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T Cell Subsets Profiling in Unexplained Infertile Women with Successful and Unsuccessful in Vitro Fertilization Outcome: Focus on the Effect of Seminal Plasma

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ABSTRACT

Unexplained infertility (UI) is one of the most common diagnoses in the fertility care. Seminal plasma (SP) plays a crucial role in the regulation of female immune responses and the success of a pregnancy. In vitro fertilization (IVF) is a well-known method for the treatment of UI. In this study, we aimed to investigate the effect of SP on the differentiation of T helper (Th) cell subsets and the relationship between these subsets with the rate of IVF success in a group of women complicated with UI compared to those with normal pregnancy.

This study was conducted on 20 UI couples (ten with successful and ten with unsuccessful IVF outcome) and 10 fertile couples as the control group. Four color flow cytometry technique was used to detect Th cell subsets in the peripheral blood mononuclear cells (PBMC) with or without stimulation by SP.

Results indicated that the frequencies of IL-17$^+$ and Foxp3$^+$ T cells after incubation with SP was significantly increased in couples with unsuccessful IVF outcome as compared to successful and healthy groups ($p<0.05$). Additionally, a positive correlation was observed between Th1 and Th2 cells in the unsuccessful IVF group (R=0.6, $p=0.03$).

In summary, the results of the present study demonstrated that exposure to SP might increase Th17 and Treg cell frequencies in infertile women with unsuccessful IVF, and might also balance inflammatory to regulatory responses to finally tune-up the Th1/Th2/Th17/Treg balance and support the success of IVF.

Keywords: Flow cytometry; In vitro fertilization; Seminal plasma; T helper subsets; Unexplained infertility

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INTRODUCTION

Infertility is the major problem of the reproductive system defined as a failure in achieving a successful

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conception after at least one year of regular unprotected intercourse. Infertility affects up to 15% of the reproductive-aged population worldwide.[1]In recent years, although, many causes for infertility have been defined, the reasons for approximately 25% of infertilities have still remained unknown that is called "unexplained infertility".[2] Various cellular and molecular defects in the endometrium are believed to be the primary cause of unexplained infertility.[3-5]Considering the importance of the immune system in the success of pregnancy, it is believed that at least 50% of unexplained infertilities are due to an imbalance in the immune system activation during the pregnancy course.[1]

Adaptive immune responses led by T helper cells play a critical role in the pregnancy period. In recent years, several subsets of T $CD4^+$ helper cells regulating the immune responses in a network manner have been introduced. Four most important subsets of $CD4^+$Th cells includeTh1/Th2/Th17/Treg that play important roles in normal pregnancy and its complications. In this regard, there are several reports indicating the importance of Th2 and Treg cells in the success of pregnancy.[6-9]Decreased number of $CD4^+Foxp3^+$ T cells has been demonstrated to be associated with spontaneous abortion.[10,11]Furthermore, increases in Th1 and Th17 cell subsets are important in the context of recurrent spontaneous abortion and in preeclampsia.[6,12]

Seminal plasma (SP) is considered an important male factor for the success of pregnancy. In line with the importance of SP in a successful pregnancy, some studies indicated that patients underwent SP insemination around the time of ovum pick-up or embryo transfer, showed higher implantation and clinical pregnancy rates compared to the controls.[13-16] SP plays important roles in spermatozoa capacitation and quality,[17] formation of tubal sperm reservoir, sperm-zona pellucida interaction, and sperm-egg fusion.[18]In addition, SP contains several immune-modulator factors influencing pregnancy from implantation to even offspring's health after labor.[19] SP can affect the expression of various immune factors such as cytokines (GM-CSF, IL-1A, IL-1B, IL-6, IL-8, IL-10, LIF, TGF-β) and chemokines (CCL2, CSF2, CSF3, CXCL8, VEGF-A).SP also promotes recruitment and activation of the immune cells, including macrophages, dendritic cells, and T cells.[20] Moreover, SP plays a pivotal role in the induction of immune tolerance to paternal alloantigen through expanding the pool of inducible Treg cells.[21]

Clinical studies at Assisted Reproductive Technology (ART) provide evidence that exposure to the conceiving partner's seminal plasma improves the rate of successful IVF and the treatment of choice for unexplained infertility. Furthermore, the absence of seminal plasma may be one factor constraining the endometrial receptivity and implantation success rates.[22] We have recently reported the effect of seminal plasma exposure on T helper subsets to shift at the mRNA level.[23] To complete the previous work and by considering the role of SP and T helper subsets on the success of pregnancy, the present study aimed to investigate the impact of SP incubation on peripheral blood Th1, Th2, Th17, and Treg cells subsets deviation. Using the flow cytometry technique, we investigated Th1/Th2/Th17/Treg cells balances in a group of unexplained infertile women as candidates for IVF treatment and a group of healthy women as controls.

MATERIALS AND METHODS

Subjects

This study was conducted on 20 unexplained infertile couples as candidate for IVF treatment (infertile group) and 10 healthy fertile women as the control group. All infertile couples had undergone IVF treatment at the IVF Center at Mother and Child Hospital, Shiraz University of Medical Sciences, Shiraz, Iran. Among them, 10 had successful and 10 had unsuccessful IVF outcome. A successful outcome was defined as a positive pregnancy test, while an unsuccessful outcome was defined as a negative HCG test after transfer of the embryo. All infertile couples had no history of previous positive pregnancy, autoimmune or immunodeficiency, and active infectious diseases. Moreover, men with male tract infections, inflammation, and varicocele were excluded from the study. Infertility was diagnosed by the same gynecologist and infertility specialist in couples with regular unprotected intercourse for at least one year. The diagnosis was based upon previously established criteria,[24-26] which state that the male partner should exhibit a normal semen profile ($>20\times10^6$ spermatozoa per mL,>40% progressive motility, >40% normal morphology, <10% agglutination, volume 1.5-6.0 mL) on at least two independent occasions, while the female partners should have been subjected to diagnostic laparoscopy, establishing tubal patency by the passage

of methylene blue and excluding other pelvic abnormalities, such as endometriosis or adhesions. All women had no sexual intercourse at least two weeks before IVF; moreover, they were not exposed to SP before/during the IVF procedure. The controls included 10 healthy multiparous non-pregnant women with uncomplicated previous pregnancies. All the healthy women were interviewed, during which personal and family histories were ascertained. None had an active disease, including an autoimmune disorder. All had a history of one or more normal deliveries. None had a history of infertility, pregnancy loss, preeclampsia, or other pregnancy-related disorders. Serum blood hormones tests were performed for all the participating women. Moreover, semen analysis was conducted for all male partners in infertile as well as healthy groups. Informed consent forms were obtained from all the participants, and the study was approved by the local Ethics Committee of Shiraz University of Medical Science (N. IR.SUMS.REC.1394.S632).

Semen Collection and Processing

Seminal fluid was obtained from all the participating men. At first, the semen samples were collected by masturbation using sterile containers. All the semen samples had a normal sperm count, motility, and morphology according to 2010 WHO's semen analysis criteria (5^{th} edition) [27] and were free of white blood cells (checked by light microscope). To separate seminal plasma, we allowed the semen samples to liquefy for 30 min, and the seminal plasma was separated by centrifugation of ejaculates at 3000 RPM (15 min, room temperature). The second round of centrifugation was performed at 14,000 RPM for 30 min to completely remove all the spermatozoa. Finally, seminal plasma was collected from the supernatants and stored at -70°C until it was co-cultured with PBMC.

PBMC Isolation and Cryopreservation

Blood samples were obtained from all the participating women. PBMCs were separated from the whole blood by a density gradient centrifugation method using Lymphoprep (Axis-Shield, Oslo, Norway). Isolated PBMC was washed using RPMI 1640 media (Gibco by Thermo Fisher Scientific, New York, USA), counted and re-suspended to cryovials containing freezing media, and preserved in liquid nitrogen tank at -80^0C until the time of experiments.

PBMC Stimulation

PBMCs were thawed and then washed two times with complete RPMI 1640 supplemented with 10% FBS (Shell max, China) and 1% penicillin-streptomycin (Shell max, China). The Trypan blue exclusion test is used for evaluation of the number of viable cells present in the cell suspension. Viability was greater than 90%. A total of 1×10^6 cells per well were co-cultured in the presence of diluted seminal plasma (0.3% V/V total concentration prepared in RPMI 1640) in 24-well flat-bottom plates in 1 ml complete RPMI 1640 media and incubated at 37°C in a humidified 5% CO2 incubator for 18 hours. As Golgi stop agent, monesin (Biolegend, San Diego, California, USA) was used in a concentration of 0.0016 mg/mL or 0.25x and added 1 hour after stimulating the cultured cells with seminal plasma(0.3% V/V total concentration). It should be noted that an unstimulated condition was considered for each panel of T cell subset.[28]

Flow Cytometry

7×10^5cells were removed from the culture and then washed with phosphate buffered saline (PBS). Then ,the cells were re-suspended in 100µl cold PBS and incubated with monoclonal antibodies directly conjugated with different fluorochromes, including CD3-PerCP (BD, pharminogen, USA), CD4-APC(BD, pharminogen, USA), CD25-FITC (BD, pharminogen, USA) for 20 minutes at 4°C in the dark for surface antigen staining. In the intracellular cytokine staining process, the cells were first treated with fixation solution A (BD, pharminogen, USA) for 15min, and then followed by addition of permeabilization solution B (BD, pharminogen, USA) for 30min to determine the intracellular cytokine expression. FITC-conjugated anti-human IFN-γ (BD, pharminogen, USA), PE-conjugated anti-human- IL-4 (BD, pharminogen, USA), IL-17 (BD, pharminogen, USA), and Foxp3 (BD, pharminogen, USA) were added and incubated for 30 min at room temperature in the dark to detect Th1/Th2/Th17/Treg subsets, respectively. After incubation, the cells were washed and re-suspended in 1% formaldehyde fixation solution. Data were analyzed using the FlowJo software 7.6.1 (FlowJo LLC, Ashland, OR, USA). The number of events acquired for each sample was 1×10^5. A region based on forwarding light scatter versus side light scatter was drawn around the major lymphocyte population. $CD3^+CD4^+$ cells were selected from the lymphocyte

gate in each of unstimulated and stimulated samples. Then, the percentage of un-stimulated, cytokine-positive cells and regulatory T cells was determined in the $CD3^+CD4^+$ cells.

Statistical Analysis

Statistical analysis was conducted using SPSS 18software (SPSS Inc, Chicago, Illinois, USA). Comparisons between two or more than two groups were performed using the non-parametric Mann Whitney-U test and Kruskal-Wallis test, respectively. Spearman's rank correlation coefficient was used to analyze the relationship between the frequencies of the two variables. Wilcoxon signed ranks test was used for comparing two metric variables measured on one group of cases. P values less than 0.05 were considered statistically significant.

RESULTS

Comparisons of the Characteristics of the Studied Groups

Table 1 summarizes the demographic and clinical features of all 30 couples participating in the present study. As indicated, the mean ages of male partners, semen analysis parameters, including semen volume, sperm concentration, sperm motility, and sperm morphology were all comparable to no significant differences for the three studied groups (Table 1). Furthermore, maternal parameters such as age, hormonal patterns including Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH), Thyroid-Stimulating Hormone (TSH), Prolactin (PRL), and free testosterone levels were compared among the three groups, and no significant difference was found among the groups in all the studied parameters (Table1).

The Frequencies of $CD3^+CD4^+$T Cells

Figure 1 depicts our strategy to detect Th subsets in the peripheral blood. At first, the percentages of peripheral blood $CD3^+CD4^+$ T lymphocytes were determined by flow cytometry analysis. Results indicated no statistically significant differences in percentages of $CD3^+CD4^+$ cells between successful IVF, unsuccessful IVF and healthy fertile women (Mean%±SD: 45.7%±7.1; 44.2%±9 and 43.4%±5.3, P=0.82, respectively). The similarity in the percentages of $CD3^+CD4^+$ T cells between infertile and healthy fertile women indicated that the total population of $CD4^+$ T cells in three studied groups was the same.

Table 1.Demographic and clinical features of infertile patients and healthy control group (Mean±SD)

Subjects	Control (n=10)	Successful IVF(n=10)	Unsuccessful IVF(n=10)	*p* value
Male partner				
Age (year)	38±5.2	33.8±3.7	34±3.9	0.24
Semen Volume(ml)	3.8±1.1	3.5±1.0	3.5±1.0	0.82
Sperm Concentration(million sperm/ml semen)	39±17.4	41±27	71±37	0.11
Sperm motility (% progressively motile sperm)	49±16.8	56±13.4	48.7±9.1	0.59
Sperm normal morphology (%)	14.3±5.5	13.2±5.4	10.6±5.6	0.46
Female partner				
Age(year)	31±3.4	29.2± 3.2	31.3±3.6	0.54
FSH level (mIU/mL)	4.7±2.1	6.6±1.8	5±1.6	0.17
LH level (mIU/mL)	5.8±3.6	4.1± 1.5	4.1 ±1.8	0.83
TSH level (mIU/mL)	2.2±1.1	2.4 ±1.5	2.09 ±0.6	0.83
Prolactin level (ng/mL)	16.07±8.7	13.8 ±4.7	12.6 ± 3.2	0.83
Free Testosterone level (ng/dL)	0.59±0.24	1.9 ±1.8	0.68 ±1.8	0.17

Exact *p* values calculated by the Kruskal- Wallis test. *p*-value<0.05 was considered statistically significant.

FSH=Follicle-stimulating hormone; LH=Luteinizing hormone; TSH=Thyroid-stimulating hormone; PRL=Prolactin; SD=Standard deviation; IVF=In vitro fertilization.

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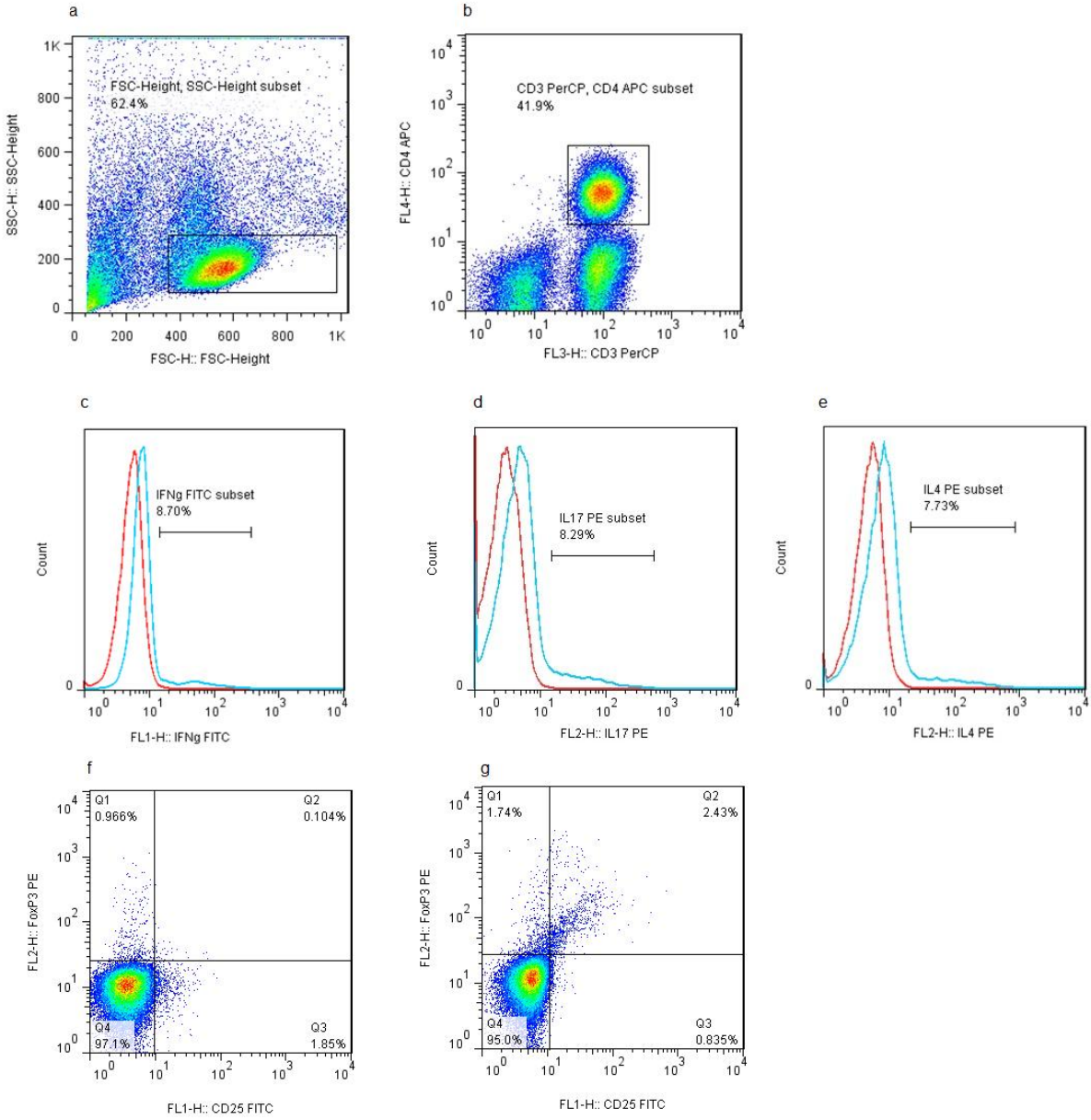

Figure 1. Four color flow cytometric analysis of T cell subsets in the peripheral blood. For the gating strategy, (a) the lymphocytes were gated based on the forward and side scatter parameters, (b) then CD3+CD4+ T cells were selected in the lymphocyte gate. IFN-γ (c), IL-17 (d), IL-4 (e) positive cells were selected from CD3+CD4+ gate and are shown in unstimulated (Red graph) and stimulated (Blue graph) conditions. CD25 and Foxp3 markers were plotted against each other, and the frequency of the unstimulated (f) and stimulated (g) CD3+CD4+CD25+Foxp3+ regulatory were determined in the pool of T cells. IFN=Interferon; IL=Interleukin; CD=Cluster of differentiation; Foxp3=Forkhead box p3; IVF=In vitro fertilization.

Frequencies of Unstimulated T Helper Subsets

Table 2 summarizes the data regarding the distribution and comparison of the four studied lymphocyte subsets before SP exposure. As this Table shows, no significant difference was found between the studied groups regarding the frequencies of four T helper subsets.

Table 2. The mean frequencies of unstimulated T helper subsets from infertile patients and healthy fertile group (mean±SD)

Th cell subsets	Control (n=10)	Successful IVF(n=10)	Unsuccessful IVF(n=10)	*p* value
$CD3^+CD4^+IFN\text{-}\gamma^+$	1.08±1.03	0.87±0.64	0.9±0.53	N.S.
$CD3^+CD4^+IL\text{-}4^+$	1.51±1.21	1.5±0.61	2.69±1.6	N.S.
$CD3^+CD4^+IL\text{-}17^+$	0.78±0.47	0.6±0.11	0.92±0.56	N.S.
$CD4^+CD25^+Foxp3^+$	0.18±0.09	0.13±0.05	0.6±0.98	N.S.

Nonparametric Kruskal-Wallis test was used for data analysis. P values less than 0.05 were considered to be statistically significant.

N.S. = not significant; SD= Standard deviation; IVF= In vitro fertilization; Th= T helper cell; Foxp3= Forkhead box p3; IL= Interleukin; CD= Cluster of differentiation.

The difference in the Frequencies of T Helper Subsets before and after Seminal Plasma Exposure in Patient and Control Groups

As Figure 2 (a-c) shows, seminal plasma exposure induced a change in some Th subsets in the studied groups. In the control group, Th1, Th2, Th17, and Treg cells were increased after SP exposure (4.7±1.5; 5.1±2.7; 4.7±1.6and 3.4±1.6, respectively; p=0.01 for all comparisons, Figure 2a).In the unsuccessful IVF group, Th1, Th17, and Treg were significantly increased after SP exposure (4.6±2.3, p=0.007; 5.1±2.5, p=0.005 and 4.7±0.8, p=0.005, respectively; Figure2b), while Th2 subsets were not changed significantly. Th1 and Treg cell subsets were increased after SP exposure in the successful IVF group (2.5±0.9; 2.8±1.4, respectively; p=0.05 for both comparison, Figure2c), while Th2 and Th17 up-regulation were not statistically significant.

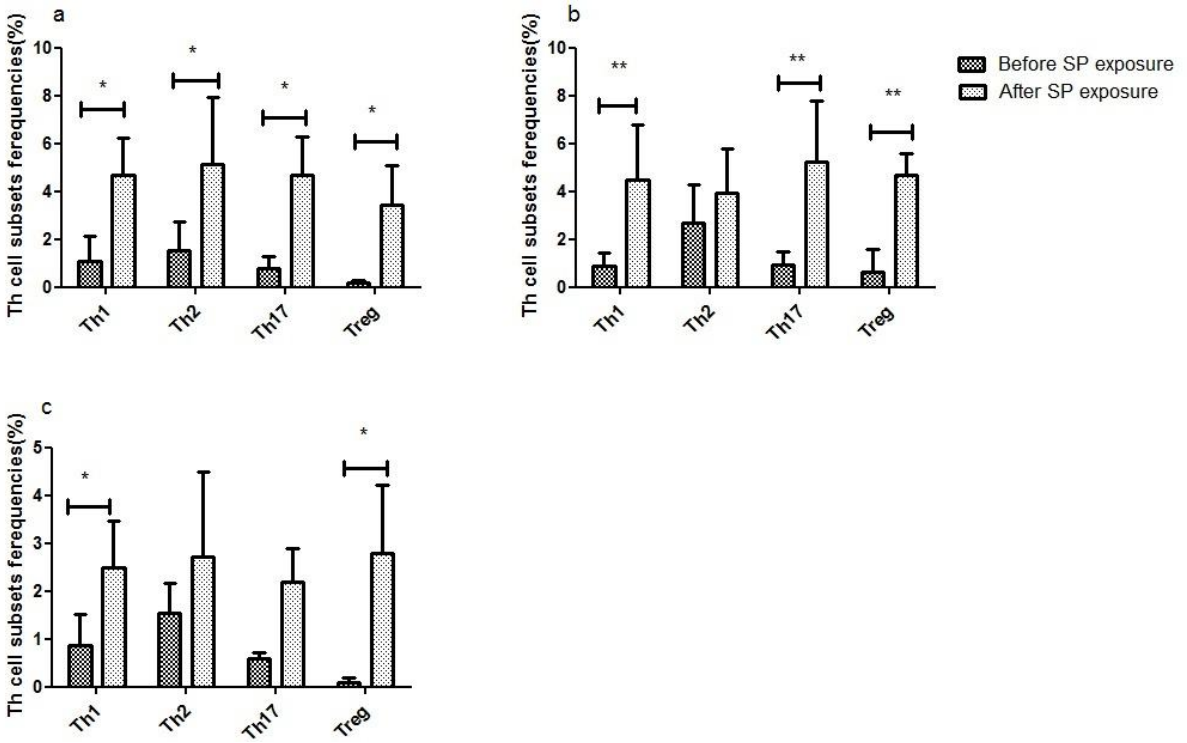

Figure 2. Comparison of Th cell subsets frequencies (%) before and after SP exposure. Differences in the frequencies of the peripheral blood lymphocyte subsets were compared before and after seminal plasma exposure in the control (a), unsuccessful IVF (b) and successful IVF groups (c).Graphs show mean±SD. The data were analyzed by nonparametric Wilcoxon signed ranks test. p<0.05 was considered statistically significant. *<0.05, **<0.01, ***<0.001. SD=Standard deviation; SP=Seminal plasma; IVF=In vitro fertilization; Th=T helper; Treg=T regulatory.

Table 3. The mean frequencies of stimulated T helper subsets from infertile patients and healthy fertile group (mean±SD)

Th cell subsets	Successful IVF(n=10)	Unsuccessful IVF(n=10)	Control (n=10)	*p*value
$CD3^+CD4^+IFN\text{-}\gamma^+$	2.5±0.9	4.6±2.3	4.7±1.5	N.S.
$CD3^+CD4^+IL\text{-}4^+$	2.7±1.7	3.9±1.8	5.1±2.7	N.S.
$CD3^+CD4^+IL\text{-}17^+$	2.2±0.6	5.1±2.5	4.7±1.6	0.05
$CD4^+CD25^+Foxp3^+$	2.8±1.4	4.7±0.8	3.4±1.6	0.03

Nonparametric Kruskal- Wallis test was used for data analysis. P values less than 0.05 were considered to be statistically significant. N.S. = not significant; SD= Standard deviation; IVF= In vitro fertilization; IFN= Interferon; IL= Interleukin; CD= Cluster of differentiation; Foxp3= Forkhead box P3; Th= T helper.

The difference in the Frequencies of Stimulated T Helper Subsets from Infertility and Fertile Groups

Table 3 shows the mean frequencies of T helper subsets after stimulation with seminal plasma. There were significant differences between the three groups in Treg and Th17 cells frequencies after seminal plasma exposure (p=0.03, p=0.05, respectively; Table 3). After seminal plasma exposure, the frequencies of Treg cells was significantly increased in the unsuccessful group when compared to the control and successful IVF groups (p=0.05 and p=0.02, respectively; Figure 3a). In addition, the frequencies of Th17 cells were significantly increased in the unsuccessful compared to the successful IVF group (p=0.05, Figure 3b).

Th1/Th2 and Th17/Treg Cytokine Ratios

To compare the frequencies of T cell subsets expressing antagonist cytokines, the ratios of Th1/ Th2 and Th17/Treg subsets were calculated and compared within different groups. As Table 3 indicates, there was no significant difference between the three studied groups regarding the Th1/Th2 and Th17/Treg ratios (Table4).

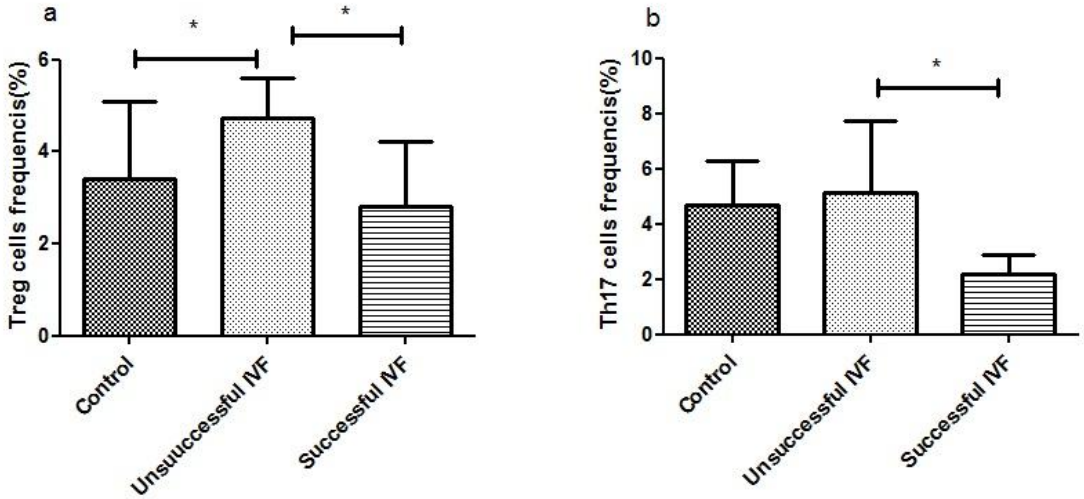

Figure 3. Comparison of Treg and Th17 cells frequencies (%) after SP exposure in the infertile patients and healthy control group. The difference in the frequencies (%) of Treg cells (a) and Th17 (b) were shown in the control, unsuccessful and successful IVF groups. Nonparametric Mann Whitney-U test was used for data analysis. P values less than 0.05 were considered to be statistically significant. *<0.05, **<0.01, ***<0.001. IVF=In vitro fertilization; Th=T helper; SP=Seminal plasma; Treg=T regulatory.

Table 4.The ratio of Th1 to Th2 and Th17 to Treg cell frequency from infertile patients and healthy fertile group (mean±SD)

Ratio	Successful IVF (n=10)	Unsuccessful IVF (n=10)	Control (n=10)	*p* value
Th1/Th2	1.3±0.7	1.06±0.6	1.1±0.4	N.S.
Th17/Treg	0.8±0.4	1±0.4	1.4±0.4	N.S.

Nonparametric Kruskal-Wallis test was used for data analysis. P values less than 0.05 were considered to be statistically significant.
N.S. = not significant; SD= Standard deviation; IVF= In vitro fertilization; Th= T helper; Treg= T regulatory.

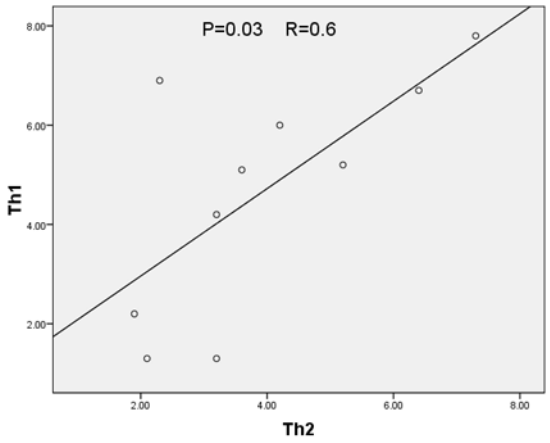

Figure 4. Correlation between the frequencies Th1 to Th2 cells in the unsuccessful IVF group. The frequency of Th1 to Th2 was positively correlated in the unsuccessful IVF group. Statistical analysis was conducted using Spearman's rank correlation test. *p*<0.05 was considered significant. IVF=In vitro fertilization; Th=T helper.

Correlation between the Frequency of Th17 and Treg Cell Subsets, Th1 and Th2 cell Subsets

Spearman's rank correlation coefficient was used to analyze the relationship between the frequencies of Th17 to Treg cell and Th1 to Th2 subsets in all the studied groups. Results indicated a positive correlation between the peripheral blood Th1 and Th2 subsets in the unsuccessful IVF group (R^2=0.6, *p*=0.03, Figure4).

DISCUSSION

The present study compared the frequency of four Th cell subsets within the peripheral blood lymphocyte of women stimulated with her husband's seminal plasma in a group of infertile and a group of healthy women. The present study results showed that the frequencies of Th17 and Treg cells were increased after seminal plasma exposure in women with IVF failure as compared to those with successful IVF as well as healthy control groups. The th17 subset is a recently described effector $CD4^+$Tcell playing an important role in several autoimmune diseases as well as in defense against fungal infection.[29] Although Th17 subset is introduced as an inflammatory subset in pregnancy, there is limited information regarding the role of these cells in reproductive immunology. Studies showed thatTh17 cells may be potentially involved in the inflammatory process in the early period of pregnancy that is required for successful implantation.[30,31]Concerning the role of seminal plasma in induction of inflammatory responses during implantation, some studies reported that seminal plasma could induce T cells to secrete IL-17, cause the neutrophils to migrate and accumulate around the uterine epithelium following insemination.[32]It is hypothesized that seminal plasma exposure in the early period of pregnancy may improve the implantation rate in patients with IVF failure by increasing the number of $IL\text{-}17^+$ T cells and subsequent activation of inflammation, but a regulatory response is needed to modulate this inflammation.[31] Our results in this study demonstrated that seminal plasma exposure might increase $IL\text{-}17^+$cells,being in line with our previous experiment.[23]Moreover, we showed that seminal plasma exposure significantly increased the $Foxp3^+$ T cells in the unsuccessful IVF group, being in line with our previous report regarding the increasing of Foxp3 at mRNA level after seminal plasma exposure.[23] Seminal plasma components such as TGF-β and PGE2-related prostaglandins along with steroid hormones are reported to be implicated in the regulation of the suppressive function of Treg cells in the pre-implantation phase of early pregnancy.[21] Thus, increasing $Foxp3^+$ T cells following exposure might be considered a positive effect of the seminal plasma and candidate the use of seminal plasma as supplementary treatment in IVF protocol in women with unsuccessful IVF outcome. So, seminal plasma exposure via

increasing Th17 induced an inflammatory response that is necessary for implantation and maintenance in early pregnancy and concurrently, modulates the maternal immune response by increasing Treg subsets.

We also examined the frequencies of Th1 and Th2 subsets within the peripheral blood cells incubated with seminal plasma. Recent studies have reported that adequate balance for Th1/Th2 type immunity slightly shifted toward Th2-type immunity may be suitable to maintain a normal pregnancy. Overstimulation of Th1 or Th2 immunity might be harmful to a successful pregnancy.[9]In this regard, injection of IFN-γ, TNF-α, and IL-2 in abortion-prone murine mating has been shown to increase the abortion rates and this has been reduced by IL-10.[33] However, the results of our study did not show statistically significant differences regarding the frequencies of Th1 or Th2 subsets in different groups. Additionally, we did not observe any significant differences regarding the ratio of Th17/Treg and Th1/Th2 subsets in the studied groups. Nevertheless, the positive correlation between inflammatory (Th1, Th17) and regulatory (Th2, Treg) responses in women with IVF failure might suggest that the use of seminal plasma can improve the IVF outcome through balancing immune system responses. However, our present study examined a relatively small population of patients and healthy women and a study with a larger population is warranted to identify whether seminal plasma insertion to IVF process can improve treatment outcome or not.

We hypothesize that the seminal plasma exposure might increase Th17 and Treg cell frequencies in women with IVF failure and balance inflammatory to regulatory responses to finally tune-up the Th1/Th2/Th17/Treg balance and support the IVF success.

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