ORIGINAL ARTICLE

Seminal plasma and CD4+ T-cell cytokine profiles in the *in vitro* fertilization success

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Background: Abnormal female immune response is one of the potential causes of unexplained infertility (UI). Seminal plasma (SP) is an important regulator of female immune responses during pregnancy. This study investigated a SP effect on the expression of CD4+ T-cell-related cytokines in a group of UI woman candidates for *in vitro* fertilization (IVF) and healthy fertile women. **Materials and Methods:** This was a semi-experimental study that performed on 20 UI couples (ten unsuccessful and ten successful IVF outcomes) and 10 fertile couples as the healthy group. CD4+ T-cells were separated from peripheral blood mononuclear cells of women by magnetic-activated cell sorting technique and incubated with (stimulated condition) or without (unstimulated condition) SP of their husbands. After incubation, real-time polymerase chain reaction method was used to investigate interleukin (IL)-23, IL-17, IL-4, IL-10, transforming growth factor (TGF)-β, and interferon (IFN)-γ gene expression. Mann–Whitney U-test, Kruskal–Wallis test, and Wilcoxon signed-rank test were used for statistical analysis. **Results:** Baseline TCD4+ mRNA levels of IL-23 (P = 0.03) and TGF-β (P = 0.01) were different between healthy and infertile groups. However, IL-17, IL-4, IFN-γ, and IL-10 were expressed similarly regardless of fertility status. Comparing mRNA expression before and after SP exposure, our results have shown that relative expression of IL-23 significantly increased in successful (P = 0.04) and unsuccessful IVF groups (P = 0.01), whereas IL-10 expression increased only in the IVF failure group (P = 0.01). **Conclusion:** SP can make a positive effect on IVF outcome through alteration in CD4 + T-cell-related cytokines expression, especially IL-10 and IL-23.

Key words: CD4-positive T-lymphocytes, fertilization *in vitro*, infertility, real-time polymerase chain reaction, seminal plasma

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INTRODUCTION

Infertility is a failure of the reproductive system for achievement of a successful pregnancy after 1 year of regular unprotected intercourse. This condition has an effect on approximately 7%–15% of couples worldwide.[1] The reasons for infertility remain unknown in 25% of infertile patients who belong to unexplained infertile group.[2] The immune system has the most important effect on pregnancy outcome, and an immune imbalance was considered as the main reason for 50% of unexplained infertility (UI) cases.[3] *In vitro* fertilization (IVF) is a potential treatment method for UI while the results of it are varied.[4] Several studies have shown that poor response to ovarian stimulation and also some immunological factors are related to IVF failure.[5,6]

T-helper (Th) cells, which are one of the main immune cell subsets of the adaptive immune system, play an important role in pregnancy period.[6] Four CD4+ T-cells subsets including Th1, Th2, Th17, and regulatory T-cells (Treg) are recognized as the main players of the immune system during pregnancy and also its health problems.[7] In recent years, several studies have shown that immune hemostasis between Th cell subsets contributes to fertility and successful outcomes.[7] Different Th cell subsets secrete distinct types of cytokines that act together as a regulatory pathway during successful pregnancy.[8]

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Th1 cells secret some inflammatory cytokines including interferon (IFN)-γ and tumor necrosis factor (TNF)-α which play a significant role in inflammatory and cell-mediated immune responses. On the other hand, Th2 cells are included in anti-inflammatory and humoral immune responses by producing interleukin (IL)-4, IL-5, and IL-13.[7] Evidence from studies on murine and human pregnancy indicates an association between successful pregnancy and Th2-type immune responses, while Th1 immune response is associated with pregnancy failure.[8] Th17, a subset of $CD4^+$ T-cells, is identified by expressing retinoic acid receptor-related orphan receptors (Rorc) and IL-17 production. Th17 cells are involved in many pregnancy disorders including preeclampsia and recurrent spontaneous abortion.[9] Tregs, which originated from naive $CD4^+$ T-cells under transforming growth factor (TGF)-β effect, are the main type of T-cells that are crucial for the maternal immune tolerance to the fetus. Treg disabilities are reported in UI, recurrent abortion, and also some pregnancy diseases such as preeclampsia and fetal growth restriction.[10]

Successful implantation needs to some cellular and molecular changes in the uterine tissue.[11] Some studies suggested that seminal plasma (SP) component such as TGF-β through providing the antigenic and environmental signals in uterine may have a critical role in successful implantation.[11] In this line, some evidence showed that patients who received intravaginal SP treatment in the time of ovum pick-up or embryo transfer had higher implantation and clinical pregnancy rates compared to those who did not receive such treatment.[12] SP components have an effect on the expression of a wide range of female immune factors such as some cytokines and chemokines that led to activation and regulation of the immune cells in the uterus.[13,14] In a previous study, we have shown that SP might increase Th17 and Treg cell frequencies in infertile women with IVF failure and might also balance inflammatory to regulatory responses to finally tune-up the Th1/Th2/Th17/Treg balance and support the success of IVF.[15] Furthermore, we have recently reported the SP effects on cytokine expression level which released by peripheral blood mononuclear cells (PBMCs).[16] We showed that after SP exposure, the mRNA level of forkhead box protein 3 (Foxp3), Rorc, IL-23, IL-17, IL-6, TGF-β, and IL-35 increased in women with successful IVF outcome.[16] In line with our previous studies, we aimed to elucidate the probable role of SP mediated by cytokine expression in success of fertilization following IVF treatment. To attain this goal, for the first time, the mRNA level of $CD4^+$ T-cell-related cytokines was investigated before and after SP exposure in healthy and unexplained infertile women with successful and unsuccessful IVF outcomes.

MATERIALS AND METHODS

Subjects

This semi-experimental study was approved by the local Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1394.S632). All individuals signed informed consent. Individuals enrolled in the present study were selected from our subject's bank and were the same as our previous study.[15]

Blood and semen samples were collected from 20 unexplained infertile couples who were candidates for IVF treatment as an infertile group and 10 fertile couples as a healthy group. Among infertile couples, 10 had successful IVF outcome and so were considered as a successful group and the remaining 10 had unsuccessful IVF outcome so were considered as an unsuccessful group. The infertile women who participated in this study had no intercourse 2 weeks before IVF treatment. No random allocation was performed in this study.

Moreover, all unexplained infertile couples were checked for history of previous positive pregnancy and immunological disorders at the time of sampling. Participants with such problems besides the men with male tract infections or varicocele were excluded from the study. The healthy fertile group was interviewed for the recorded history of fertility problems and selected if they had one previous termed pregnancy. Other excluding criteria were the same as the fertile group. Hormone level was measured for all women. Moreover, semen analysis test was performed for all male partners in the infertile and healthy groups.

SP preparation

Semen was obtained from all participating men. Fresh semen was liquefied for 30 min at 37°C and then centrifuged for 15 min at 3000 RPM (room temperature). The supernatant was collected and centrifuged again at 14,000 RPM for 30 min in 4°C to remove all remaining spermatozoa. Finally, SP was collected and stored at −70°C.

Peripheral blood mononuclear cell isolation

PBMCs were isolated from whole blood samples by a density gradient centrifugation method using Lymphoprep (Axis-Shield, Oslo, Norway) and then washed in RPMI 1640 media (Gibco by Thermo Fisher Scientific, New York, USA). Cells were counted and resuspended to cryovials containing freezing media and preserved in liquid nitrogen tank until the time of experiments.

Isolation and purification of $CD4^+$ T-cells by magnetic-activated cell sorting

In our experiment, a negative selection strategy was used to separate $CD4^+$ T-cells. The $CD4^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with

95%–99% reproducibly was used for fast isolation of these cells from PBMCs. 8 × 10^6 PBMCs were resuspended in magnetic-activated cell sorting (MACS) buffer and $CD4^+$ T-cell biotin-antibody cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation, the $CD4^+$ T-cell microbead cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the mixture and incubated for 20 min in the refrigerator. Then, the cell suspension was applied onto the MS column (Miltenyi Biotec, Bergisch Gladbach, Germany) in order to achieve $CD4^+$ T-cells. Approximately 47% of cells were positive for CD4 before MACS isolation while this percentage increased to 97% after MACS purification [Figure 1]. For each individual, the viability and the number of cells were up to 97% and 3 × 10^6, respectively. Subsequently, the cells were cultured in RPMI for SP stimulation.

Cell culture

A total of 1 × 10^6 female PBMCs/well were cultured without exposure to male SP (unstimulated condition or before intervention) and also co-cultured with SP (stimulated condition or after intervention) (0.3% V/V total concentration) in the 24-well plates in 1 ml RPMI 1640 media supplemented with 10% fetal bovine serum (Shell Max, China) and 1% penicillin-streptomycin (Shell Max, China) and incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After culture, cells were collected and stored in −70°C until the time of experiments.

Real-time polymerase chain reaction

Cellular RNA was extracted from isolated $CD4^+$ T-cells by RNA isolation kit (Pars tous, Mashhad, Iran). Then, the RNA was quantified by spectrophotometry (Eppendorf BioPhotometer D30 Hamburg, Germany). 150–200 ng of total RNA template was reversed transcript into cDNA by the ABI high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a sensitivity of 0.02–0.2 µg, according to the manufacturer's protocol, and cDNA was stored at −70°C. SYBR Green Master Mix reagents (Applied Biosystems, Foster City, California, USA) was used for amplification of cDNAs by real-time polymerase chain reaction (PCR) technique using QuantStudio™ instrument (Applied Biosystems, Foster City, California, USA). Amplification program was same for all studied mRNAs: 95°C for 10 min (Hot start) and 45 cycles consisting of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The dissociation protocol was added after the final PCR cycles to assure specificity. The sequence of all corresponding primers was derived from related published studies.[17,18] All used primers are summarized in Table 1. The ΔΔCt method, normalized target gene to RPL13a expression as a housekeeping gene, was applied to quantify the mRNA expression of IL-23, IL-17, IL-10, IL-4, IFN-γ, and TGF-β genes.

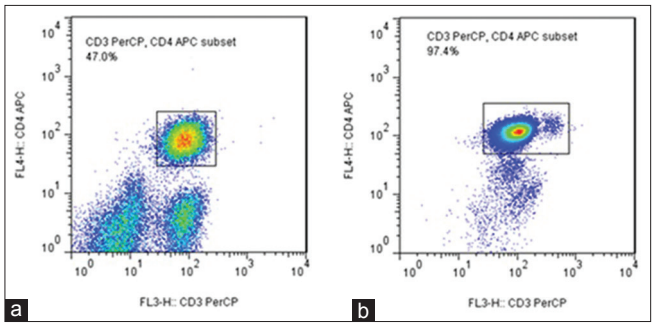

Figure 1: Purity of magnetic-activated cell sorting isolated T-cell. (a) CD3+CD4+ T-cell before and (b) after magnetic-activated cell sorting isolation

Statistical analysis

Statistical analysis was performed by SPSS software version 18 (SPSS Inc., Chicago, Illinois, USA). Mann–Whitney U-test and Kruskal–Wallis test were used for mean comparison between two or more than two groups, respectively. Wilcoxon signed-rank test was used for comparing two paired groups (stimulated and unstimulated conditions). $P < 0.05$ was considered as statistically significant.

RESULTS

Unstimulated $CD4^+$ cultures

Baseline $CD4^+$ mRNA level (unstimulated condition) of IFN-γ, TGF-β, IL-4, IL-17, IL-23, IL-10, and RPL13a genes were assessed using SYBR Green real-time PCR method [Table 2]. The level of IL-23 and TGF-β was significantly different between the healthy fertile and infertile groups ($P = 0.03$, $P = 0.01$, respectively) [Table 2]. The expression of IL-23 ($P = 0.008$) decreased in successful IVF, whereas the expression of TGF-β ($P = 0.01$) decreased in women with unsuccessful IVF as compared with the healthy group [Table 2]. There are no significant differences in unstimulated levels of IFN-γ, IL-17, IL-10, and IL-4 among different infertile and healthy groups [Table 2].

The difference in the mRNA levels of $CD4^+$ T-cell-related cytokines in seminal plasma stimulated and nonstimulated conditions

Mean fold change in cytokine expression after SP stimulation (stimulated condition) in comparison to unstimulated condition (calibrator) is presented in Table 3. There were no significant differences in the relative expression of cytokines in the healthy group after SP treatment. The relative expression of IL-23 significantly increased in the successful IVF group after SP stimulation ($P = 0.04$) while there were no significant differences regarding the expression of other cytokines in this group [Table 3]. The relative expression of IL-23 and IL-10 in the IVF failure group was significantly higher compared with an unstimulated condition (before treatment with SP) ($P = 0.01$ and 0.01, respectively).

Table 1: Primer sequences used for real-time polymerase chain reaction

Gene	Accession number	Primer sequence 5'□ 3'	Amplicon (bp)	Tm of amplicon (°C)
IL-4	M13982	TCCGATTCCTGAAACGGCT TCTGGTTGGCTTCCTTCACAG	81	83
IL-17	NM_002190	CAATGACCTGGAAATACCCAA TGAAGGCATGTGAAATCGAGA	52	71
IL-23	NM_016584	GGACAACAGTCAGTTCTGCTTGC AGGCTCCCCTGTGAAATATCC	91	78
TGF-β	M38449	GGTGGAAACCCACAACGAAAT TCTCGGAGCTCTGATGTGTTGA	85	75
IFN-γ	NM_0006192	GTGTGGAGACCATCAAGGAAGACA TTGGACATTCAAGTCAGTTACC	110	75
IL-10	NM-000572.2	TGAGAACAGCTGCACCCACT GGCAACCCAGGTAACCCTTA	164	81.4
RPL-13	NM-012423	CATAGGAAGCTGGGAGCAAG GCCCTCCAATCAGTCTTCTG	157	85

Bp=Base pair; IL=Interleukin; TGF=Transforming growth factor; IFN=Interferon; RPL=Ribosomal protein L

Table 2: Fold change in relative expression of cytokine in unstimulated CD4⁺ from infertility and fertile group

Cytokine	Mean (SD)			$P^{\dagger}$
	Healthy (*n*=10)	Successful IVF (*n*=10)	Unsuccessful IVF (*n*=10)	
IFN-γ	0.9 (0.2)	1.3 (0.9)	0.9 (0.7)	NS
IL-17	2.9 (1.2)	0.6 (0.1)	6.2 (2.2)	NS
IL-23	1.4 (0.5)[a]	1 (0.03)[a]	1 (0.7)	0.03
TGF-β	1 (0.1)[b]	0.9 (0.2)	0.1 (0)b	0.01
IL-10	0.5 (0.1)	1.8 (1)	2.4 (2.2)	NS
IL-4	1 (0.1)	0.6 (0.1)	4 (2.5)	NS

[†]The data were assessed using Kruskal-Wallis and Mann-Whitney U-tests.
[a]$P \leq 0.05$ (Healthy vs. Successful IVF), [b]$P \leq 0.05$ (Healthy vs. Unsuccessful IVF).
IFN=Interferon; TGF=Transforming growth factor; IL=Interleukin; NS=Not significant; IVF=*In vitro* fertilization; SD=Standard deviation

Table 3: Fold change in relative gene expression of cytokines in different studied groups before and after stimulation with seminal plasma

Cytokine	Treatment	Group, mean (SD)		
		Healthy	Successful IVF (*n*=10)	Unsuccessful IVF (*n*=10)
IFN-γ	Unstimulated	1 (0.4)	0.6 (0.2)	0.3 (0.1)
	Stimulated	1 (0.6)	0.8 (0.4)	2.6 (2)
IL-23	Unstimulated	1.4 (1.1)	1.5 (1)	1.2 (0.7)
	Stimulated	2.2 (1.6)	3 (1.7)[a]	2.4 (1.6)[a]
TGF-β	Unstimulated	1 (0.3)	1.1 (0)	0.7 (0.6)
	Stimulated	0.6 (0.4)	1 (0.7)	1.3 (1.5)
IL-10	Unstimulated	1.4 (0.3)	0.7 (0.5)	0.5 (0.2)
	Stimulated	1.7 (1.5)	1.3 (0.5)	10 (6.9)[a]
IL-4	Unstimulated	1 (0.2)	1.1 (0.7)	1.2 (0.7)
	Stimulated	0.5 (0.7)	0.3 (0.1)	0.9 (1.5)
IL-17	Unstimulated	1.5 (1.4)	2.9 (1.7)	1.6 (1.3)
	Stimulated	0.08 (0.04)	0.5 (0.1)	0.6 (0.3)

Nonparametric Wilcoxon signed-rank test was used for data analysis. [a]$P \leq 0.05$.
SP=Seminal plasma; IFN=Interferon; IL=Interleukin; TGF=Transforming growth factor; IVF=*In vitro* fertilization; SD=Standard deviation

DISCUSSION

Previous studies showed that infertility might be associated with an inappropriate function of the female immune system. The adaptive immune response plays an important role in etiology of UI.[16] In the first part of this study (baseline CD4⁺ mRNA level of cytokines in unstimulated condition), the results showed that IL-23 and TGF-β levels increased in the fertile group as compared to infertile ones. IL-23 induces the differentiation of naive CD4⁺ T-cells into Th17 cells producing IL-17A, IL-17F, IL-6, and TNF-α, which leads to pro-inflammatory processes and neutrophil-mobilization.[19,20] In addition to inducing inflammatory responses, increasing local concentration of matrix metallopeptidase-9 and stimulating angiogenesis for implantation are induced by IL-23.[21] In line with our results, recent evidence has shown that the proportion of Th17 cells and their related cytokines (IL-17 and IL-23) was higher in the first trimester than in the postnatal period. Furthermore, Th17 cells may be involved in the inflammatory process which is essential for successful implantation.[22] However, some clinical studies showed that IL-23 had a destructive effect after implantation. A study by Darmochwal-Kolarz *et al*. showed that increased expression of IL-23 in the first trimester is associated with recurrent pregnancy losses.[21] TGF-β has an important role in the proliferation and differentiation of trophoblast cells at the human feto-maternal interface.[23] Evidence showed that TGF-β level, in trophoblast-stimulated PBMC cultures, was significantly lower in individuals who experienced failed pregnancy compared with normal pregnant women.[24] Therefore, increased expression of IL-23 and TGF-β in the healthy group can be considered as positive factors in favor of a normal pregnancy.

In the second part of this study, we showed that SP stimulation can lead to increase in expression of IL-23 in the healthy and infertile groups, although these changes were not statistically significant in healthy couples. We previously showed that SP exposure might increase IL-23 level in PBMCs of infertile patients.[16] Previous studies have shown that villous and extravillous trophoblast cells

express IL-23 in early period of pregnancy, and this cytokine modulates the expression of other cytokines and also helps cell survival in endometrial cells.[25] Therefore, increased expression of IL-23 by SP stimulation may be in favor of IVF outcome in the infertile patient.

Furthermore, our data indicated that SP stimulation could increase IL-10 expression in women with IVF failure. IL-10 is a pleiotropic anti-inflammatory cytokine with the ability to suppress the pro-inflammatory milieu. Increased expression of IL-10 during the first and second trimesters but not in the third trimester has been reported. Although IL-10 is not essential for the growth and development of the fetus, it has a main role in the inhibition of increased inflammatory responses.[26] In line with our results, it is reported that SP fractions such as prostaglandin E2 (PGE2) and 19-hydroxy-PGE2 might stimulate IL-10 release from a monocytic cell line.[27] Hence, increased IL-10 level after SP insertion might improve IVF outcome in women with unsuccessful IVF outcome. It is also reported that SP might induce a significant increase in intracellular expression of the immunosuppressive cytokine such as TGF-β in women with unsuccessful IVF outcome.[28,29] In line with the mentioned studies, our data indicated that the level of TGF-β may increase after SP stimulation in women with unsuccessful IVF outcome, although not significant. The sample size can be considered as the major reason for this discrepancy and also the main limitation of the current study.

CONCLUSION

In summary, SP might have a positive effect on IVF outcome through changes in $TCD4^+$-related cytokine levels, especially IL-10 and IL-23. However, we worked on a small population of infertile and healthy women, and a larger sample population should be provided for identifying certain effects of SP on IVF treatment outcome.

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Conflicts of interest

There are no conflicts of interest.

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