

1-1-2018

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ÖZKAN, ZEHRA SEMA; DEVECİ, ŞÜKRİYE DERYA; AKPOLAT, NUSRET; İLHAN, NAFİYE FULYA; ŞİMŞEK, MEHMET; and TİMURKAN, HÜSEYİN (2018) "The impact of plasma SOCS3 levels and endometrial leukocytes on unexplained infertility," *Turkish Journal of Medical Sciences*: Vol. 48: No. 3, Article 9. <https://doi.org/10.3906/sag-1707-103>

Available at: <https://journals.tubitak.gov.tr/medical/vol48/iss3/9>

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The impact of plasma SOCS3 levels and endometrial leukocytes on unexplained infertility

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Received: 17.07.2017 • Accepted/Published Online: 03.04.2018 • Final Version: 14.06.2018

Background/aim: To investigate the possible differences in endometrial leukocyte subtype distribution between women with unexplained infertility and normal fertile women and to determine whether there is a correlation between endometrial leukocyte counts and plasma cytokine levels in unexplained infertility.

Materials and methods: This case-control study involved 79 infertile and 40 fertile women. Peripheral venous blood samples and endometrial samples were obtained on day 21 of the menstrual cycle. Plasma interleukin-4 (IL4), IL6, IL10, IL17, IL35, interferon-gamma (IFN-g), tumor necrosis factor-alpha (TNF-a), transforming growth factor-beta (TGF-b), and suppressor of cytokine signaling-3 (SOCS3) levels were determined by enzyme-linked immunosorbent assay. Endometrial CD8, CD56, and CD163 counts were detected by immunohistochemistry.

Results: CD8 and CD56 counts were significantly higher, while CD163 count was significantly lower in infertile women than in fertile women. Plasma SOCS3, IL35, and IL4 levels of the infertile group were significantly lower than those of the fertile group ($P < 0.01$); the remaining cytokine levels were significantly higher in the infertile group than in the fertile group ($P < 0.01$).

Conclusion: We observed aberrant cytotoxic immune activity in infertile women. The interaction between plasma SOCS3 levels and staining degree of endometrial leukocytes may be either the reason for or result of infertility leading to unavailability of the environment for implantation.

Key words: CD56, CD163, CD8, unexplained infertility, SOCS3

1. Introduction

Uterine receptivity, a complex and multifactorial event, is affected by several growth factors and cytokines during the midluteal phase of the menstrual cycle (1,2). Endometrial macrophages and cytotoxic T cells influence embryo attachment and implantation during this period (3). Maternal immune modulation is important in protecting the fetus, given its semiallogenic condition. Researchers suggest that an increased ratio of prorejective cytokines to antirejective cytokines has a negative influence on implantation (4). Impaired endometrial immune cells are thought to be responsible for some cases of infertility in which standard work-up fails to yield a reason (3,5). Furthermore, implantation of an embryo to the proper depth of the endometrium requires proteolytic activity. Highly localized endometrial degradation with

active, targeted, and strict regulated proteolysis is essential for successful implantation (6).

Natural killer (NK) cells with CD56 antigen are the dominant immune cell types in the endometrium (1). The number of NK cells in the endometrium significantly increases in the late secretory phase, with further increment during early pregnancy (7). The main role of NK cells is secretion of angiogenic factors, cytokines, and growth factors such as tumor necrosis factor-alpha (TNF-a), interleukin-10 (IL-10), transforming growth factor-beta (TGF-b), and interferon-gamma (IFN-g) (8). CD8+ cells form a large proportion (66%) of endometrial T cells (9). The cytotoxic activity of endometrial CD8+ T cells is retained during the proliferative phase, but this activity decreases in the secretory phase without any change in the quantity of the cells (10). IL-10-secreting

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endometrial macrophages play an important role in the acceptance of fetal tissue during early pregnancy (11). The proportion of CD163+ macrophages increases in the superficial endometrial stroma during the secretory phase of the cycle (5). Researchers suggest that the proportion of endometrial macrophages is not obviously correlated with the diagnosis of infertility (3). Suppressor of cytokine signaling 3 (SOCS3) plays an important regulatory role in inflammation, and SOCS3-defective macrophages were found to produce fewer inflammatory cytokines than normal ones (12). In addition, SOCS3 expression is dependent on IL-6, IL-10, and TNF- α production (13).

In the present study, we firstly aimed to investigate the possible differences in endometrial leukocyte subtype distribution between women with unexplained infertility and normal fertile women. Secondly, we explored the possible relation between endometrial leukocyte staining levels and plasma cytokine and SOCS3 levels in unexplained infertility.

2. Materials and methods

2.1. Study subjects

This case-control study was conducted with study subjects and controls at the In-Vitro Fertilization (IVF) Unit of Firat University Hospital, Elazığ, Turkey, from October 2011 and October 2012 after gaining approval from the local ethics committee (2011/107). The study group comprised 79 infertile women planning to undergo controlled ovarian hyperstimulation–intracytoplasmic sperm injection (COH–ICSI) treatment owing to unexplained infertility. All patients underwent gynecological examination, antral follicle count, FSH–LH–estradiol–TSH–prolactin level evaluation on day 3 (D3) of the menstrual period, hysterosalpingography/hysteroscopy, and semen analysis before starting COH. Unexplained infertility is defined as infertility lacking a reason. Couples with a history of two unsuccessful gonadotrophin-induced ovulation cycles combined with intrauterine insemination were referred for the COH–ICSI cycle.

The inclusion criteria for the study group were as follows: 21–38 years old, primary infertility, regular menstrual cycle (24–35 days), body mass index (BMI) <30 kg/m², FSH <10 mIU/mL, and E2 <50 pg/mL on day 3 of the menstrual cycle (D3). Pituitary downregulation was carried out using the gonadotropin-releasing hormone (GnRH) agonist according to the long luteal protocol.

The control group comprised 40 fertile women. The inclusion criteria for the control group were as follows: 21–38 years old; 1 live birth less than 2 years ago; regular menstrual cycle (24–35 days); no history of D&C, abortion, or endometrial biopsy; BMI <30 kg/m²; FSH <10 mIU/mL; and E2 <50 pg/mL on D3. The indication for endometrial biopsy in the control group was suspicion

of endometrial polyp. Office hysteroscopy was performed during the luteal phase (D21) after confirming sexual abstinence to exclude the possibility of pregnancy. If endometrial polyp was excluded on office hysteroscopy, an endometrial biopsy was conducted.

Women with endometriosis (laparoscopic diagnosis or endometrioma evaluation with transvaginal sonography), tubal factor infertility, ovulatory dysfunction, anatomical uterine pathological conditions, male factor infertility, previous implantation failure, recurrent spontaneous abortion, myomectomy or dilatation and curettage history, and positive screening for bacterial or viral infection were excluded. Informed and signed consent was obtained at the time of blood sampling.

2.2. Ovarian hyperstimulation protocol

Pituitary downregulation with GnRH agonist was started on D21 of the menstrual cycle and ovarian stimulation was started on D3 of the proceeding cycle with recombinant FSH. Gonadotropin at a dose of 225–300 IU was administered from stimulation day 1 until the day the human chorionic gonadotropin (hCG) trigger was administered. When at least 3 follicles reached a diameter of 17 mm, hCG was administered. Estradiol level and endometrial thickness were measured on the day the hCG trigger was administered. The oocytes were retrieved 35–36 h after the trigger was administered. Grade 1 embryo transfer (ET) was performed on day 3 or 5 after retrieval (according to Turkey Republic Health Laws, mandatory single and conditional two ETs were performed). There was no frozen-thawed embryo transfer, and fresh embryos were used for all performed cycles. Twelve days after embryo transfer, blood beta-hCG analysis was conducted to check for pregnancy. If the result was positive, the beta-hCG test was repeated 48 h later. If a regular increment was observed, transvaginal ultrasound examination was carried out to detect the gestational sac 10 days later.

2.3. Blood sampling

Peripheral venous blood samples were drawn from all women on D21 of their natural menstrual cycle and the samples were centrifuged at 2500 rpm at 4 °C for 15 min and stored at –80 °C until run time. In the study group, GnRH agonist treatment was started on the same day after obtaining the sample and COH was started at the following menstrual period. The extracted plasma samples were assayed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits for IL17, IFN- γ , TNF- α , TGF- β , IL6, IL4, IL10 (Boster Biological, Fremont, CA, USA), SOCS3, and IL35 (USCN Wuhan/China) according to the manufacturer's instructions. The theoretical sensitivity or lowest detection limits for IFN- γ , TNF- α , TGF- β , IL6, IL4, IL10, IL17, IL35, and SOCS3 were, respectively, as follows: 0.2 pg/mL, 1.7 pg/mL, 1.5 pg/mL, 0.4 pg/mL, 3.6 pg/mL, 7.3 pg/mL, 1 pg/mL, 1 pg/

mL, and 9.3 pg/mL. The samples were analyzed by the same staff under the same laboratory conditions. Within- and between-assay variations were less than 6% and 8%, respectively, for all ELISA assays. TNF- α , IFN- γ , TGF- β , and IL-17 were categorized as prorejective cytokines and IL4, IL6, IL10, and IL35 were categorized as antirejective cytokines.

2.4. Immunohistochemical study

Endometrial biopsies were carried out on D21 of the natural menstrual cycle (the same day that blood samples were taken). The tissues were fixed in neutral 10% formaldehyde, embedded in paraffin, cut in 5-mm sections, and stained with hematoxylin–eosin. For immunohistochemical staining, 5-mm paraffin sections were deparaffinized in xylene, rehydrated, and then placed in a phosphate-buffer saline (PBS) bath (pH 7.6). Antigen retrieval was performed using a 15-min bath in boiling citrate buffer (pH 6.0) solution. The sections were treated with 3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity, rinsed with deionized water, and then placed in the PBS. The sections were incubated first with 1% pre-immune rabbit serum to reduce nonspecific staining and then with monoclonal antibodies to CD8, CD56, and CD163 for 45–60 min each at room temperature (Table 1). Immune detection was performed using a biotin-streptavidin detection system (BioGenex, San Ramon, CA, USA) with 3,3'-diaminobenzidine chromogen (Dako, Carpinteria, CA, USA). The tissues were counterstained with Mayer's hematoxylin, dehydrated, and cover-slipped with permount on glass slides; then they were evaluated under a light microscope. Positive cells were counted randomly at 320 $\times$ magnification in 3 fields.

2.5. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences version 16.0 (SPSS Inc., USA). The results were presented as mean $\pm$ SE. Differences in continuous variables between groups were analyzed by Student's t-test or Mann–Whitney U-test according to the distribution of data. The differences for categorical variables were analyzed by chi-squared test or Fisher's

exact test where applicable. The relationship among plasma cytokine levels, endometrial leukocyte subtypes, and IVF outcomes (fertilization rate, implantation rate, pregnancy rate, live birth rate) was evaluated by Spearman correlation analysis. Receiver operating characteristic (ROC) curves and likelihood ratios (LR+) were applied to determine the sensitivity and specificity of cytokines in predicting pregnancy. After classifying the infertile women according to the cytokine level determined with LR+ value, binary logistic regression analysis was performed to determine the cytokines that had an influence on conception. Logistic regression analysis was performed to determine the cytokine that had an influence on leukocyte subtype staining. P values of <0.05 were considered statistically significant. Power analysis of the study was performed using the G Power 3 program; the power of our study was 82%.

3. Results

The baseline characteristics of the study population are presented in Table 2. The mean age and BMI of the study population were 30.2 ± 0.4 years and 25 ± 0.4 kg/m², respectively. There was no significant difference between infertile and fertile women in terms of age, BMI, D3 FSH-TSH-prolactin levels, or antral follicle count.

In the infertile group, 60% (48/79) of the patients became pregnant. The implantation rate was 55%. The live birth and abortion rates were 40% (32/79) and 20% (16/79), respectively. The comparison of stimulation characteristics of infertile women according to whether they conceived or not revealed no significant difference (Table 3).

The plasma cytokine levels of all women in the study are presented in Table 4. While plasma SOCS3 ($P = 0.008$), IL35 ($P = 0.002$), and IL4 ($P = 0.003$) levels of the infertile group were significantly lower than those of the fertile group, the remaining cytokine levels were significantly high in the infertile group ($P < 0.01$). In the infertile group, a comparison of cytokine levels according to whether they conceived or not revealed no significant difference.

The comparison of prorejective to antirejective cytokine ratios between groups revealed the following

Table 1. Details of immunohistochemical analysis.

Primary antibody	Manufacturer	Clone	Dilution	Pretreatment
CD8	Thermo (USA)	SP16	1:50	Microwave pressure cooker, 10 min, 10 mM citrate buffer (pH 6.0)
CD56	Thermo (USA)	123C3.D5	1:100	Microwave pressure cooker, 20 min, 10 mM citrate buffer (pH 6.0)
CD163	Leica-Novocastra (Germany)	10D6	1:100	Microwave pressure cooker, 10 min, 10 mM citrate buffer (pH 6.0)

Table 2. Baseline characteristics of all women in the study.

	Infertile (n = 79)	Fertile (n = 40)	P value
Age (years)	29.2±0.3	30±0.5	0.23
BMI ^a (kg/m ²)	24.6±0.4	25±0.3	0.47
D3 FSH ^b (IU/L)	6±0.4	6.3±0.6	0.51
TSH ^c	1.6±0.1	0.9±0.2	0.19
Prolactine	16.4±1.5	20±0.5	0.34

Note: Values are presented as mean ± SE. a = body mass index;
b = day 3 follicle stimulating hormone; c = thyroid stimulating hormone.

Table 3. Comparison of demographic and stimulation characteristics of infertile women according to becoming pregnant.

Parameter	Pregnancy (–) (n = 31)	Pregnancy (+) (n = 48)	P value
Age (years)	29.74 ± 0.45	29.11 ± 0.75	0.12
Male age (years)	33.36 ± 0.79	33.72 ± 0.70	0.24
BMI ^a (kg/m ²)	24.1 ± 0.8	24.9 ± 0.7	0.45
Infertility duration (years)	5.7 ± 0.5	5.9 ± 0.5	0.78
D3 FSH ^b (mIU/mL)	6.6 ± 0.2	5.7 ± 0.4	0.64
Antral follicle count	13.2 ± 2.0	14.3 ± 3.1	0.21
Stimulation duration (days)	9.17 ± 0.11	9.06 ± 0.6	0.58
Estradiol level on the day of hCG ^c administration (pg/dL)	2645 ± 255	2322 ± 231	0.29
Progesterone level on the day of hCG administration (ng/dL)	0.89 ± 0.10	0.79 ± 0.08	0.34
Retrieved oocyte number	14 ± 1	14.6 ± 1.2	0.16
Mature oocyte number	10.4 ± 1	10.7 ± 0.9	0.09
Fertilized oocyte number	8.7 ± 0.6	8.5 ± 0.6	0.08

Note: Values are presented as mean ± SE; a = body mass index; b = day 3 follicle stimulating hormone; c = human chorionic gonadotrophin.

findings: TNF- α /IL10, IFN- γ /IL10, IFN- γ /IL6, and IFN- γ /IL4 ratios were significantly high ($P < 0.01$), but the ratios of TNF- α /IL6 ($P = 0.03$) and IL35/IL17 ($P = 0.02$) were significantly low in the infertile group.

According to the ROC curve analysis and LR+ values of cytokines for predicting pregnancy, the area under curve (AUC) of SOCS3 was under 0.5, meaning SOCS3 showed no potential in predicting pregnancy. All the remaining cytokines except IL35 showed high sensitivity (>90%) and specificity (>90%) for predictive values. Binary logistic regression analysis revealed that IL10 (RR = 2, 95% CI: 1.548–2.665, $P = 0.02$) and IL17 (RR = 2, 95% CI: 1.531–2.661, $P = 0.01$) were the prominent cytokines that had an influence on conception. SOCS3 plasma levels showed

an inverse association with plasma IL4, IL6, IL10, IL17, IFN- γ , TGF- β , and TNF- α levels. SOCS3 did not show an association with IL35.

Immunohistochemical staining results for CD8, CD56, and CD163 antigens on endometrial biopsy specimens are presented in Table 5 and Figure 1. The staining dominancy of leukocyte subtypes on endometrial biopsy specimens was observed for CD8 antigen. The staining counts of CD8 ($P = 0.004$) and CD56 ($P = 0.006$) antigens on the endometrial biopsy specimens of the infertile group were significantly higher than those of the fertile group. On the other hand, the CD163 antigen staining count of the infertile group was significantly lower than that of the fertile group ($P = 0.003$). The comparison of leukocyte

Table 4. Cytokine levels of all women in the study.

Cytokine	Infertile (n=79)	Fertile (n=40)	P value
IFNg (pg/mL)	26.48 ± 1.57	0.47 ± 0.20	0.005
IL35 (pg/mL)	83.38 ± 10.83	89.36 ± 33.56	0.002
IL17 (pg/mL)	62.13 ± 7.54	1.58 ± 0.07	0.002
SOCS3 (pg/mL)	13.80 ± 2.46	158.73 ± 26.36	0.008
TGFb (pg/mL)	43.45 ± 8.05	2.92 ± 0.48	0.003
IL6 (pg/mL)	6.23 ± 0.25	0.61 ± 0.19	0.004
TNFa (pg/mL)	28.03 ± 2.48	2.73 ± 1.55	0.007
IL10 (pg/mL)	55.29 ± 3.45	9.49 ± 0.11	0.001
IL4 (pg/mL)	29.16 ± 0.12	32.10 ± 0.78	0.003

Note: Values are presented as mean ± SE.

Table 5. Immunohistochemical study of endometrial biopsies in all women.

Antigen	Fertile ^a (n = 40)	Infertile ^a (n = 79)	P value
CD163	36.92 ± 3.92	11.68 ± 0.54	0.003
CD56	7.75 ± 3.36	35.29 ± 5.22	0.006
CD8	41.36 ± 5.57	61.32 ± 5.1	0.004

Note: a = Mean number and standard error of positive cells in 3 random fields (320×).

subtype staining of infertile women according to whether they conceived or not revealed no significant differences.

The correlation analysis results showed that none of the endometrial leukocyte subtypes was correlated with implantation rate. CD8, CD56, and CD163 staining counts showed a positive correlation with the IFN-g/IL10 ratio ($P < 0.01$). CD163 staining count showed a correlation with the plasma cytokine levels except IL35, IFN-g, and TGF-b. CD56 and CD8 staining counts showed a correlation with the plasma cytokine levels except IL35 and IFN-g. The linear regression analysis results showed that only plasma SOCS3 levels had an influence on endometrial CD163 staining count (OR = 6.3, 95% CI = 0.085–0.163, $P = 0.001$). None of the cytokines had an influence on CD56 staining. On the other hand, SOCS3 (OR = 2.9, 95% CI = 0.036–0.186, $P = 0.002$) and TGF-b (OR = 2.5, 95% CI = 0.05–0.439, $P = 0.01$) levels have had a weak influence on endometrial CD8 staining.

4. Discussion

In the present study we observed that 1) endometrial CD8 and CD56 counts were significantly higher and

endometrial CD163 counts were significantly lower for infertile women than for fertile women. 2) Plasma SOCS3 levels had an influence on the staining count of endometrial macrophages with surface antigen CD163 during the luteal phase of the menstrual cycle. 3) Plasma SOCS3 and TGF-b levels showed a significant association with the staining count of cytotoxic T cells with CD8 antigen on the luteal endometrium. 4) Plasma IL10 and IL17 levels were the covariates that approached statistical significance in the regression model fitted for conception.

Russell et al. analyzed 1989 endometrial biopsy specimens of 222 women with recurrent miscarriage or IVF failure to provide reference ranges for CD8+, CD163+, and CD56+ cells for individual days of a normalized menstrual cycle. The number of CD8+ T cells showed a moderate increment in the luteal phase. A significant accumulation of CD163+ macrophages was observed in the superficial stroma after day 20 of the cycle. In addition, CD56+ uterine NK cells showed a dramatic increment from day 22 of the standardized 28-day cycle (5). The CD163+ cell count was significantly lower in our infertile population than in our fertile population. This

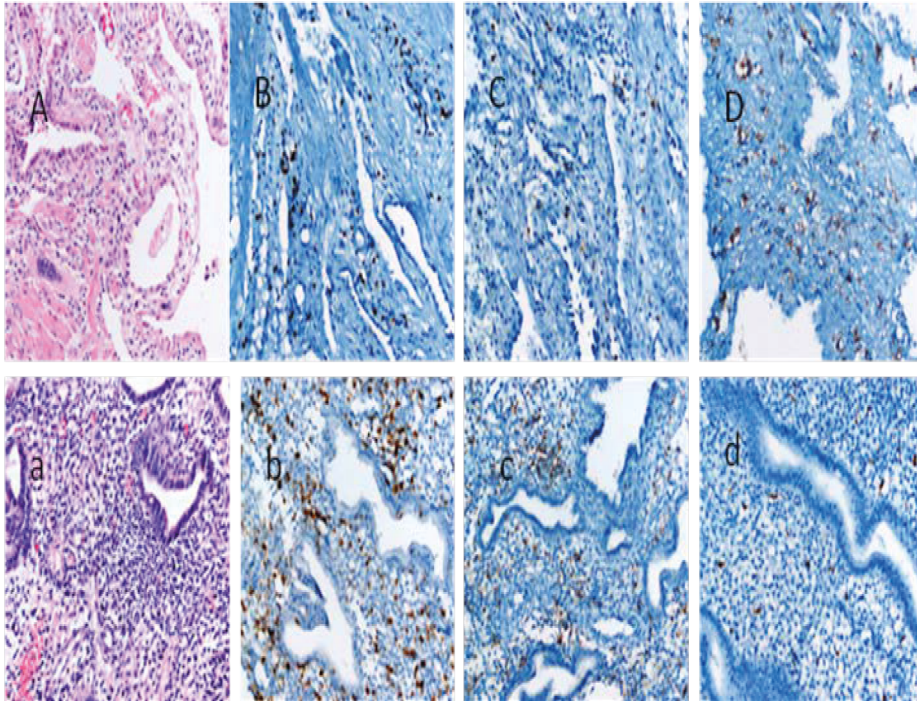


Figure 1. Immunohistochemical staining of CD8, CD56, and CD163 on luteal endometrium of fertile and infertile women. Fertile group: A) Secretory endometrium, B) Endometrial CD8 positive lymphocytes, C) Rare endometrial CD56 positive NK cells, D) High endometrial CD163 positive macrophages (A: H&E, 200 $\times$; B, C, D: immunoperoxidase, 200 $\times$). Infertile group: a) Endometrial lymphocyte aggregations and eosinophils, b) High CD8 positive lymphocytes in endometrial stroma and glands, c) CD56 positive NK cells, d) Rare CD163 positive macrophages in endometrium (a: H&E, 200 $\times$; b, c, d: immunoperoxidase, 200 $\times$).

finding can be attributed to the presence of unexplained infertility in our study population. Rizzo et al. observed that the proportion of CD56+ NK cells in endometrial flushing samples was lower in primary infertile women than in secondary infertile women (1). Matteo et al. found that the level of NK cells in the secretory endometrium was significantly lower in infertile women with polycystic ovary syndrome than in fertile women (14). However, Lynch et al. reported an increased expression of CD56 antigen in the endometrium of infertile women compared to that in the endometrium of fertile controls (15). In an endometrial culture study, it was observed that low-dose mifepristone increased the number of CD56+ NK cells during the receptive period to prevent pregnancy (16). Klentzeris et al. observed that the infertile women had fewer CD56+ cells in the luteal endometrium than the fertile controls (17). Fukui et al. studied the endometrial CD56+ NK cell positivity in an infertile population with mixed etiology. They observed increased CD56 positivity in patients with failed implantation (18). In our study, as compared to the fertile population, the infertile population showed an increase in the level of CD56+ NK cells in the luteal endometrium. The differences in endometrial NK

cell expression among the studies can be attributed to the heterogeneity of the populations.

Researchers have reported that infertile women have lower peripheral blood total NK cell percentages than fertile women (19). Some investigators observed decreased peripheral NK activity in infertile women that became pregnant compared to in nonpregnant infertile women (20). Peralta et al. found that, compared with infertile women, fertile women showed increased integrin molecule production by the peripheral blood NK cells in the preovulatory period (21). McGrath et al. reported increased circulating NK cells in infertile women with unexplained infertility in the secretory phase (22). In our study, we could not investigate the peripheral blood leukocyte levels and so we could not make a comparison on this topic with other studies. Interestingly, researchers have suggested that increased preconceptional TNF- α /IL-10 ratio is related to IVF failure (23). In our study, we did not observe any relation between TNF- α /IL-10 ratio and IVF outcome. This difference may have been caused by the sampling period. We obtained blood samples in the luteal phase of the natural cycle just before COH and not in the stimulated cycle. Fornari et al. compared natural and

stimulated cycle plasma IL-10 levels of infertile women with unexplained infertility. They did not observe a significant difference. The comparison of infertile women with fertile women revealed no significant difference either (24). The discussion on IL-10 is complex because it is produced by either CD4+ cells or mononuclear phagocytes.

Russell et al. observed a modest increment in the number of endometrial CD8+ T cells during the luteal phase in infertile women (5). Michimata et al. observed similar endometrial CD8+ T cell positivity in the endometrial samples of patients with recurrent pregnancy failure and male factor infertility (25). Fukui et al. observed increased peripheral blood CD8+ leukocyte percentage in patients with failed implantation compared to that in patients with successful implantation during the midsecretory phase of the menstrual cycle prior to the IVF cycle (18). Klentzeris et al. studied luteal endometrial CD8 positivity in 24 women with unexplained infertility and found that they had significantly lower numbers of CD8+ cells than fertile women (17). Galgani et al. observed increased levels of CD8+ cells in the midsecretory endometrium of women who experienced repeated IVF failure (26). In our study, we observed that, as compared to fertile women, infertile women had increased CD8+ leukocyte counts in the luteal endometrium. Different results on endometrial CD8 positivity among studies can be attributed to the variety of populations and methods. In our study, the lack of correlation between leukocyte subtypes and implantation rate can be attributed to the limited study population or to

the fact that endometrial sampling was carried out during the natural cycle and not during the stimulated cycle.

Carvalho et al. found that SOCS3 gene expression had an influence on receptivity and implantation in bovine endometrium (27). Xu et al. showed that increased SOCS3 expression inhibited decidual CD56+ NK cell cytotoxicity in the implantation period (28). Another study demonstrated increased decidual CD56 cytotoxicity by SOCS3 silencing (29). In our study, we observed that plasma SOCS3 levels had an influence on endometrial CD8 and CD163 staining counts.

Our study has the following limitations: 1) We could not investigate the endometrial tissue and intracellular levels of cytokines, 2) the plasma and tissue results were obtained from the natural cycle not stimulated cycle, and 3) we could not investigate the peripheral blood leukocyte levels.

In conclusion, increased cytotoxic activity with decreased macrophage activity in the luteal endometrium and decreased plasma SOCS3 levels in infertile women may be either the reason for or result of unexplained infertility. This condition may affect the therapeutic results of IVF. We suggest that modifications in the endometrial leukocyte subpopulations can contribute to the improvement in IVF outcomes. Further studies with larger populations are needed to confirm this finding.

Acknowledgement

This study was financially supported by Firat University Scientific Research Foundation (FÜBAP).

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