

The role of expressed T-cells cytokines mRNAs from endometrial tissue in patients with unexplained infertility

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The aim of this study was to investigate the immunomodulatory process and cytokines parameters that potentially modulates unexplained infertility (UI) by monitoring changes in the mRNA expression levels. From 52 UI patients who underwent biopsy, we collected endometrial tissue specimens between 21st-24th days of the menstrual cycles. Specimens were also collected from 34 matched healthy-fertility women, which served as control. Blood hormone levels were evaluated on 2nd and 3rd day of the menstrual cycles. We measured mRNAs of pro-inflammatory cytokines [tumor necrosis factor- α (TNF- α), interleukin (IL)-8], anti-inflammatory cytokine [IL-10], and other cytokines [IL-12A, IL-17A, IL-20, leukocyte inhibitory factor (LIF), transforming growth factor- β (TGF- β)]. Real-time q-PCR was used to examine immunomodulatory effects on UI cases. There was no significant difference between UI and control groups in terms of age, body-mass index (BMI), follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, thyroid-stimulating hormone (TSH), free T4 (FT4), and progesterone levels ($P > 0.05$). Estradiol (E₂) levels were significantly different between the groups ($P < 0.01$). Compared to fertile-controls, mRNA expression levels of IL-12A, IL-17A, TNF- α , and LIF were significantly lower in UI specimens ($P < 0.05$). IL-10 mRNA expressions were higher in UI than fertile-controls ($P < 0.05$). Spearman test demonstrated positive correlation between cytokines in the UI group ($P < 0.05$). Our findings highlight that depending on the patients' immunity, decreased expressions of IL-12A, IL-17A, LIF, TNF- α , and higher expressions of IL-10 in the endometrium of UI patients may be one of the potential cellular/molecular alteration mechanisms of infertility. Better understanding of these complex mechanisms may provide newer therapeutic targets for managing UI.

Keywords: Immunomodulators, mRNA, q-PCR, Therapeutic targets, Unexplained infertility

Infertility is a reproductive disease characterized by the absence of a clinical pregnancy after 12-months or more despite unprotected and/or regular intercourse¹. Infertility has been reported to be a growing problem that affects approximately 15% of the reproductive age couples². The estimated prevalence is reported to be between 10-15%, but the actual prevalence is estimated to be higher as diagnosis of the disease creates a social stigma in the community³. When the etiology of infertility was evaluated, approximately 75% of patients had ovulatory disorders, tubal obstruction and semen abnormalities; 25% of the patients were reported

to have endometriosis or unexplained infertility (UI). The UI is a condition in which no reason is found related to infertility parameters considered normal⁴. Worldwide, UI prevalence is between 22%-28%⁵.

The acceptance of a fetus by a woman's body results in an immunological environment/system that clears the condition of pregnancy necessary for the maternal immune system to tolerate successful pregnancy⁶. Recently, it has been argued as to what affects the UI, and several possible underlying causes have been proposed; however, the "immune system" was defined as a "natural" immunosuppressive condition that regulates the immune system to maintain a healthy pregnancy^{6,7}.

Cytokines, which can be defined as hormones of the immune system, play important role(s) in the

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regulation of immune system, thereby representing as pleotropic molecules^{8,9}. These molecules are synthesized from the immune and non-immune cells by the action of a stimulus, and are usually linked to their specific target cell receptors, involving various mechanisms/targets¹⁰. Cytokines are, for example, pro- and anti-inflammatory, or associated with different helper T-cells (Th) such as Th1, Th17, and regulatory (Treg) cells¹¹. Th1 is mainly responsible for the production of interleukins (IL), such as IL-1, IL-2, IL-12, IL-15, IL-18, interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α), whereas Th2 cells produce IL-5, IL-6, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF)¹². Th17 cells are the source of IL-17A and IL-17E. IL-17 induces inflammatory reactions and plays vital roles in immunity against fungal infections under physiological conditions¹³. Th17 has a direct involvement in pathological immune processes such as autoimmunity, allergy, transplant rejection, and pregnancy disorders¹⁴. Examples of cytokines secreted by Treg cells are IL-10, TGF- β , which are known to increase during immunosuppression and play critical roles during pregnancy.

Studies showing any relationship between immune system cells and UI have limitations depending on the number of cytokines investigated, the level of cytokine in serum, sperm, and endometrial tissue, or depending on the patient group selection^{15,16}. Previous studies focused immune system cells at the beginning and continuation of a successful pregnancy, and a relationship between immune system and infertility has been attempted^{17,18}. It also suggested to focus on Th1 and Th2 cells; but the relationship between infertility and immune system cells did not consist solely of these cells. Additional studies also hypothesized that Th17 and Treg cells may play role(s) in UI^{19,20}. Immunological marker(s), which can be used in the diagnosis of UI has not yet been established, therefore in this study, we explored different cytokines involved in the endometrial tissue samples of UI cases. Specifically, we aimed to compare the mRNA expression levels of IL-8, IL-10, IL-12, IL17-A, IL-20, TGF- β , TNF- α , and LIF from T-cells, and cytokines' levels in the endometrial tissues of UI and normal-fertile subjects.

Materials and Methods

Patients

The Ethics Committee approval was obtained from the Health Sciences University of Zeynep Kamil

Women and Children Diseases Training and Research Hospital, Istanbul, Turkey. Informed consents were obtained from all the subjects. The study consists of 52 UI patients whose biopsy was performed to collect endometrial tissue specimens between the days 21st-24th of the menstrual cycles during December-2015 and August-2017. The causes of infertility is defined as UI because all parameters were normal. For comparison as control, 34 matched fertility women were included for all conditions who were considered healthy and had at least one live birth within 3 years (with proven fertility and had regular menstrual cycles). Blood hormone levels were evaluated on the 2nd and 3rd day of the menstrual cycles. Key parameters such as the age, BMI, FSH, LH, PRL, TSH, FT4, and progesterone levels of all the subjects were examined.

Patients were included to study whose ovarian reserve was normal, which refers to the reproductive potential within the two ovaries measured by the number and quality of the woman's eggs, was determined on the second or third day of menstruation by the amount of FSH in serum and the number of antral follicles.

Selection Criteria

Inclusion Criteria

The study included cases whose diagnosis of UI has been established and were infertile for at least one-year, had regular ovulation and menstruation, had normal spermogram results and whose tubal patency was confirmed by hysterosalpingography (HSG) as normal. The healthy volunteers with progesterone levels above 3 ng/mL were included in the study.

Exclusion Criteria

Cases that presented tubal factor, polycystic ovary syndrome (PCOS), total progressive sperm count of less than 20 million, and who had low ovarian reserve were excluded from this study. Also, patients with chronic drug use, chronic diseases, and BMI >25 kg/m² were excluded. Patients with progesterone levels below 3 ng/mL were also excluded. Other exclusion criteria were that none of the subjects had used steroidal contraceptives for at least 3-months before the study, or used any intrauterine device for at least 6 months before the study enrolment.

Preparation of Tissue Specimens

After the endometrial biopsy, the tissues were fixed in paraffin and taken into a 1.5 mL tube. The RNA, which inhibits the degradation of the RNA in the

tissue and retards the disintegration process for a long time, was treated with the lateral solution at +4°C for 1-day in accordance with the laboratory protocol. The RNA lateral solution around the tissue was removed by pipette and the specimens were stored at -80°C until the RNA was purified.

Target Biomarkers Selection

Key target biomarkers were selected based on the published peer-reviewed literatures that showed possible links to induce/alter its association with the UI, and the reasons (rationale) for their selection is summarized in (Table 1). These biomarkers include LH, FSH, prolactin, TSH, estradiol, FT4 and progesteron. Additional biomarkers included are: pro-inflammatory cytokines (TNF- α), chemokine (IL-8), anti-inflammatory cytokine (IL-10), and other cytokines/growth factors (such as IL-12A, IL-17A, IL-20, TGF- β 2, and LIF)²¹.

Determination of Total RNA Concentration

The concentration of RNA in samples were evaluated using spectrophotometric absorbance values

at 260 and 280 nm wavelength using a NanoDrop 2000 Spectrophotometer. As shown in the formula below, the RNA concentration was calculated by multiplying the absorbance value and multiplying the RNA coefficient and the dilution coefficient:

$$\text{RNA concentration } (\mu\text{g/mL}) = \text{OD at 260 nm} \times \text{CC} \times 40 \mu\text{g/mL}$$

where: OD = optical density, nm = nanometer, CC = coefficient, and an absorbance of 1 Unit at 260 nm corresponds to 40 μg of RNA per mL ($A_{260} = 1 = 40 \mu\text{g/mL}$)²².

Gene Expression Analysis

Real-Time-PCR

For quantification of mRNA expression in tissues, total RNAs were isolated by using the Total RNA Purification Kit-Column Kit (Jena Bioscience, Germany) as per the manufacturer's instructions. After completion of RNA isolation, its purity and concentration were calculated utilizing NanoDrop 2000 (Thermo Fisher Scientific, Pleasanton, CA,

Table 1 — Selection of target biomarkers for the assessments of cytokines' levels and mRNA expression that potentially modulates unexplained infertility (UI)

Protein with UI	Gene Name	Gene ID	Accession Number	Rationale for Selection	Reference(s)
Interleukin-17A	IL-17A	3605	Q16552	Th17 cells, a sub-group of helper T-cells, produce IL-17A, which is pro-inflammatory and they play important roles in initiating inflammation and acute transplant rejection	[24,25]
Interleukin-10	IL-10	3586	P22301	During early pregnancy stage, IL-10 and its receptors produced by Treg cells should be present in the endometrium and decidua. IL-10 causes proliferation of decidual cells and secretion of TNF- α	[26]
Interleukin-12 Subunit Alpha	IL-12A	3592	P29459	At the beginning of the inflammatory response, phagocytic cells produce IL-12, a cytokine that provides an important functional bridge between the innate resistance and immune responses	[27]
Transforming Growth Factor- β	TGF- β	7040	P01137	TGF- β levels increase during immunosuppression and play important roles during pregnancy	[28]
Interleukin-20	IL-20	50604	Q9NYY1	IL-20 regulates proliferation and differentiation of keratinocytes during inflammation, particularly dermal inflammation. In addition, IL-20 also causes cell expansion of multipotent hematopoietic progenitor cells	[29]
Interleukin-8	IL-8 (CXCL8)	3576	P10145	In humans, natural-killer cells in the decidua during early pregnancy are the main sites where CXCL8 is produced which is a chemokine. Elevated plasma level of IL-18 is correlated with endometriosis and UI	[30,31]
Tumor Necrosis Factor- α	TNF- α	7124	P01375	A pro-inflammatory cytokine that simplify fertilization by suppressing the immune system	[32]
Leukemia Inhibitory Factor	LIF	3976	P15018	LIF belongs to the IL-6 family, considered one of the cytokines necessary for the successful completion of human pregnancy. Implantation in cases with UI of recombinant LIF, it has been suggested that it can help improve the speed	[33,34]

Table 2 — The list of primers used for real-time PCR analysis

Gene Name	Gene Bank #	Primer Sequences	Product Size (bp)
IL-17A	NM_002190.3	Forward 5'-GAACGTGGACTACCACATGAA-3' Reverse 5'-TCCCCAAGCCCAGAATCTTATC-3'	532
IL-10	NM_001382624.1	Forward 5'-GAACCAAGACCCAGACATCAA-3' Reverse 5'-CCAAGCCCAGAGACAAGATAAA-3'	2789
IL-12A	NM_001354583.2	Forward 5'-GACAGAAAGCAAGAGACCAGAG-3' Reverse 5'-GGCACAGGGCCATCATAAA-3'	
TGF- β	NM_000660.7	Forward 5'-CGCGCCCATCTAGGTTATTT-3' Reverse 5'-GCCAGGAATTGTTGCTGTATTT-3'	1170
IL-20	NM_018724.3	Forward 5'-GGATGGAGGAGACAGAATAGGA-3' Reverse 5'-CCTGGCTGGAAGATACACAATTA-3'	626
IL-8	NM_000584.4	Forward 5'-GTGCCTTGGTTTCTCCTTTATTT-3' Reverse 5'-CAATGACAAGACTGGGAGTATCA-3'	452
TNF- α	NM_000594.4	Forward 5'-CTCAACCTCTTCTGGCTCAAA-3' Reverse 5'-CCACTGAATAGTAGGGCGATTAC-3'	697
LIF	NM_002309.5	Forward 5'-TGTCGCTCTCTAAGCACTTTAC-3' Reverse 5'-GGTCCACACCAGCAGATAAA-3'	562

Abbreviations: PCR = Polymerase Chain Reaction; bp = Basepair; IL = Interleukin; TGF- β = Transforming Growth Factor- β ; TNF- α = Tumor Necrosis Factor- α ; LIF = Leukemia Inhibitory Factor

USA). For this, 1 μ L RNA samples were pipetted in the device for determination of 260/280 and 260/230 ratios. Concentrations of all RNA samples were equalized before reverse transcription. RNAs were reverse transcribed into cDNA according to the kit protocol utilising Script cDNA Synthesis Kit (Jena Bioscience, Germany). The resulting cDNA was amplified by quantitative real-time-polymerase chain reaction (qRT-PCR) using qPCR GreenMaster with the UNG/Low ROX Kit (Jena Bioscience, Germany). The real-time conditions were carried out on the Stratagene MX3005P Real-Time PCR System (Agilent Technologies, Inc., Santa Clara, CA, USA) as follows: 50°C, 2 min; 95°C, 2 min; followed by 40 cycles of 95°C, 15 s; 58°C, 20 s; and 72°C, 30 s. The relative mRNA transcript levels were calculated according to the $2^{-\Delta\Delta CT}$ method²³ and the relative expression of each gene was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers were obtained from the LGC Biosearch Technologies (Denmark). The specific primers used are shown in (Table 2). All measurements were performed at least in triplicate.

Statistical Analyses

In our study, mean and standard deviation (SD) values were calculated as descriptive statistics for continuous variables. Frequency distributions are disclosed as ratios. Student's *t*-test was used to compare mean values of the control and case samples. The Spearman test was used to compare the correlation between the cytokines ΔCt values. During

Table 3 — Comparison of demographic characteristics and biochemical parameters between the unexplained infertility and fertile-control groups of subjects

Parameters	Unexplained Infertility Group (n= 52)*	Fertile Group (n= 34)*	<i>p</i> -Value
Age (year)	27.08 \pm 4.84	29.64 \pm 3.47	0.12
BMI (kg/m ²)	23.98 \pm 4.01	24.51 \pm 3.24	0.65
FSH (IU/L)	5.99 \pm 1.36	5.66 \pm 1.93	0.52
E ₂ (pg/mL)	38.62 \pm 12.07	51.62 \pm 18.42	0.01
LH (U/L)	5.24 \pm 1.80	6.77 \pm 3.76	0.47
PRL (pmol)	17.26 \pm 7.21	19.40 \pm 19.27	0.61
TSH (mIU/L)	1.92 \pm 0.87	2.57 \pm 1.91	0.14
FT4 (ng/dL)	1.04 \pm 0.22	1.03 \pm 0.19	0.88
Progesterone (ng/L)	8.65 \pm 7.53	6.46 \pm 4.96	0.28

*The values are expressed as mean \pm standard deviation (SD). Parametric comparisons were made using the student's *t*-test, and non-parametric comparisons were made using the Mann-Whitney *U* test.

p-Value in bold represents significant.

Abbreviations: BMI, body mass index; FSH, follicle stimulating hormone; E₂, estradiol; LH: luteinizing hormone; PRL, prolactin; TSH, thyroid stimulating hormone; FT4, free T4

the analyses, it was decided that the hypothesis was bidirectional and $p \leq 0.05$ as meaningful/significant. For all the statistical analyses, SPSS (Statistical Package for Social Sciences for Windows) program 17.0 was used.

Results

This study included 52 subjects with UI and 34 healthy fertile women as control. Table 3 shows a comparison of demographics of the participants.

There was no significant difference between the two groups in terms of subjects' age ($P= 0.122$) and BMI ($P= 0.647$). Also, there was no significant difference between the two groups when comparing the levels of FSH, LH, PRL, TSH, FT4, and progesterone levels ($P> 0.05$). Only estradiol (E_2) levels were found to be significantly different between the groups ($P< 0.01$) (Table 3).

Cytokines' levels of patients with UI and fertile groups were interpreted according to the Δ Ct values, as summarized in (Table 4). Notably, IL-12A mRNA levels in patients with UI were significantly lower than the fertile group cases ($P= 0.002$). We also observed significantly decreased levels of LIF, IL-17A, and TNF- α mRNA levels in patients with UI as compared with the fertile group. In contrast, IL-10

Table 4 — Comparison of cytokine (Δ Ct) profiles in patients with unexplained infertile (UI) and fertile groups

Cytokines mRNA (Δ ct)	Unexplained Infertile Group (n= 52)*	Fertile Group (n= 34)*	<i>p</i> -Value
IL-17A	7.26 \pm 1.63	8.53 \pm 1.74	0.03
IL-10	6.0 \pm 1.82	4.62 \pm 1.66	0.02
IL-12A	3.91 \pm .98	5.93 \pm 1.21	0.002
TGF- β	6.08 \pm 1.85	6.47 \pm 1.84	0.50
IL-20	6.0 \pm 1.87	5.94 \pm 2.25	0.93
IL-8	6.36 \pm 1.91	6.0 \pm 2.31	0.56
TNF- α	4.11 \pm 1.57	5.15 \pm 1.21	0.05
LIF	4.36 \pm 1.55	5.2 \pm 2.042	0.01

*The values are expressed as the mean \pm standard deviation (SD). *p*-Values in bold represent significant.

Abbreviations: Δ ct, change in cytokine; IL, interleukine; TGF- β , transforming growth factor; TNF- α , tumor necrosis factor-alpha; LIF, leukemia inhibitory factor

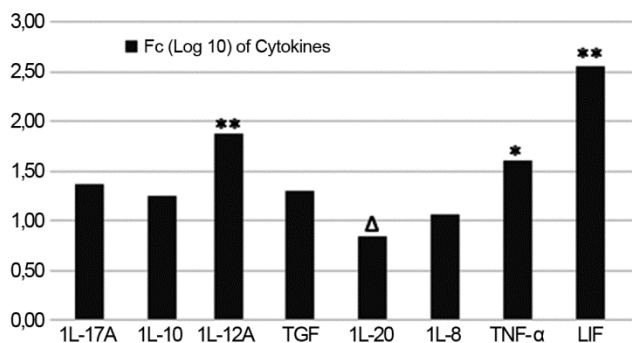


Fig. 1 — Comparison of patients with unexplained infertility (UI) groups with control (fertility) group of Fc of cytokines by taking the value of Fc (log 10) in the control group as 1,00. (For up-regulation $**P< 0.001$; $*P< 0.05$; for down-regulation $\Delta P< 0.05$). Abbreviations: IL = Interleukin; TGF- β = Transforming Growth Factor- β ; TNF- α = Tumor Necrosis Factor- α ; LIF = Leukemia Inhibitory Factor

mRNA levels in patients with UI were significantly higher as compared to the control-fertile group cases ($P< 0.02$). No significant difference was noted between the two groups for the mRNAs levels of IL-8, TGF- β , and IL-20 [Table 4].

Figure 1 shows fold change (Fc-Log 10) values of cytokines, chemokine, and growth factor. We observed significant changes in the LIF and IL-12A levels ($P< 0.001$). The level of Fc of TNF- α was also significantly changed ($P< 0.05$), and Fc of IL-20 is shown as downregulated ($\Delta P< 0.05$). Figure 2 shows a comparison of the studied cytokines between the two groups based on Δ Ct values. The IL-12A and IL-17A mRNA expression levels in patients with UI group were significantly lower than the fertile-control group cases ($P= 0.002$, $P< 0.03$, respectively). Likewise, the levels of TNF- α and LIF mRNA levels in patients with UI were significantly lower as compared with the fertile-control cases ($P< 0.05$; $P< 0.01$, respectively). In contrast, the IL-10 mRNA expression in patients with UI was significantly higher than the control cases ($P= 0.02$). No significant difference was noted when the two groups of TGF- β , IL-8, and IL-20 mRNAs levels were compared (Fig. 2).

Table 5 compares the values for IL8, IL-10, IL-12A, IL-17A, IL-20, TGF- β , TNF- α , and LIF in UI and fertile-control groups' cases on the basis of progesterone level >3 years. No significant difference was observed between the cytokine levels in the two groups ($P> 0.05$). The comparative values of IL-8, IL-10, IL-12A, IL-17A, IL-20, TGF- β , TNF- α , and LIF in the UI and fertile group are given in Table 6 (based on infertile duration >2 years and

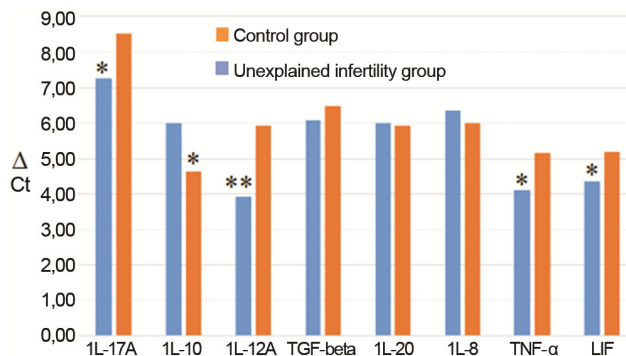


Fig. 2 — Comparison of patients with unexplained infertility (UI) groups with control group in terms of Δ Ct value of cytokines ($*P< 0.05$; $**P< 0.002$). Abbreviations: Δ ct = Change in Cytokine; IL = Interleukin; TGF- β = Transforming Growth Factor- β ; TNF- α = Tumor Necrosis Factor- α ; LIF = Leukemia Inhibitory Factor

Table 5 — Comparison of cytokines according to progesterone >3-years in patients with unexplained infertility (UI)

Cytokines (Δ ct)	Progesterone Level (> 3 Years)*	<i>p</i> -Value
IL-17A	7.23 \pm 1.92	0.90
IL-10	4.57 \pm 1.65	0.86
IL-12A	3.94 \pm 1.84	0.93
TGF- β	6.33 \pm 1.78	0.28
IL-20	6.78 \pm 1.99	0.08
IL-8	5.54 \pm 1.27	0.23
TNF- α	4.23 \pm 1.59	0.39
LIF	4.67 \pm 1.33	0.12

*The values are expressed as mean \pm standard deviation (SD).

Abbreviations: Δ ct, change in cytokine; IL, interleukine; TGF- β , transforming growth factor; TNF- α , tumor necrosis factor-alpha; LIF, leukemia inhibitory factor

Table 6 — Comparison of the levels of cytokines in infertile group with unexplained infertility (UI) over the duration of infertility

Cytokines (Δ ct)	Infertility Duration (> 2 Years)*	Infertility Duration (< 2 Years)*	<i>p</i> -Value
IL-17A	7.19 \pm 1.72	7.67 \pm 1.16	0.65
IL-10	4.72 \pm 1.74	4.0 \pm 1.0	0.50
IL-12A	3.90 \pm 2.10	4.0 \pm 1.0	0.94
TGF- β	6.09 \pm 1.95	6.0 \pm 1.0	0.94
IL-20	6.45 \pm 1.92	5.67 \pm 2.08	0.52
IL-8	5.29 \pm 1.49	5.0 \pm 1.41	0.79
TNF- α	3.94 \pm 1.98	4.0 \pm 2.0	0.96
LIF	4.41 \pm 1.44	4.0 \pm 2.65	0.82

*The values are expressed as mean \pm standard deviation (SD).

Abbreviations: Δ ct, change in cytokine; IL, interleukine; TGF- β , transforming growth factor; TNF- α , tumor necrosis factor-alpha; LIF, leukemia inhibitory factor

infertility duration < 2 years). Also, no significant difference was found in the mRNA expression levels of these cytokines ($P > 0.05$) when comparing the two groups of infertility time > 2 years and infertility time < 2 years.

Discussion

Unexplained fertility is a unique disease, and generally no specific cause has been identified yet. However, studies implicate that some cytokines/growth factors may be associated during immunobiological process of the disease course^{35,36}. Therefore, in this study we sought to evaluate the levels of mRNAs expression in the samples of women with UI in comparison to fertile (normal) women for key immunologic biomarkers. We also compared the hormone levels in the age- and BMI-matched cases with UI to better understand any correlatable relationship with immunological defects. Our preliminary findings suggest that the

underlying pathophysiological mechanisms of these biomarkers in UI may be relevant because of the altered levels of cytokines, chemokines, hormones, and growth factors, etc.

In this study, hormone (progesterone) effect was evaluated in patients with UI and correlated with fertile women, and both groups did not show any difference in terms of FSH, LH, PRL, TSH, FT4, and progesterone levels. Only estradiol (E₂) levels were found to be significantly different between the groups (decreased E₂ levels in UI cases). Landgren *et al*³⁷ also showed that E₂ level was changing before the final menstrual period compared with the FSH, LH, and progesterone levels. We still do not know as to why E₂ levels persist in follicular senescence; however, Welt *et al*³⁷ hypothesized that E₂ levels remain firm because of the increased ovarian aromatase function in the late menopause transition. It is widely recognized that E₂ has several functions to maintain the reproductive system in the female body. Notably, E₂ levels are increased during the menstrual cycle to support maturation and release of the egg, and this hormone levels are decreased significantly during early menopause³⁸. Therefore, significantly decreased E₂ levels in our patients with UI is interesting observation and we suggest that the altered E₂ levels in patients UI could be a new aspect to further explore mechanistically. In contrast, the levels of FSH, LH, PRL, and progesterone remained unchanged, and interestingly a strong negative correlation was observed in regards to E₂ levels versus other hormones ($P < 0.001$).

Cytokines and growth factors are thought to play key immunoregulatory roles in the production of successful implantation during the UI. Likewise, the immune system also play important roles during a successful pregnancy^{16,39-41}. Therefore, in the current study, we aimed to investigate whether various cytokines, chemokine (IL-8), and growth hormones have any effect on the UI cases. We found that IL-12A, IL-17A, TNF- α , and LIF levels were decreased in patients with UI, and the level of IL-10 increased as compared to the fertile group of women. The increased levels of IL-10 and decreased levels of TNF- α may be indicative of an inflammatory response during UI. It is therefore plausible that increased IL-10 may impair successful pregnancy due to suppressed immunity.

Also, we observed a significant and positive correlation between IL-10 Δ Ct levels and IL-12A, TGF- β , TNF- α and LIF Δ Ct levels in patients with UI,

respectively ($P < 0.001$). In contrast, we found a significantly positive correlation between ΔCt levels of TNF- α and all of the studied parameters except LIF in the fertility (control) group ($P < 0.001$). TNF- α levels were directly connected with ΔCt level of other parameters in the fertility group, however this discrepant observation in our UI cases may be questionable. We determined Fc levels that changed LIF and IL-12A levels significantly ($P < 0.001$). Level of Fc of TNF- α was also significantly changed, and Fc of IL-20 was down-regulated. When we evaluated IL-8, IL-10, IL-12A, IL-17A, IL-20, TGF- β , TNF- α , and LIF values of UI cases in comparison to fertile cases on the basis of progesterone level > 3 years, no statistical significant was observed.

Our findings suggest that decreased levels of four biomarkers (i.e., IL-12A, IL-17A, TNF- α , and LIF) could be an indication of UI, and the increased level of IL-10 may also be associated with the UI pathobiology. Reduced LIF levels may affect normal endometrium growth in women with UI, which may lead to failure of implantation⁴⁰. It is well-known that the anti-inflammatory cytokine (IL-10) is associated with angiogenesis and defense against extracellular pathogens. Our UI cases showed increased IL-10 levels, which may potentially be responsible for signaling during pathogenic activities of UI. Disruption of the endometrial condition is therefore suggested among the main problems of UI, as our study focused on immune factors playing any role(s) in the UI. The potential limitations of our research study are relatively small number of cases in both groups, and that the additional parameters (biomarkers) have not been studied and/or correlated with different methodological (instrumental) techniques.

Conclusion

Unexplained infertility (UI) is a condition in which generally no reason is found related to infertility parameters being considered normal. We investigated the immunomodulatory process and cytokines' parameters that potentially modulates UI by monitoring changes in the mRNA expression levels. We observed that there was no significant difference between UI and fertile (control) groups of women in terms of age, BMI, FSH, LH, prolactin, TSH, FT4, and progesterone levels. Estradiol levels were significantly different between the groups. Compared to fertile-controls, mRNA expression levels of IL-12A, IL-17A, TNF- α , and LIF were significantly lower in UI specimens, and IL-10 mRNA expressions

were higher in UI group. Spearman test demonstrated positive correlation between cytokines in the UI group. We conclude that depending on the patients' immunity, decreased expressions of IL-12A, IL-17A, LIF, TNF- α , and higher expressions of IL-10 in the endometrium of UI patients may be potential immunobiological alteration mechanisms of infertility. Better understanding of these processes may provide newer therapeutic targets for managing UI, hence further mechanistic studies pertaining to immunopathobiology of UI would be desirable to further validate our research findings.

Conflict of interest

All authors declare no conflict of interest.

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