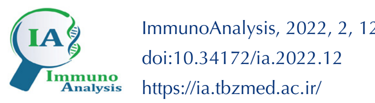

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Original Article

Study of LIF, NR3C1, BCL6, miR-494, and miR-30d in Endometrial Epithelial Cells of Repeated Implantation Failure and Healthy Women Treated with Calcitriol and Letrozole in a Fibrin Scaffold

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Abstract

Background: Determining the exact time of window of implantation (WOI) in individuals with repeated implantation failure (RIF) is necessary for successful implantation. The present study aimed to compare the effects of letrozole and/or calcitriol on the expression of receptivity markers in endometrial epithelial cells (EECs) of RIF patients and healthy fertile women.

Methods: Endometrial tissue was obtained from RIF patients (n=5) and healthy women (n=5) in the proliferation phase. EECs were isolated and cultured in a fibrin scaffold in 10 groups. The cells were treated with letrozole and/or calcitriol in three types of treatment. To check the proliferation and migration of cells in the scaffold, immunofluorescent and H&E staining was used, and gene expression was checked using real-time polymerase chain reaction (PCR).

Results: This study showed that the expression levels of pro-implantation genes (NR3C1, LIF, and miR-30d) were higher in healthy women than in RIF patients, and the expression levels of anti-implantation genes (BCL6 and miR-494) were lower in healthy women than in RIF patients. Calcitriol and letrozole increased the expression level of pro-implantation genes (NR3C1, LIF, and miR-30d) in RIF patients. Furthermore, they reduced the expression level of anti-implantation genes (BCL6 and miR-494) in RIF patients.

Conclusion: Our findings suggest that treatment with letrozole is more effective than co-treatment with letrozole and calcitriol or alone treatment with calcitriol on receptivity markers in RIF patients.

Introduction

Repeated implantation failure (RIF) is a significant challenge faced by 15% of couples undergoing assisted reproductive technology (ART) treatment.[1] RIF is determined as the absence of pregnancy following at least 3 times high-grade embryo transfers, which is due to the decrease in endometrial receptivity.[2] Approximately 7-10 days after ovulation in a 28-day cycle, endometrial receptivity for embryo implantation occurs, which involves some molecular and cellular events in the uterine epithelium. Since many of these implantation-related processes must occur within a particular time (window of implantation, WOI), determining the exact WOI time in individuals with RIF is crucial for successful implantation.[3] A wide range of molecules such as adhesion molecules, growth factors, cytokines, and transcription factors, have the main role in implantation.[4] These molecules can be divided into two categories: Anti- and pro-implantation markers. The expression of pro-implantation markers is positively related to implantation success. Moreover, the expression of anti-implantation markers is negatively related to implantation success.[5]

nuclear receptor subfamily 3 group C member 1 (NR3C1) is a glucocorticoid receptor and a pro-implantation marker whose low expression caused implantation failure. The role of this receptor in endometrial receptivity, implantation,

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and decidualization makes it a candidate marker for RIF.[6] B-cell lymphoma 6 (BCL6) is an oncogene and an anti-implantation marker that is increased in pathologies such as endometriosis, pre-eclampsia, and infertile women and can be a good candidate for predicting endometrial dysfunction and implantation.[7] Leukemia inhibitory factor (LIF) is a pro-implantation cytokine that has maximum secretion in WOI. This gene is involved in implantation, decidualization, and blastocyst invasion, which turns LIF into a candidate marker to investigate endometrial receptivity.[8]

Other candidates in implantation failure are low and high-expression endometrial miRNA.[5,9] miR-494 (anti-implantation) and miR-30d (pro-implantation) have different expressions in the endometrium of RIF patients compared to fertile women.[10]

The use of calcitriol and letrozole in the treatment of infertility is common. These two drugs are involved in processes such as endometriosis, ovulation, polycystic ovary syndrome (PCOS), and cancer.[11,12] Calcitriol (the active form of Vit-D, 1,25(OH)2-vitamin D3) increases the rate of pregnancy. Furthermore, the role of calcitriol or letrozole on the expression of endometrial receptivity genes such as osteopontin, LIF, HOXA10, L-selectin, and αvβ3 has been reported.[13,14] However, more molecular studies are needed to investigate the role of calcitriol and letrozole in the endometrial preparation of RIF women.

As far as we know, no study has been done on epithelial cells from the endometrium of RIF and healthy women under the influence of letrozole and calcitriol in three-dimensional (3D) culture. Therefore, in this study, the expression of genes in the endometrial epithelial cells (EECs) of RIF and healthy women treated with letrozole and calcitriol in fibrin 3D culture was investigated so that the results can help the treatment of RIF patients.

Materials and Methods

Sample collection

Endometrial biopsies were obtained from healthy fertile women and patients with RIF who were referred to the infertility clinic of Al-Zahra hospital at Tabriz University of Medical Sciences, Tabriz, Iran. All of the participants were selected according to the criteria[13] in Table 1.

Endometrial samples were collected during the proliferative phase of the menstrual cycle using a biopsy curette (Pipelle® Endometrial Suction Curette, Cooper Surgical, USA). One section of the tissue sample was sent to the pathology laboratory, and the other part was transferred to the research laboratory in Dulbecco's modified eagle medium (DMEM)-F12 (DMEM/F12, Gibco, Denmark) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Invitrogen, Stockholm, Sweden). Moreover, estradiol, AMH, FSH, and vitamin D were measured by the ELISA technique.

Epithelial cells isolation and characterization

EEC isolation was carried out using the procedure described in prior research.[15,16] In summary, the endometrial sample was washed three times with phosphate-buffered saline (PBS) containing a 2% antibiotic-antimycotic (Gibco, USA) solution. Then the endometrial sample was cut and incubated in 2 mg/mL collagenase I (Cat. No.C9891, Sigma, USA) solution in DMEM/F12 for 90 minutes at 37°C. The obtained cell suspension was passed through filters with mesh sizes of 70 and 40 µm (SPL, South Korea), and then the 40-µm filter mesh was washed to acquire epithelial glands. Using the trypsin enzyme (Sigma, USA) (0.025%), epithelial glands were isolated as single epithelial cells. To assess the vitality of cells, trypan blue staining was employed and cell vitality was estimated at 90%. The cells were plated onto 10-cm culture dishes(Sigma, USA) coated with collagen I and cultured at 37°C in a humid environment with 5% CO2 in the air for 1 hour. After 1 hour, the remaining stromal cells in suspension from the previous step (which had a small amount) were attached to the bottom of the coated plate, but the epithelial cells were suspended. At this stage, the supernatant was collected to transfer the cells to the fibrin scaffold of the 3D cell culture. Immunofluorescence staining for cytokeratin8 and vimentin was utilized to verify the purity of the isolated epithelial cells. For this purpose, 4% paraformaldehyde and subsequently 0.2% Triton X-100 were used to fix and permeabilize epithelial cells. Unspecific binding sites of cells were blocked using 1% bovine serum albumin (BSA) and 22.52 mg/mL glycine dissolved in PBS containing 0.1% Tween 20. Then, anti-cytekeratin8 and anti-vimentin primary antibodies (DAKO, Glostrup, Denmark) as well as DAPI (blue) and FITC 493 (green) or PE 488 (red)-conjugated secondary

Table 1. Participant's inclusion and exclusion criteria

Group	Inclusion criteria	Exclusion criteria	Reference
Healthy	• Complaints of abnormal bleeding with a normal uterine cavity in diagnostic hysteroscopy • Regular menstrual cycles of 25 to 35 days • Fertility (at least one healthy live delivery in the previous three years) • had received any steroids or vitamin D3 supplementation in the previous three months. • Equal to or less than 35 years of age	Polyps, myomas, endometrial hyperplasia, endometrial cancer, previous infertility, miscarriage, history of ART treatment, preeclampsia	13
RIF	• Three unsuccessful intra-cytoplasmic sperm injection cycles and the subsequent implantation of grade A high-quality embryos. • had received any steroids or vitamin D3 supplementation in the previous three months. • Equal to or less than 35 years of age	Hydrosalpinx, immunological abnormalities, hormonal or anatomical conditions, polyps, adhesions, history of miscarriage, ectopic pregnancy, myomas, male factor infertility	13

antibodies were added to the cells.

Preparation of fibrinogen and thrombin

Fibrinogen and thrombin were prepared based on the methods described in previous studies with slight modifications.[17-19] Fresh frozen plasma (FFP) and fibrinogen were acquired from the Blood Transfusion Organization of Tabriz. All the prepared bags had the same blood group (AB positive). Due to the different concentrations of fibrinogen in different bags, first, the fibrinogen content of all the used bags was mixed in a glass container. Then, fibrinogen was aliquoted in 1-mL microtubes and kept at -80°C until use.

The FFP bag was thawed at 37°C and 16 mL of FFP was mixed with 10 mL of calcium gluconate. The resulting solution was incubated for 3 hours at 37°C followed by centrifugation at 2500 rpm for 10 minutes and the supernatant was used as thrombin. The extracted thrombin was first collected in a falcon tube (to equalize the thrombin concentration in all scaffolds) and then aliquoted in microtubes and stored at -80°C until use.

Preparation of fibrin scaffold

To obtain the appropriate ratio of fibrinogen (F) to thrombin (T) to prepare the scaffold, different ratios of these two compounds were mixed in a 24-well plate (Nunclon; Nunc, Roskilde, Denmark) and monitored at room temperature for 5-10 and 15 minutes. After that, the ratios that did not form any gel were excluded from the study. This step was repeated three times and throughout all these iterations, gel formation was only observed in the wells with F: T ratios of 1:1 and 3:1. Next, the ratios that formed the gel were incubated for two weeks. During this period, the culture medium was changed every 48 hours. After two weeks, it was observed that the gels of neither ratio were destroyed. In the next step, we prepared gels with these two ratios again, but this time, after the formation of the gels, we added cells to their surface. After five days, the gel with a ratio of 1:1 started to degrade, whereas the gel with a ratio of 3:1 remained intact until the end of the second week. As a result, the ratio of 3:1 was chosen for our study. Next, staining was performed to prove cell proliferation and migration in the fibrin scaffold with a ratio of 3:1.

Evaluation of cell migration in scaffold

After preparing paraffin sections and slides from the fibrin scaffold containing epithelial cells, immunofluorescence and H&E staining were performed to assess the existence of epithelial cells in the fibrin scaffold. In summary, the sections were deparaffinized using xylene and rehydrated by ethanol and deionized water. H&E staining was carried out on the sections followed by rinsing, dehydration, and clearance. For immunofluorescence staining, the scaffold was separated from the attachment point to the cell plate and was immediately stained with acridine orange (1 mg/mL). Then, it was studied and its image was captured using a fluorescent microscope.

Drug treatment

Epithelial cells were cultured in the fibrin scaffold for four days for proliferation and migration of cells in the scaffold. During these four days, the M199 culture medium (Sigma, USA) modified with estrogen (0.3 nmol/L) (Merck Serono, Milan, Italy)and progesterone (900 nmol/L) (Merck Serono, Milan, Italy) was used for cell culture.[20] Then the studied drugs were added to the treatment groups. The epithelial cells of each sample were divided into five groups: (1) no-treatment group (did not receive any drugs), (2) solvent group (received ethanol), (3) calcitriol group, (4) letrozole group, and (5) calcitriol and letrozole group (co-treatment). The dosages of calcitriol (1,25(OH)2-vitamin D3) (10^{-7}M) (Sigma-Aldrich, St. Louis, MO, USA) and letrozole (1 µmol/L) (Ivy Fine Chemicals, Cherry Hill, NJ) were selected based on previous studies.[13,21] Scaffold with Cells received drugs for 7 days, that was mimics seven days of luteal phases until reaching the WOI time. During this period, the M199 culture medium modified with estrogen and progesterone was used again and it was replaced every 48 hours. Finally, the cells were prepared for analysis of gene expression.

Gene expression

Preparation and evaluation of mRNAs

Total RNA was extracted from epithelial cells using the TRIzol LS reagent (Invitrogen, Carlsbad, CA). Then, using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis, the quantity, purity, and integrity of total RNA were assessed. Next, cDNA was synthesized during the reverse transcription (RT) reaction based on the instructions of the kit, Thermo Fisher Scientific kit (Cat. No. EP0441; Waltham, MA). Moreover, to perform the real-time PCR assay (Roche, Pleasanton, CA), the required materials including primer pairs, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (as a control gene), and SYBR green master mix (Cat. No. 45323402; Odense, Denmark) were prepared (Table 2). The prepared 10-µL mixture contained 6 µL of SYBR green master mix, 0.5 µL of forward and reverse primers, 2 µL of diethyl pyrocarbonate H_2O, and 1 µL of cDNA. Real-time PCR was performed as follows: Initial enzyme activation at 95°C for 3 min followed by 45 cycles of 94°C for 10 seconds (denaturation), 62°C for 40 seconds (annealing), and 72°C for 60 seconds (extension). Finally, the relative expression level was assessed using the formula $2^{-\Delta\Delta Ct}$. All the evaluations were conducted in triplicate.

Preparation and evaluation of miRNAs

After extracting total RNA, a 10.5-µL mixture containing 1.5 µL of stem-loop RT primer (50 nM), 5 µg of extracted miRNA modified to contain at least 5 µg of total RNA, and 4 µL of distilled water was prepared. This mixture was incubated at 65°C for 10 minutes in a thermocycler

(Bio-Rad Inc., San Francisco, CA). Then the microtubes were placed on ice and the following ingredients were added to each microtube: 1 μL of expanding reverse transcriptase (50 U), 2 μL of dNTP (10 mM), 0.5 μL of RNase inhibitor (20 U), 2 μL of dithiothreitol (DTT) (10 mM), and 4 μL of RT buffer (Thermo Fisher Scientific). The thermocycler was set for cDNA synthesis under the following conditions: 25°C for 15 minutes, 37°C for 15 minutes, 42°C for 40 minutes, and lastly, 95°C for 2 minutes. Additionally, to perform the real-time PCR assay, a 10-μL mixture (1 μL of cDNA, 6 μL of qPCR master mix, 0.5 μL of each oligonucleotide primer, and 2 μL of ddH_2O) was prepared (Table 2). The real-time PCR device was set to perform the enzyme activation phase at 95°C for 10 minutes followed by 45 cycles of the thermal amplification phases at 94°C for 10 seconds and 58°C for 40 seconds . U6 was employed as the internal control.

Statistical analysis

GraphPad Prism 9.4 software was used for statistical analysis and graphing the assumptions of this research. All mean comparison tests were two-way. Two-way analysis of variance (ANOVA) tests with Tukey's post hoc test were used in this study. Data were expressed as mean ± SEM. We employed Pearson's correlation test for parametric and the Spearman correlation for non-parametric data to study the association between miRNAs and gene levels. The $P < 0.05$ was considered statistically significant.

Results

The average age of all the participants was equal to or less than 35 years old. Comparing body mass index (BMI), age, anti-Müllerian hormone (AMH), serum levels of estradiol, serum levels of follicle-stimulating hormone (FSH), and serum levels of vitamin D between the two groups, no significant difference was observed (Table 3).

Table 2. mRNA and miRNA primer sequences (5′–3′) used in quantitative real-time PCR

mRNA and miRNA		
Primer name		**Primer sequence (5′–3′)**
NR3C1	Forward	CTGCCATTTCTGTTCATGGTGT
	Reverse	GTCCCCAGAGAAGTCAAGTTGT
BCL6	Forward	GTGTAGGCAGACACAGGGA
	Reverse	CATGACGCAGAATGGGATGAG
LIF	Forward	TGCAGTGAACAAACTACACA
	Reverse	CCCACCTAAGTCCCAACCTAA
GAPDH	Forward	AAGGTGAAGGTCGGAGTCAAC
	Reverse	GGGGTCATTGATGGCAACAA
MiR-494	STL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGAAGAGAA
	Forward	CGTGCTCATAGGTTGTCCGT
MiR-30d	STL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACTTCCA
	Forward	CGTGCTCATTGTAAACATCC
Universal	Reverse	GTGCAGGGTCCGAGGT
U6	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT
	STL	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA AAA ATAT

STL, stem loop

EEC characterization

The isolated epithelial cells were cultured on a collagen-coated petri dish. The masses of epithelial cells were initially in the form of colonies that grew and showed the squamous morphology of the normal epithelium. To further confirm the epithelial nature of the isolated cells, using immunofluorescence staining for cytokeratin8/ vimentin. The results demonstrated that more than 90% of the cells were positive for cytokeratin8 (epithelial markers), and almost all were negative for vimentin (stromal marker) (Figure 1A-D).

Cell migration in scaffold

After two weeks, the presence of cells in the scaffold layers (pores inside the scaffold) was confirmed by immunofluorescent and H&E staining (Figure 1E and 1F).

MRNA expression levels in EEC of RIF and Healthy women

The LIF and NR3C1 gene expression was significantly lower in the RIF no-treatment group compared to the healthy no-treatment group ($P < 0.05$) (Figure 2A). The miR-30d gene expression was lower in the RIF no-treatment group compared to the healthy no-treatment group and there was no significant difference between these groups ($P > 0.05$). The expression of BCL6 and miR-494 genes was significantly higher in the RIF no-treatment group compared to the healthy no-treatment group ($P < 0.05$) (Figure 2A).

Effect of calcitriol on mRNA expression in EEC

The expression of LIF, BCL6, and miR-494 genes in the healthy calcitriol group decreased significantly compared to the healthy no-treatment group as well as in the RIF calcitriol group compared to the RIF no-treatment group ($P < 0.05$) (Figure 2B). The expression of NR3C1 and

Table 3. Baseline characteristics of two groups

Parameter	RIF group	Control group	*P* value
Number of patients	5	5	
Age	34.00 ± 4.30	35.60 ± 2.50	0.498
BMI	19.00 ± 1.41	23.00 ± 2.82	0.056
AMH (pmol/L)	42.35 ± 1.65	44.86 ± 3.44	0.194
Serum estradiol (pg/mL) day 3	47.26 ± 3.30	46.21 ± 3.71	0.648
Serum FSH (IU/l) day 3	5.06 ± 0.47	5.49 ± 0.99	0.417
Serum vitamin D (ng/mL)	28.42 ± 2.87	28.96 ± 3.49	0.797

AMH, anti-Mullerian hormone; BMI, body mass index; FSH, follicle stimulating hormone; NS, non-significant; RIF, repeated implantation failure. Data are presented as mean ± SD.

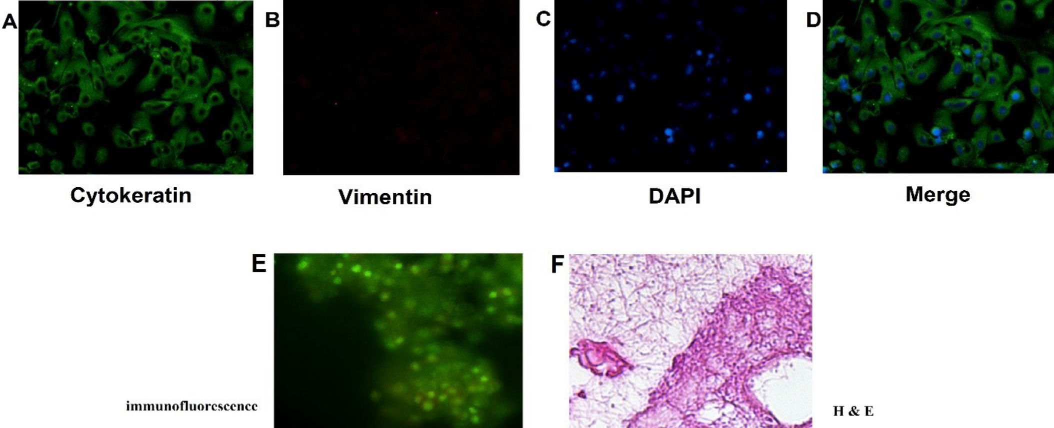

Figure 1. Immunofluorescence and H&E staining. Green, red, and blue fluorescence represents antibody reactivity against cytokeratin 8 and vimentin, and nuclear DAPI, respectively. (A) Cytokeratin 8 positive EEC. (B) Vimentin negative EEC. (C & D) DAPI and Merge. (E & F) Immunofluorescence and H & E staining for scaffold characterization

miR-30d genes in the healthy calcitriol group increased significantly compared to the healthy no-treatment group ($P<0.05$). The expression of the miR-30d gene in the RIF calcitriol group was higher compared to the RIF no-treatment group and there was no significant difference between these groups ($P>0.05$). The expression of the NR3C1 gene in the RIF calcitriol group was significantly higher than in the RIF no-treatment group ($P<0.05$) (Figure 2B).

Effect of letrozole on mRNA expression in EEC

The expression of LIF, NR3C1, and miR-30d genes in healthy letrozole groups increased significantly compared to the healthy no-treatment group as well as in the RIF letrozole group compared to the RIF no-treatment group ($P<0.05$) (Figure 2C). The expression of BCL6 and miR-494 genes in healthy letrozole groups decreased significantly compared to the healthy no-treatment group as well as in the RIF letrozole group compared to the RIF no-treatment group ($P<0.05$) (Figure 2C).

Effect of co-treatment with letrozole and calcitriol on mRNA expression in EEC

The expression of the NR3C1 and miR-30d genes in the healthy co-treatment group increased significantly compared to the healthy no-treatment group as well as in the RIF co-treatment group compared to the RIF no-treatment group ($P<0.05$) (Figure 2D). The expression of BCL6 and miR-494 genes in the healthy co-treatment group decreased significantly compared to the healthy no-treatment group as well as in the RIF co-treatment group compared to the RIF no-treatment group ($P<0.05$). The expression of the LIF gene in the healthy co-treatment group was lower compared to the healthy no-treatment group as well as in the RIF co-treatment group compared to the RIF no-treatment group ($P>0.05$) (Figure 2D).

Comparison of mRNA expression levels in EEC of the same treatment groups

The expression of LIF, NR3C1, and miR-30d genes was significantly lower in the RIF calcitriol group compared to the healthy calcitriol group as well as in the RIF letrozole group compared to the healthy letrozole group ($P<0.05$) (Figure 2E). The expression of BCL6 and miR-494 genes was significantly higher in the RIF calcitriol group compared to the healthy calcitriol group ($P<0.05$). The expression of the miR-494 gene was significantly higher in the RIF letrozole group compared to the healthy letrozole group ($P<0.05$). The expression of the BCL6 gene was higher in the RIF letrozole group compared to the healthy letrozole group and there was no significant difference between these groups ($P>0.05$) (Figure 2E, F).

LIF and NR3C1 gene expression in the RIF co-treatment group was significantly lower than in the healthy co-treatment group ($P<0.05$). BCL6 and miR-494 gene expression in the RIF co-treatment group was significantly higher than in the healthy co-treatment group ($P<0.05$). miR-30d gene expression in the RIF co-treatment group was lower than in the healthy co-treatment group ($P>0.05$) (Figure 2E, F).

Effect of solvent on mRNA expression in EEC

There was no significant difference in the expression of any of the genes (LIF, BCL6, NR3C1, miR-494, and miR-30d) in both healthy women and those with RIF between the solvent group (received ethanol) (Ethanol was used as a solvent for drugs), and the no-treatment group ($P>0.05$).

Correlations between miRNAs and mRNAs levels in EEC of healthy women

In the no-treatment group, there was a positive correlation between miR-494 and miR-30d (Table 4). In the calcitriol group, there was a positive correlation between LIF and NR3C1, also there was a negative correlation between LIF and BCL6. In the co-treatment group, there was a negative correlation between NR3C1 and miR-30d.

Correlations between miRNAs and mRNAs levels in EEC of RIF patients

In the calcitriol group, there was a positive correlation

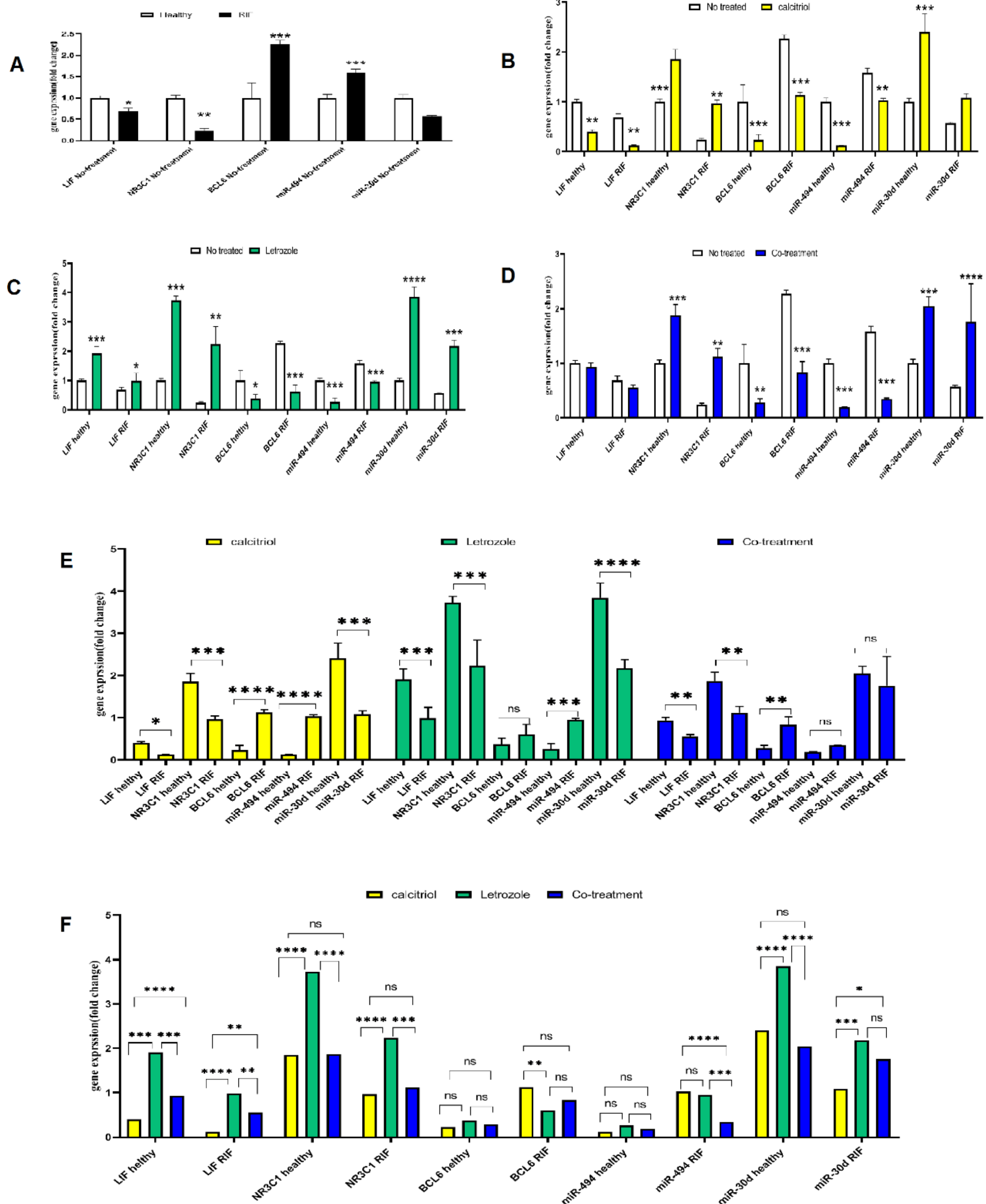

Figure 2. Real-time PCR analysis results. (A) mRNA expression levels in EEC of RIF and Healthy women. (B) Effect of calcitriol on mRNA expression in EEC of RIF and healthy women. (C) Effect of letrozole on mRNA expression in EEC of RIF and Healthy women. (D) Effect of co-treatment with letrozole and calcitriol on mRNA expression in EEC of RIF and Healthy women. (E, F) comparison of mRNA expression levels in EEC of the same or different treatment groups. Repeated implantation failure (RIF); non-significant (NS); *P* value < 0.0332 (*); *P* value < 0.0021 (**); *P* value < 0.0002 (***); *P* value < 0.0001 (****)

between LIF and miR-30d (Table 4). In the letrozole group, there was a positive correlation between LIF and NR3C1. In the co-treatment group, there was a positive correlation between LIF and miR-30d, also there was a negative correlation between LIF and NR3C1.

Discussion

RIF is a significant challenge that has prompted reproductive specialists to focus more on the molecular mechanism of WOI and the effect of drugs used for these patients. The present study is the first research conducted

Table 4. Correlations between miRNAs and mRNAs levels in EEC of healthy and RIF women

	LIF		BCL6		NR3C1		miR-494		miR-30d	
	r	*P* value	r	*P* value	r	*P* value	r	*P* value	r	*P* value
Healthy No-treatment										
LIF	-	-	0.464	0.431	0.801	0.103	0.261	0.672	0.154	0.805
BCL6	0.464	0.431	-	-	0.133	0.831	0.514	0.375	0.122	0.845
NR3C1	0.801	0.103	0.133	0.831	-	-	-0.153	0.806	-0.169	0.786
miR-494	0.261	0.672	0.514	0.375	-0.153	0.806	-	-	**0.911**	**0.031**
miR-30d	0.154	0.805	0.122	0.845	-0.169	0.786	**0.911**	**0.031**	-	-
Healthy calcitriol										
LIF	-	-	**-0.781**	**0.055**	**0.935**	**0.02**	0.367	0.544	-0.376	0.533
BCL6	**-0.781**	**0.055**	-	-	-0.804	0.101	0.038	0.952	0.728	0.163
NR3C1	**0.935**	**0.02**	-0.804	0.101	-	-	0.152	0.807	-0.491	0.401
miR-494	0.367	0.544	0.038	0.952	0.152	0.807	-	-	0.709	0.18
miR-30d	-0.376	0.533	0.728	0.163	-0.491	0.401	0.709	0.18	-	-
Healthy letrozole										
LIF	-	-	-0.211	0.733	0.675	0.211	0.725	0.166	0.412	0.491
BCL6	-0.211	0.733	-	-	-0.068	0.914	-0.049	0.937	0.612	0.273
NR3C1	0.675	0.211	-0.068	0.914	-	-	0.333	0.584	0.406	0.498
miR-494	0.725	0.166	-0.049	0.937	0.333	0.584	-	-	0.709	0.18
miR-30d	0.412	0.491	0.612	0.273	0.406	0.498	0.709	0.18	-	-
Healthy co-treatment										
LIF	-	-	0.442	0.457	0.252	0.683	-0.523	0.366	0.062	0.921
BCL6	0.442	0.457	-	-	0.08	0.898	0.395	0.511	0.106	0.865
NR3C1	0.252	0.683	0.08	0.898	-	-	0.125	0.841	**-0.936**	**0.019**
miR-494	-0.523	0.366	0.395	0.511	0.125	0.841	-	-	-0.192	0.757
miR-30d	0.062	0.921	0.106	0.865	**-0.936**	**0.019**	-0.192	0.757	-	-
RIF No-treatment										
LIF	-	-	-0.656	0.229	0.639	0.246	0.447	0.45	0.034	0.957
BCL6	-0.656	0.229	-	-	-0.073	0.907	-0.092	0.883	0.045	0.942
NR3C1	0.639	0.246	-0.073	0.907	-	-	0.082	0.895	-0.101	0.872
miR-494	0.447	0.45	-0.092	0.883	0.082	0.895	-	-	0.823	0.087
miR-30d	0.034	0.957	0.045	0.942	-0.101	0.872	0.823	0.087	-	-
RIF Calcitriol										
LIF	-	-	0.167	0.788	-0.366	0.544	0.362	0.55	**0.914**	**0.03**
BCL6	0.167	0.788	-	-	0.179	0.773	-0.491	0.401	-0.132	0.833
NR3C1	-0.366	0.544	0.179	0.773	-	-	-0.572	0.313	-0.184	0.767
miR-494	0.362	0.55	-0.491	0.401	-0.572	0.313	-	-	0.315	0.606
miR-30d	**0.914**	**0.03**	-0.132	0.833	-0.184	0.767	0.315	0.606	-	-
RIF Letrozole										
LIF	-	-	-0.395	0.51	**0.932**	**0.021**	0.133	0.832	0.215	0.729
BCL6	-0.395	0.51	-	-	-0.495	0.396	-0.012	0.985	0.805	0.101
NR3C1	**0.932**	**0.021**	-0.495	0.396	-	-	0.184	0.767	0.081	0.897
miR-494	0.133	0.832	-0.012	0.985	0.184	0.767	-	-	-0.036	0.954
miR-30d	0.215	0.729	0.805	0.101	0.081	0.897	-0.036	0.954	-	-
RIF co-treatment										
LIF	-	-	0.156	0.802	**-0.872**	**0.054**	0.253	0.682	**0.874**	**0.052**
BCL6	0.156	0.802	-	-	-0.325	0.594	-0.744	0.149	-0.12	0.847
NR3C1	**-0.872**	**0.054**	-0.325	0.594	-	-	-0.319	0.601	-0.563	0.323
miR-494	0.253	0.682	-0.744	0.149	-0.319	0.601	-	-	0.241	0.697
miR-30d	**0.874**	**0.052**	-0.12	0.847	-0.563	0.323	0.241	0.697	-	-

Bold font indicates a statistically significant correlation.

on the effect of letrozole and calcitriol on the expression of genes related to endometrial receptivity in women with RIF. We discuss our findings in four subtitles.

MRNA expression levels in EEC of RIF and Healthy women

Our findings showed that pro-implantation genes (LIF, NR3C1, miR-30d) expression levels were lower in the RIF patients than in healthy women. Furthermore, anti-implantation genes (BCL6, miR-494) expression levels were higher in the RIF patients than in healthy women. Decreased expression of LIF in RIF has been already reported by Stewart et al.[22] This group showed that implantation did not occur in LIF-deficient mice, but these mice were able to fertilize. Hu et al [23] showed that implantation success increased after the addition of exogenous LIF. Song et al [24] also reported that the decrease in LIF expression can lead to a decrease in the synthesis of prostaglandins and thus lead to implantation failure, which supports our finding regarding decreasing LIF in the RIF. In WOI, LIF through binding to its receptor (LIFR) and activating STAT suppresses proliferation, increases response to progesterone, and decreases response to estrogen. There is also a positive correlation between P53 and LIF. Hence, the reduction of P53, LIF, and STAT in RIF patients can justify implantation failure during IVF.[25] In keeping with our findings, Wang et al[6] also observed decreased levels of NR3C1 expression in the endometrium of RIF patients. Anti-inflammatory mechanisms should neutralize primary inflammation around embryo implantation. During the WOI, NR3C1 is expressed in EECs. Interaction of NR3C1 with adrenal cortisol leads to the inactivation of the anti-inflammatory process across implantation and pregnancy.[6] Moreover, in the uterine natural killer (NK) cells, which underwent a significant elevation in RIF patients, the expression of NR3C1 is together with 11b-Hydroxysteroid dehydrogenases (HSD11B1), implying that NR3C1 can influence NK cells. Given the substantial contribution of NR3C1 in embryo implantation and its aberrant downregulation in RIF, it can be concluded that NR3C1 has the potential capacity to serve as a therapeutic target for RIF.[26,27] On the other hand, Almquist et al[28] and Evans-Hoeker et al[29] reported considerably higher levels of BCL6 expression in endometriosis and unexplained infertility patients. In their study, the increased BCL6 expression level in unexplained infertility women was directly related to implantation failure and decreased live birth rate. Gong et al[30] also reported that BCL6 protein and mRNA expression levels are significantly higher in RIF women, which confirms our results. The interaction of BCL6 with SIRT1 leads to the suppression of GLI1 in the Hedgehog pathway, which will result in progesterone resistance. Progesterone during implantation helps to connect the embryo to the endometrium by suppressing estrogen, reducing proliferation, and inducing some genes. Therefore, the increase in resistance to progesterone due to the abnormal increase of BCL6 in RIF women indicates the potential role of BCL6 in implantation failure and failure of IVF in RIF.[7] Altmäe et al[10] reported decreased expression of miR-494 and increased expression of miR-30d in the receptive endometrium of healthy fertile women. Another group also showed increased expression of miR-494 and decreased expression of miR-30d in the mid-secretory phase of the endometrium of endometriosis women compared to healthy women, which confirms our findings.[31] Overexpression of miR-494 through suppressing the PI3K/Akt/mTOR pathway and reducing the expression of HOXA 10 (one of the important genes in implantation) leads to implantation failure.[32] Increasing the expression of miR-30d leads to decreasing the expression of SOCS1 in the normal endometrium. Therefore, the increased expression of SOCS1 and its effect on the inactivation of the LIF-STAT3 pathway can explain the implantation disorder in RIF women under IVF.[33]

Effect of calcitriol on mRNA expression in EEC of RIF and healthy women

In the current study, NR3C1 and miR-30d expression levels were higher in the calcitriol treatment groups than in the no-treatment groups both RIF and healthy women. Furthermore, LIF, BCL6, and miR-494 levels were lower in the calcitriol treatment groups than in the no-treatment groups both RIF and healthy women. Vitamin D binds to its nuclear receptor (VDR), then forms a heterodimeric complex with the retinoid X receptor (RXR) and interacts with vitamin D response regions (VDRE) on DNA, then regulate cell proliferation and differentiation.[11] In previous studies, the existence of VDR and the enzyme that converts vitamin D into its active form (mitochondrial enzyme 1α-hydroxylase) has been reported in the secretory endometrium phase and during pregnancy.[34,35] Du et al[36] also reported that calcitriol increases the expression of HOXA10 in endometrial stromal cells during the secretory phase. On the other hand, Tesic et al[37] reported that maternal vitamin D deficiency leads to a decrease in glucocorticoid receptors during pregnancy, which confirms our results. In the context of the relationship between vitamin D and NR3C1 in other cells and tissues, studies have shown that calcitriol leads to an increase in NR3C1, thereby modulating the response to glucocorticoids in patients with asthma.[38,39] Furthermore, Obradovic et al[40] observed that vitamin D increased the expression of NR3C1 in cultured hippocampal neurons. Moreover, Nurminen et al[41] identified BCL6 as a primary target for vitamin D. It is puzzling that the expression of a large number of genes in examining the physiological response of monocytes to vitamin D, was induced after 24 hours, while these genes did not have a direct receptor for vitamin D. The results of their investigation showed that vitamin D affects the expression of these genes through the transcription factor BCL6, and most of the genes under the long-term effect of

vitamin D had a binding site for BCL6.[41] A previous study demonstrated that vitamin D suppresses the expression of LIF in the presence of cancer-associated fibroblast, and T-cell lines.[42] In a review study, Giangreco et al[43] reported the relationship between vitamin D and the expression level of miRNAs in cases such as prostate, breast, colon, and leukemia cancers, and their results indicate that vitamin D plays a role in regulating the expression level of many miRNAs. Unfortunately, there is no study on the relationship between LIF, BCL6, miR-494, miR-30d, and vitamin D in normal and pathologic endometrium but as mentioned above the miR-494, miR-30d, LIF, and BCL6 expression levels change might be due to vitamin D.

Effect of letrozole on mRNA expression in EEC of RIF and healthy women

Clinical and experimental data have confirmed that letrozole (non-steroidal aromatase inhibitor) does not have an antagonistic effect on endometrial estrogen receptors. Letrozole has a shorter half-life, and less effect on endometrial thickness compared to clomiphene citrate (steroidal aromatase inhibitor).[12] Letrozole has not affected the hypothalamus-pituitary axis associated with FSH negative feedback and is more suitable for endometrial implantation and receptivity.[44] Bilotas et al[45] and Khazaei et al[21] reported that letrozole has positive effect on endometrial receptivity and successful implantation through its effect on EECs. In addition, Ganesh et al[46] have indicated that the expression levels of LIF increased in endometrial epithelial and stromal cells of unexplained infertility women treated with letrozole. Moreover, the present study showed that pro-implantation genes (LIF, NR3C1, miR-30d) expression levels were higher in the letrozole treatment groups than in the no-treatment groups in both RIF and healthy women. In addition, the expression of anti-implantation genes (BCL6, miR-494) was lower in the letrozole treatment groups than in no-treatment groups in both RIF and healthy women. As mentioned above, letrozole has a positive effect on endometrial receptivity, but more studies are needed to clarify the mechanism of letrozole in the molecular process of endometrial receptivity.

Co-treatment (letrozole and calcitriol) or alone treatment (letrozole or calcitriol)

We found that NR3C1 and miR-30d expression levels were higher in the co-treatment groups than in the no-treatment groups in both RIF and healthy women. Furthermore, LIF, BCL6, and miR-494 expression levels were lower in the co-treatment groups than in the no-treatment groups in both RIF and healthy women. Moreover, we found that the expression of pro-implantation genes (LIF, NR3C1, miR-30d) were higher in the letrozole group than in the co-treatment or calcitriol group in both healthy and RIF women (Figure 2F). In addition, anti-implantation genes (BCL6, miR-494) expression levels were higher in the letrozole group than in the co-treatment or calcitriol group in both healthy and RIF women except in the case of BCL6 in RIF women. Khan et al[47] reported that the administration of vitamin D in women with breast cancer reduced musculoskeletal pain caused by letrozole. They reported that letrozole decreases estrogen production through aromatase inhibition. One of the side effects of decreasing estrogen is musculoskeletal pain and bone fractures. Taking vitamin D leads to a reduction in muscle pain and bone loss (a side effect of letrozole). We did not find any studies that reported the simultaneous effect of two drugs (letrozole, and calcitriol). Therefore, more studies are needed on the mechanism and the effects of co-treatment with letrozole and calcitriol on endometrial receptivity.

Correlations between miRNAs and mRNAs levels in EEC of RIF and healthy women

In the correlation analysis, we observed that in some of the treatment groups of this study, pro-implantation and anti-implantation genes had a positive correlation as well as in some cases, they did not have a positive correlation. Our purpose in investigating the correlation was to find a possible relationship between pro-implantation and anti-implantation genes. The different results obtained in this study can be due to the small size of the sample as well as the lack of sufficient information about the common points of the targets and pathways of the genes with each other, which requires more comprehensive investigations and bioinformatics studies.

Conclusion

In conclusion, our results showed that in all three types of treatment (letrozole, calcitriol, and co-treatment with letrozole and calcitriol), the expression level of pro-implantation genes (LIF, NR3C1, miR-30d) in RIF patients and healthy women increased. Moreover, in all three types of treatment (letrozole, calcitriol, and cotreatment with letrozole and calcitriol), the expression level of anti-implantation genes (BCL6, miR-494) in RIF patients and healthy women decreased. Therefore, it seems treatment with letrozole is more effective than co-treatment with letrozole and calcitriol or alone treatment with calcitriol on receptivity markers (LIF, NR3C1, BCL6, miR-494, miR-30d) in healthy women and RIF patients. If the use of letrozole is limited, it is suggested to use calcitriol only for the treatment of RIF patients.

Authors' Contribution

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Competing Interests
The authors declare no conflicts of interest.

Ethical Approval
The protocol used in the present study was approved by the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran, with the reference number IR.TBZMED.REC.1399.226. A written informed consent form was obtained from all the study subjects.

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