

EVALUATION OF PROGESTERONE RECEPTOR, FKBP51 AND FKBP52, ASSOCIATED WITH UTERINE RECEPTIVITY, IN ENDOMETRIAL TISSUE OF WOMEN WITH REPEATED IMPLANTATION FAILURE

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Abstract

Background. Repeated implantation failure (RIF) is the most important problem in assisted reproductive technologies (ART). In the process of embryo implantation, accurate function of progesterone through progesterone receptors (PR) is crucial for establishment of a receptive endometrium. FKBP51 and FKBP52 are two co-chaperones acting as negative and positive regulators of PR function, respectively. Studies have shown that any deficiencies in expression of PR or its co-chaperones causes reproductive disorders.

Materials and Methods. In this study we evaluated the PR protein expression by immunohistochemistry and expression of PR, FKBP51, FKBP52 genes by quantitative real-time PCR in endometrial tissue of normal and RIF women during the window of implantation.

Results. Immunohistochemical studies showed that the PR protein expression

in stromal cells is significantly higher in the endometrium of normal women than RIF women ($P < 0.001$). In addition, a significantly lower PR and FKBP52 gene expression was observed in endometrial tissue of RIF women compared to normal women ($P < 0.001$ and $P < 0.001$, respectively), whereas there was no significant difference in PR protein in epithelial cells ($P = 0.3$) and FKBP51 gene expression between the two groups ($P = 0.6$).

Conclusion. The results indicate that altered expression of PR protein in stromal compartment and gene expression of PR and FKBP52 gene in endometrial tissue can be related to endometrial receptivity defects and occurrence of RIF.

Key words: Repeated Implantation Failure, Progesterone Hormone, Progesterone Receptor, FKBP51, FKBP52.

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INTRODUCTION

After successful birth of the first newborn using *in vitro* fertilization (IVF) in 1978 (1), there have been many advances in the treatment of infertility, and different methods of assisted reproductive technologies are widely used for treatment of infertile couples, but their probability of success is approximately 30%. In most of the cases, despite transferring good quality embryos to the uterus, implantation does not occur, and so many of infertile couples suffer from repeated implantation failure (2, 3). Since this problem is the most important limiting factor in ART, research on factors related to embryo implantation failure is very valuable.

In the process of embryo implantation and establishment of a successful pregnancy, receptivity of endometrium is critical. This receptivity lasts only for a limited period of time between day 19 and 23 of menstrual cycle, and is called implantation window (4). The accurate function of progesterone hormone as a predominant mediator is essential for creating a receptive endometrium as well as implantation, decidualization and pregnancy maintenance (5-7). Insufficient level of this hormone or attenuated response to it results in a non-receptive endometrium, aberrant embryo implantation, defective formation of the placenta as well as unsuccessful progression of pregnancy, which contribute to RIF and infertility (8). Progesterone hormone should bind to its receptor (PR) in endometrial tissue to activate the downstream regulated transcription of genes essential for embryo implantation. In midse-

cretory phase of menstrual cycle, PR is downregulated in epithelial cells of the endometrium, whereas it is upregulated in stromal cells, and the stroma becomes the main site of progesterone action for preparing a receptive endometrium; therefore, any noticeable alteration in the level of PR expression in stroma of mid-secretory endometrium can be related to a non-receptive endometrium (9-10).

FKBP51 and FKBP52, two well known immunophilins, are co-chaperones for steroid hormone receptors. The physiological function of FKBP51 and FKBP52 has been confirmed in some steroid-dependent tissues using mouse gene knockout models (11-12). They are involved in a variety of cancers, neurological disorders, hormone-dependent diseases and especially male and female reproductive system problems (13-17). Studies on male reproductive system confirmed the role of these co-chaperones in reproductive system development, in cancers and also in fertility disorders related to androgen receptor function (13, 18-22). In the case of progesterone hormone, FKBP51 and FKBP52 act as progesterone receptor co-chaperons. These immunophilins have a similar structure, but FKBP52 increases the affinity of PR for its ligand while FKBP51 decreases this affinity, thereby reducing the response of target tissue to this hormone (17, 23-24). Studies showed that Fkbp52 null female mice are infertile due to endometrial receptivity defects, and their inability to sustain pregnancy was related to progesterone hormone resistance (25-26). In addition, decreased expression of FKBP52 gene is involved in the etiology of other infertility related problems such

as endometriosis (27-30).

Since the above-mentioned statements provide evidences for the critical role of PR as well as FKBP51 and FKBP52 immunophilins in endometrium response to progesterone hormone, in the present study we investigated possible *in vivo* role of PR, FKBP51 and FKBP52 in repeated implantation failures.

MATERIALS AND METHODS

Human subjects

This case-control study was performed from May 2012 to December of 2013 and approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences. Also informed written consent for the use of endometrial tissue was obtained from all case and control subjects. The case group involved 10 women referred to infertility clinic, with 34.40 ± 1.24 years old and body mass index (BMI) of 24.25 ± 0.73 kg/m², who did not achieve clinical pregnancy after transfer of good-quality embryos in at least three fresh cycles. They did not have any problems related to male factor infertility, and had regular menstrual cycles without using any hormones or contraceptive drugs for at least 3 months before endometrial sampling. All the cases underwent comprehensive diagnostic tests for the presence of any factors associated with RIF. For this purpose, a detailed history, physical examination, chromosome analysis of peripheral blood lymphocytes and transvaginal three-dimensional ultrasound or hysterosalpingography for detection of hydrosalpinx, uterine anomalies and endometrial defects were performed for

all cases. They were also checked for endocrinology profiles on day 3 of the menstrual cycle (follicle-stimulating hormone, luteinizing hormone, prolactin and testosterone), for thyroid hormone function (T3, T4 and thyroid-stimulating hormone) and thrombophilia factors including anti-cardiolipin antibodies (IgG, IgM), lupus anticoagulant, anti-thrombin III, protein S and protein C.

The control group included 10 reproductive-age healthy women with similar age and BMI to RIF group (33.00 ± 1.16 years and 22.87 ± 0.76 kg/m²) ($P = 0.4$ and 0.2 respectively) undergoing endometrial biopsy before performing bilateral tubal ligation. Women in this group did not have any pathological problem, and had at least two successful pregnancies and live births without any ART procedure.

Endometrial Biopsy

The endometrial biopsy was performed using a standard Pipelle curette in the middle of secretory phase of the menstrual cycle, 6 to 9 days after the urinary LH surge, approved by ultrasonography. Then, each endometrial sample was divided into two pieces. One piece was fixed in a 2 ml microtube containing 4% paraformaldehyde solution for histological dating and immunohistochemical study of PR protein. The other piece was placed in a 2 ml microtube (RNase and DNase free, Greiner Bio One, Germany) containing 1 ml RNAlater stabilizer solution (Qiagen, Germany), and was maintained at -20°C until quantitative real-time PCR analysis was performed for PR, FKBP51 and FKBP52 genes. In addition, plasma

progesterone concentration was measured at the time of endometrial biopsy by ELISA (enzyme-linked immunosorbent assay) in both normal and RIF groups.

Endometrial dating

Fixed tissue biopsies embedded in paraffin blocks were cut into 5- μ m thick sections, and were stained by hematoxylin and eosin. Then, histological evaluation and dating of endometrial samples was carried out by an experienced pathologist according to Noyes criteria (31).

Immunohistochemical study

Immunohistochemistry for PR protein was performed on new tissue sections of the same paraffin blocks which were in midsecretory phase of menstrual cycle, confirmed by histological evaluation. Tissue sections were deparaffinized in xylene, and were hydrated in a series of graded ethanols (100%, 96% and 70%). Any endogenous peroxidase activity was quenched by incubating the sections in 3% hydrogen peroxide in methanol for 20 minutes, and the sections were washed by PBS. Antigen retrieval was done by placing the sections in 10 mM/L of sodium citrate buffer (pH 6.0) at 95°C for 20 minutes. Thereafter, the slides were incubated with primary antibody against rabbit anti-human progesterone receptor (DAKO, Denmark) for 60 minutes at room temperature and with the secondary anti-rabbit antibody (DAKO, Denmark) conjugated to HRP (horse-radish-peroxidase) for 30 minutes according to the manufacturer's instructions. Between each incubation, the slides were washed in PBS solution. Finally, the sections

were incubated with 3,3'-diaminobenzidine (DAB) (DAKO, Denmark) for 5-20 minutes for visualizing the antigen-antibody reaction, counterstained with hematoxylin for 6 minutes, washed with distilled water and coverslipped. Negative controls were prepared in a similar way without using primary antibodies. Each slide was evaluated by an observer blinded about whether the samples were taken from normal or RIF women. Intensity of staining was reported by analysis of each slide at $\times 400$ magnification using a light microscope. Staining intensity of PR protein was assigned as 0, 1, 2 and 3 as follows: 0= no staining, 1= weak, 2= moderate and 3= strong. Thereafter, PR protein expression in epithelial and stromal compartment was calculated by applying a semi-quantitative method, H-score, using the following equation:

H-score= $\sum P_i(i+1)$ where i denotes staining intensity and P_i the percentage of cells staining at each intensity (from 0 to 100%) (32).

The stained tissues were portrayed using an optical microscope (Nikon, Japan) equipped with a digital camera (Nikon, Japan).

Quantitative real-time PCR

Primer and Probe design

Gene-specific primer pairs and Taqman probe for PR, FKBP51, FKBP52 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, as internal control) were designed using the beacon designer software 7, at exon junctions and were checked by blast. Moreover, any case of SNPs (Single-Nucleotide Polymorphism) was determined.

The sequences of forward and

reverse primers and Taqman probes used in this study are presented in Table 1.

RNA extraction, Reverse transcription and real-time PCR

Total RNA from tissue samples stabilized in RNAlater stabilizer solution was extracted using RNeasy Plus Mini Kit containing DNA separation column (Qiagen, Germany). The purity of total RNA was assessed by measuring the absorbance ratio at 260 nm and 280 nm wavelengths (260/280). Single-stranded cDNA was synthesized using RivertAid™ First Strand cDNA Synthesis Kit (Fremontas, Canada) with the same initial concentration of RNA for all samples according to the manufacturer's protocol in final reaction volume of 20 ml. The reaction conditions included incubation of the reaction mixture at 65°C for 5min, 42°C for 60 min, followed by 5 min at 70°C. The resulting first-strand cDNA was stored at -20°C, and was used as template in the real-time quantitative PCR analysis.

Eventually, detection of target gene expression was performed using

Rotor gene Taqman Master Mix (Qiagen, Germany) and a Corbett Sequence Detection System (Corbett 6000, Australia). Data were analyzed by comparative cycle times (Ct) method. For each sample, the 2ΔCt was calculated, and the results were indicated as relative expression (33).

Statistical Analysis

For analysis of data distribution, Kolmogorov-Smirnov test was performed. As this test was positive for all the parameters evaluated in this study, the student's t-test was used to compare the two means. P value of <0.05 was considered to be statistically significant.

RESULTS

As presented in Table 2, plasma progesterone concentration did not show significant difference between normal and RIF women (P = 0.1).

Evaluation of PR protein expression and PR, FKBP51 and FKBP52 gene expression in endometrial tissue of

Table 1. Primer and probe sequences used in Real-time PCR

Gene	Accession number	Primer and Taqman probe sequence
PR	NM-000926.4	Primer forward: 5'CCC TAT CTC AAC TAC CTG3'
	NM-0012024741.1	Primer reverse: 5'CTT CCA TTG CCC TCT TAA3' Taqman probe: 5'ATT CAG AAG CCA GCC AGA GCC3'
FKBP51	NM- 001145775.1	Primer forward: 5'GCC ATT GTC AAA GAG AAG3'
	NM- 001145776.1	Primer reverse: 5'CAG CTT TGG TGT ATT CTC3'
	NM- 004117.3	Taqman probe: 5'AAC CAT ATT CCA TCT CTA ACC AGG ACA3'
FKBP52	NM-002014.3	Primer forward: 5'CTA CCC CAA CAA CAA AGC3' Primer reverse: 5'GAT GGT CTC CTG AGG AAG3' Taqman probe: 5'TGT TCT CCT CCT CAG CCA GC3'
GAPDH	NM-001256799.1	Primer forward: 5'CTC TGG TAA AGT GGA TAT TG3' Primer reverse: 5'GTG GAA TCA TAT TGG AAC A3' Taqman probe: 5'CCT TCA TTG ACC TCA ACT ACA TGG TT3'

Table 2. Age, BMI, Progesterone concentration and the PR immunohistochemical staining in endometrial tissue of normal and RIF women obtained during the implantation window

	Normal (n=10)	RIF (n=10)	P value
Age (year)	33.40 ± 1.16	34.40 ± 1.24	0.4
BMI (kg/m ²)	22.87 ± 0.76	24.25 ± 0.73	0.2
Progesterone (ng/mL)	6.0 ± 0.50	7.1 ± 0.66	0.1
Stromal PR (H-score)	306.5 ± 5.38 ^{a,b}	179.1 ± 7.70	< 0.001
Epithelial PR (H-score)	149.7 ± 6.81	159.8 ± 5.85	0.3

Values represent mean ± SEM.

a. P < 0.001 between stromal compartment of normal and RIF.

b. P < 0.001 between epithelial and stromal compartment of normal.

RIF group compared to control group showed the following results.

Fig. 2 indicates that the overall PR mRNA expression profile is significantly higher in the endometrium of normal women than of RIF women (P < 0.001).

Comparison of PR protein ex-

pression in epithelial cells did not show any significant difference between normal and RIF women (P=0.3), but the stromal compartment of endometrial tissue in normal women exhibited significantly higher PR protein expression than in RIF women (P < 0.001) (Table 2, Fig. 1).

The endometrial tissue of both

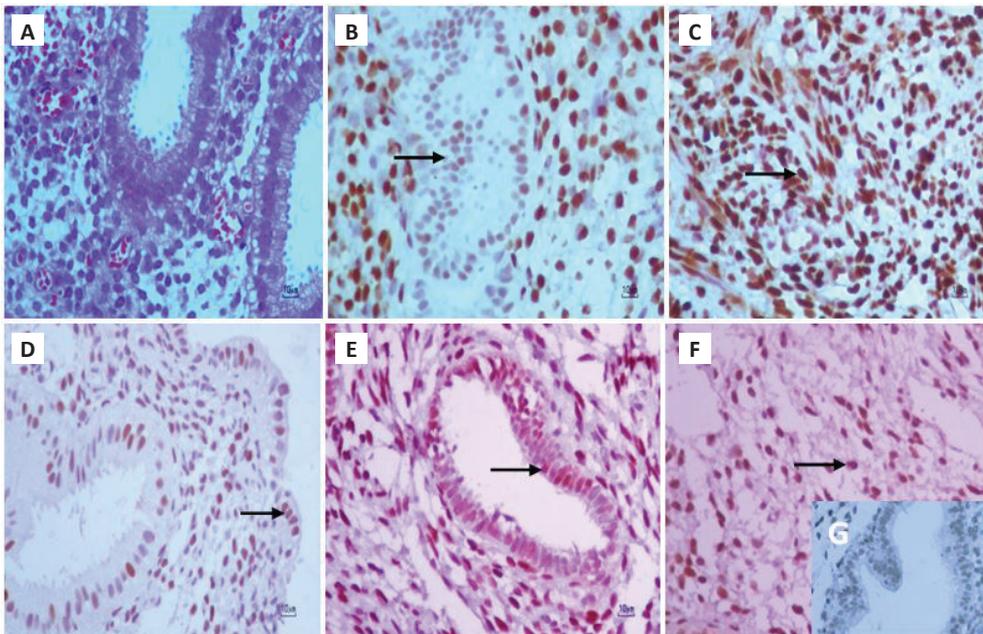


Figure 1. Hematoxylin and eosin staining of endometrial tissue in mid secretory phase (A). Immunohistochemical detection of PR protein in endometrial tissue of normal women in glandular epithelium (B), stroma (C) and from women with repeated implantation failure in luminal epithelium (D), glandular epithelium (E) and stroma (F). Endometrium without using primary antibody (negative control) (G). Arrow heads indicate positive staining of PR proteins. Magnification in all panels is x400. Scale bars represent 10 µm.

normal and RIF women showed significantly higher PR protein expression in the stromal compartment than epithelial compartment, but only in normal women was this difference statistically significant ($P < 0.001$) (Table 2, Fig. 1).

Moreover, in endometrial tissue of RIF women, expression of FKBP52 mRNA as a positive regulator of PR was significantly down-regulated compared to its expression in endometrial tissue from normal women ($P < 0.001$) (Fig. 2).

Expression of FKBP51 mRNA as a negative regulator of PR decreased in RIF women compared to normal, but it was not statistically significant ($P = 0.6$) (Fig. 2).

DISCUSSION

Nowadays, assisted reproductive technologies are widely used for the

treatment of infertile couples, but their rate of success is limited mainly because of repeated implantation failures. A main reason for decreased implantation rates is transfer of ART-derived embryos into a non-receptive endometrium due to insufficient endometrial response to progesterone, which is a critical hormone for almost every stage of pregnancy including ovulation, fertilization, implantation, decidualization and pregnancy maintenance. In the midsecretory phase of endometrium, the presence of progesterone receptors is essential for the proper function of progesterone hormone, appropriate endometrial response to progesterone and establishment of a receptive endometrium (4-7). Studies also showed that in the signaling pathway of progesterone hormone, the two co-chaperons of FKBP51 and FKBP52 act as PR regulators, decreasing and increasing the

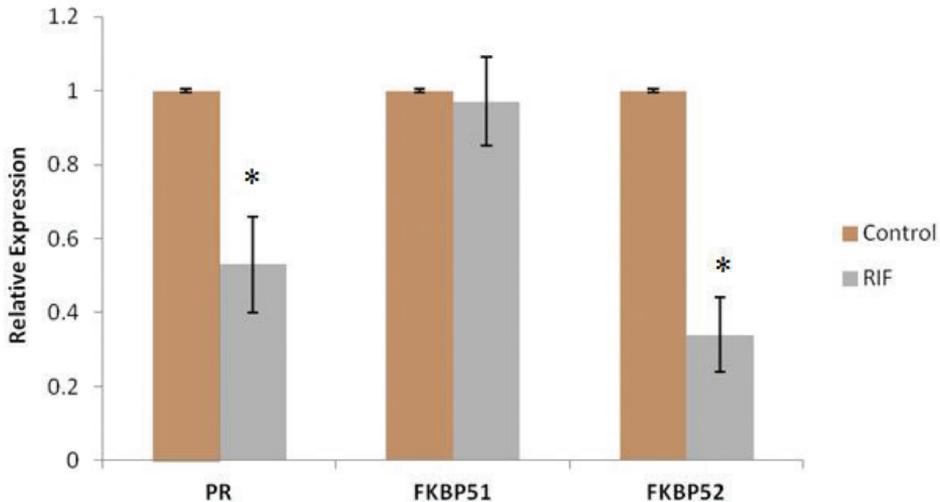


Figure 2. The relative expression pattern of PR, FKBP51 and FKBP52 genes measured by Real-time PCR in endometrial tissue of normal and RIF women obtained during the window of implantation. Values represent mean \pm SEM. * Significantly different from control. $P < 0.001$ for PR and FKBP52.

affinity of PR for its ligand, respectively, thereby altering the response of target tissue to this hormone.

Since recent genetic researches have demonstrated differences in expression of genes related to endometrial receptivity in endometrial tissue of RIF women in comparison with normal women during the implantation window, in the present study we investigated the possible *in vivo* role of PR, FKBP51 and FKBP52 by expression evaluation of PR protein and PR, FKBP51 and FKBP52 genes in endometrial tissue of RIF women.

Based on our results, plasma progesterone concentration did not show any significant difference in normal and RIF women, but endometrial tissue of RIF women showed significantly lower levels of PR for both messenger RNA (mRNA) and protein. Many studies have reported that decreased expression of PR causes progesterone resistance and some gynecological problems such as endometriosis (33-34). It has been also suggested that luteal phase deficiency, which leads to inadequate endometrial receptivity and pregnancy wastage, is mostly because of the difference in response of endometrium to progesterone, resulting from reduced or altered PR expression rather than absolutely insufficient levels of plasma progesterone hormone (35).

In normal midsecretory phase of endometrium, PR protein is mainly expressed in stromal cells in but not in epithelial cells under the effect of progesterone hormone, resulting in stroma predecidualization and preparing the endometrium to become receptive to embryo (9-10). In our study similar PR

protein expression pattern was observed in the endometrium of normal women. In this group, higher stromal PR protein expression compared to epithelial compartment is statistically significant, but although there is higher stromal PR protein expression compared to epithelial compartment in RIF endometrium, it is not statistically significant.

Comparing the epithelial PR protein expression in endometrium shows no significant difference between the two groups but compared to normal endometrium, PR expression in stromal compartment of RIF women is significantly lower, and this attenuated expression of PR protein in the stromal compartment, as the main site of progesterone action at the window of implantation may indicate an alteration in uterine response to progesterone, which results in unreceptivity of endometrium to embryo in this group.

In our study, FKBP51 and FKBP52 genes were expressed in endometrial tissue of both groups at window time of implantation. Expression of FKBP51 mRNA as a negative regulator of PR showed no significant difference in RIF and normal groups. However, based on our researches, no other studies have been conducted for evaluation of FKBP51 gene expression in endometrial tissue of women with gynecological problems to be compared with our study results. Only one study showed that a higher level of FKBP51 gene expression in decidual cells of pregnant women facilitates their labor process by reducing sensitivity of these cells to progesterone, indicating its contribution to progesterone signaling pathway and response of

target tissues to progesterone (23).

Gene expression analysis of FKBP52, a molecule with an important regulatory action in endometrial function, particularly during the implantation window, showed reduced expression of its gene in endometrial tissue of RIF women. This finding is in agreement with other researchers indicating attenuated expression of FKBP52 gene in some gynecological problems related to signaling pathway of progesterone. Tranguch *et al.* observed that female mice with targeted deletion of the *Fkbp52* gene are infertile due to implantation failure resulting from uterine receptivity defects, and concluded that reduced P4 binding affinity to PR, lower response of endometrial tissue to progesterone hormone and down regulation of several progesterone-regulated genes in these animals are the main causes of this problem (11, 25, 36). Other studies showed that reduction of FKBP52 gene expression is involved in the etiology of unexplained human infertility and endometriosis in humans as well as mice and baboon models, and is related to progesterone insensitivity (27-30). Wang *et al.* showed that assessment of FKBP52 gene and protein expression is positively related to ultrasonic evaluation of endometrial receptivity, and suggested that FKBP52 may have an important role in improving the receptivity of endometrium (37).

In conclusion, a lower level of PR protein expression in stromal compartment and a lower level of FKBP52 gene expression in endometrial tissue of RIF women may cause alteration in function of progesterone hormone, leading to diminished endometrial receptivity to

embryo implantation. Therefore, in addition to a proper level of PR, existence of FKBP52 as a positive regulator of PR is essential for progesterone signaling pathway and establishment of a receptive endometrium in human. Moreover, since progesterone supplementation is used as luteal phase support in some ART cycles, we should determine whether RIF patients require other treatments after embryo transfer to restore their fertility.

In addition, by expression evaluation of PR and its regulators, FKBP51 and especially FKBP52, we can evaluate receptivity of endometrium and even predict embryo implantation and pregnancy outcome prior to exposing RIF patients to expensive and complicated ART procedures. Moreover, by producing some drugs for regulation and alteration of PR and FKBP52 gene expression with the aim of improving endometrial response to progesterone, we will have a therapeutic solution for RIF patients.

Conflict of interest

We declare that there is no conflict of interest.

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