study, and Supervision. F.Gh., Z.Ch.; Patient recruitment. F.Gh.; Performed laparoscopic biopsies. N.N., S.A., Z.Ch.; Performed laboratory examinations. N.N., A.R., S.A., A.N., Z.Ch.; Formal analysis and Investigation. F.Gh., F.K., Z.Ch.; Writing-Reviewing and Editing manuscript. All authors read and approved the final manuscript.

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