

Cytokine and nitric oxide concentrations in follicular fluid and blood serum of patients undergoing assisted reproductive treatment: relationship to outcome

IVF tedavisi alan hastaların serum ve foliküler sıvı sitokin ve nitrik oksit konsantrasyonları: başarı ile ilişkisi

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Abstract

Objective: The role of cytokines and nitric oxide (NO) in ovarian folliculogenesis and the development of mature and fertilizable oocytes is controversial. The aim of this study is to determine the concentrations of interleukin (IL)-1 β , IL-6, IL-8, IL-12, tumor necrosis factor (TNF)- α and NO in the follicular fluid (FF) and blood serum (S) of patients undergoing assisted reproductive treatment (ART) and to investigate whether these cytokines could be used as a predictive parameter for ART outcome.

Material and Methods: A retrospective clinical study was performed at a university hospital including a total of 85 women who underwent ART. FF and serum samples were collected at the time of oocyte retrieval and measured for interleukin (IL)-1 β , IL-6, IL-8, IL-12, tumor necrosis factor (TNF)- α by the enzyme-linked immunosorbent assay (ELISA) technique, using commercially available kits and NO by the nitrate/nitrite colorimetric assay. The results were compared between the women who became pregnant and those who did not following ART.

Results: No significant difference was found in the FF and blood serum concentrations of the cytokines and NO between pregnant and non-pregnant women.

Conclusion: Follicular fluid and blood serum concentrations of IL-1 β , IL-6, IL-8, IL-12, TNF- α and NO do not predict pregnancy achievement following ART. (J Turkish-German Gynecol Assoc 2009; 10: 132-6)

Key words: Assisted reproductive treatment; cytokines; follicular fluid; nitric oxide; pregnancy

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Özet

Amaç: Folikül sıvısı içindeki sitokinlerin ve nitrik oksitin matur oosit gelişimi üzerindeki etkisi tartışmalıdır. Bu çalışmanın amacı, tüp bebek tedavisi alan hastaların folikül sıvısı (FF) ve serumunda (S) interleukinleri IL-1 β , IL-6, IL-8, IL-12, tümör nekrozis faktör (TNF)- α ve NO düzeylerini saptamak ve bunların başarı ile ilişkisini araştırmaktır.

Gereç ve Yöntemler: Bir üniversite hastanesinin tüp bebek merkezinde retrospektif çalışma planlandı. 85 kadın çalışmaya dahil edildi. IL-1 β , IL-6, IL-8, IL-12, tümör nekrozis faktör (TNF)- α düzeyleri ELISA ile, NO düzeyleri ise nitrat/nitrit kolorometrik assay ile saptandı.

Bulgular: Gebe kalan ve kalamayan kadınların S ve FF sitokin ve NO konsantrasyonları arasında fark bulunamadı.

Sonuç: Serum ve foliküler sıvı IL-1 β , IL-6, IL-8, IL-12, tümör nekrozis faktör (TNF)- α ve NO düzeyleri tüp bebek tedavilerinde başarıyı predikte etmez.

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Anahtar kelimeler: Yardımla üreme teknikleri tedavisi, sitokinler, foliküler sıvı, nitrik oksit, gebelik

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Introduction

A connection is suggested to be present between the endocrine and cytokine systems. Cytokines have been shown to play an important role in the regulation of follicular development and also follicular atresia (1). FF provides a microenvironment for the developing oocyte and contains immunological factors for the regulation of its development. Changes in the expression and the concentrations of certain cytokines can influence oocyte and embryo quality, resulting in a reduced ability to implant. Several FF markers were suggested to affect oocyte developmental potential in IVF (2, 3).

Different cytokines have been investigated for their effects on various steps in assisted reproductive treatment (ART) cycles, namely follicular development, fertilization, embryo development and implantation. The correlations between various cytokines and ART outcome have been sought extensively and controversial results have been reported.

Gonadotropins used in assisted reproduction were reported to induce local and systemic production of interleukin-1beta (IL-1 β , 4). Some authors suggested increased (4), some suggested decreased (5) and some suggested no different (6) FF IL-1 β concentrations in pregnant women compared to non-pregnant ones. FF IL-6, IL-8 and IL-12 levels were also investigated in IVF cycles (6-8). FF IL-6 concentrations were noted

to be significantly higher in pregnant women (6). No correlation was found between FF IL-8 concentrations and fertilization or implantation rates (8). FF IL-12 concentrations were reported to be associated with a negative outcome in IVF treatment (6, 8). On the contrary, some researchers found no significant difference in FF IL-12 concentrations between pregnant and non-pregnant patients (5, 7). Another cytokine investigated in the follicular fluid with regard to IVF parameters is tumor necrosis factor-alpha (TNF- α). Some researchers revealed no significant difference in FF TNF- α levels of pregnant and non-pregnant patients (6,9). However, it was suggested that FF TNF- α levels influenced oocyte quality, and significantly higher FF TNF- α concentrations were found in follicles containing poor quality oocytes (9). High levels of nitric oxide (NO) in follicular fluid was also proposed to be detrimental, as embryo quality was observed to decrease in patients with high FF NO levels (10). Similarly in another study, follicles that contained oocytes that fertilized and went on to divide beyond the 6 cell stage were found to have significantly lower FF NO levels as compared to follicles that contained oocytes that did not fertilize or failed to develop beyond the 5 cell stage (11). However, no significant difference was found in FF NO levels of pregnant and non-pregnant patients (9).

As seen from the literature, a number of factors found in the follicular fluid can influence assisted reproduction outcome. This study was undertaken to investigate whether FF and serum concentrations of cytokines IL-1 β , IL-6, IL-8, IL-12, TNF- α and NO differ according to the pregnancy achievement following ART. The results might lead to the use of these factors as the determinants of pregnancy after IVF. To the best of our knowledge, our patient cohort is one of the largest series reported so far.

Materials and Methods

Patients and protocols

This retrospective clinical study was performed at the Center for Reproductive Medicine of Yeditepe University Hospital, Istanbul, Turkey between July 2006 and October 2008. FF and serum samples obtained during oocyte retrieval from 85 women who underwent ART were analyzed for this study. FF samples contaminated with blood were not included. Patients with endometriosis, PCOS, recurrent abortions and immunological disorders were excluded from the study. Informed consent was obtained from all patients, allowing future scientific investigations on the collected FF and serum samples. The ethics committee of the hospital approved this study.

Patients were stimulated with either GnRH agonist long or antagonist protocol. Patients given GnRH agonist long protocol were administered leuprolide acetate (Lucrin®, Abbott, France), starting on the 21st day of the preceding cycle. On the third day of menses gonadotropins recombinant FSH (Puregon®, Organon, Netherlands or Gonal-F®, Serono, Switzerland) were initiated at a dose of 225-450 IU/day according to age, ovarian reserve and previous cycles. Patients given GnRH antagonist protocol were administered the same gonadotropins mentioned above at the same doses on the second day of menses after the ultrasound evaluation. When the leading follicle

reached 14 mm, GnRH antagonist (Orgalutran®, Organon, Netherlands or Cetrotide®, Serono, Switzerland) was added at a dose of 0.25 mg daily until the day of HCG injection. In both stimulation protocols, ovarian response was monitored by serial ultrasound scans and serum estradiol measurements and daily gonadotropin doses were adjusted accordingly. When the leading follicle reached 20 mm, HCG (Pregnyl®, Organon, Netherlands) 10000 IU was administered and oocyte pick-up was performed 35 hours later. ICSI was the usual method of fertilization. Two to five days after oocyte retrieval, the embryos were transferred into the uterus under ultrasound guidance. Luteal support was given starting on the day of oocyte pick-up with i.m. progesterone (Progynex®, Kocak, Turkey) 50 mg daily plus vaginal progesterone gel (Crinone®, Serono, Switzerland) once daily and continued up to the pregnancy test; in women who were pregnant only the vaginal gel was continued until the 12th gestational week. About 12 days after the transfer procedure, serum beta HCG measurement >10 IU/l determined the pregnancy.

Biochemical assays

FF and blood serum samples were obtained simultaneously from 85 women during the oocyte pick-up procedure. FF samples were collected in each woman from the leading follicles and pooled. Only the visually blood-free samples of follicular fluid were included in the analysis. FF and serum samples obtained were immediately centrifuged at 350 g for 10 min and supernatants were then collected and stored in tubes at -70°C until assay. In FF and serum samples, the concentrations of IL-1 β , IL-6, IL-8, IL-12, TNF- α and NO were measured with commercially available kits. IL-1 β was measured with The AssayMax Human IL-1beta ELISA kit (Assaypro, USA), intra-assay CV 5.1%, inter-assay CV 7.5% and minimum detectable level <3 pg/ml. IL-6 with The AssayMax Human IL-6 ELISA kit (Assaypro, USA), intra-assay CV 5.1%, inter-assay CV 7.0% and minimum detectable dose <10 pg/ml. IL-8 with The AssayMax Human IL-8 ELISA kit (Assaypro, USA), intra-assay CV 5.0%, inter-assay CV 7.2% and minimum detectable dose <1 pg/ml. IL-12 with The BioSource Human IL-12 +p40 ELISA kit (BioSource International Inc., USA), intra-assay CV 3.9%, inter-assay CV 4.0% and minimum detectable level <2 pg/ml. TNF- α with The AssayMax Human TNF-alpha ELISA kit (Assaypro, USA), intra-assay CV 5.5%, inter-assay CV 7.0% and minimum detectable level <10 pg/ml. NO with Nitrate/Nitrite Colorimetric Assay Kit Cat. No. 780001 (Cayman Chemical Company, USA), intra-assay CV 2.7%, inter-assay CV 3.4% and the detection limit is 2.5 μ M.

Statistical analysis

To determine whether serum and FF markers could distinguish the patients who became pregnant from those who did not, FF and blood serum cytokine and NO measurements were compared between pregnant and non-pregnant patients following ART. Statistical calculations were made using the Statistical Package for the Social Sciences (version 12.0, SPSS Inc., Chicago, IL, USA). For continuous variables Student's t-test and for categorical variables Chi-squared test and Fisher's exact test were used, where applicable. Results are expressed as

means±SD or percentages (counts) as appropriate. Statistical significance was defined as a value of P <0.05.

Results

Demographic features and cycle characteristics evaluated in 85 women constituting the studied groups are shown in Table 1. The patients who became pregnant were significantly younger than patients who did not (29.7±4.7 years vs. 32.2±4.8 years, respectively; p=0.02). In the 85 women recruited for the study, the indication for ART was unexplained infertility in 26% (n=22), tubal disease in 8% (n=7), and male factor in 66% (n=56). No significant difference was found between pregnant and non-pregnant patients regarding infertility etiology (Table 1). When cycle characteristics are concerned, significantly lower amounts of gonadotropins were required in patients who became pregnant compared with the patients who did not (2500±967 IU vs. 3278±1265 IU, respectively; p=0.002) and the number of total oocytes retrieved was significantly higher in patients who achieved pregnancy compared to non-pregnant ones (13.0±6.2 vs. 10.0±6.2, respectively; p=0.03). No significant difference was observed between the two groups regarding other cycle parameters (Table 1).

FF cytokine and NO concentrations in both groups are presented in Table 2. No significant difference was found in values between the women who became pregnant and those who did not (Table 2).

Blood serum concentrations of cytokines and NO in the two groups of patients are shown in Table 3. There was no significant difference between pregnant and non-pregnant women (P>0.05).

Discussion

Numerous studies have attempted to find differences between the serum and/or FF cytokine concentrations of pregnant and non-pregnant women following IVF treatment. Given the ambiguous findings regarding the association of FF/serum cytokine levels and IVF outcome, we evaluated whether the concentrations of various cytokines and NO were altered in pregnant cycles following IVF. To the best of our knowledge, our study is one of the largest series to assess multiple rel-

evant cytokines in serum and follicular fluid simultaneously in relation to the pregnancy achievement following IVF. In the present study, we observed no significant differences in the FF and blood serum cytokine and NO concentrations investigated between pregnant and non-pregnant cycles.

Table 2. Follicular fluid cytokine and nitric oxide concentrations of pregnant versus non-pregnant women following ART

Variable	Pregnant	Non-pregnant	P
FF IL-1β (pg/ml)	3.9±1.6	4.3±1.6	NS
FF IL-6 (pg/ml)	27.3±15.7	23.5±12.7	NS
FF IL-8 (pg/ml)	21.1±9.4	20.1±8.7	NS
FF IL-12 (pg/ml)	61.6±28.8	60.0±24.8	NS
FF TNF-α (pg/ml)	31.1±8.2	29.3±2.9	NS
FF NO (μM)	0.5±0.2	0.5±0.2	NS

(FF, follicular fluid; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; IL-12, interleukin 12; TNF-α, tumor necrosis factor alpha; NO, nitric oxide; NS, not significant, p>0.05).
Student's t-test

Table 3. Blood serum cytokine and nitric oxide concentrations of pregnant versus non-pregnant women following ART

Variable	Pregnant	Non-pregnant	P
S IL-1β (pg/ml)	3.4±2.5	3.6±2.9	NS
S IL-6 (pg/ml)	17.1±10.3	15.6±5.9	NS
S IL-8 (pg/ml)	29.8±18.6	23.3±19.5	NS
S IL-12 (pg/ml)	54.4±22.9	50.9±23.6	NS
S TNF-α (pg/ml)	36.6±13.7	32.4±7.5	NS
S NO (μM)	1.7±1.6	1.7±1.1	NS

The values are given as mean±SD.
(S, serum; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; IL-12, interleukin 12; TNF-α, tumor necrosis factor alpha; NO, nitric oxide; NS, not significant, p>0.05).
Student's t-test

Table 1. Demographic features and cycle characteristics of pregnant versus non pregnant women following ART

Variable	Pregnant (n=51)	Non-pregnant (n=34)	P
Age (years)	29.7±4.7	32.2±4.8	0.02
Infertility duration (years)	6.8±4.1	8.0±5.2	NS
Cause of infertility %, (n)			
Tubal	6 (3)	12 (4)	NS
Male	69 (35)	62 (21)	NS
Unexplained	25 (13)	26 (9)	NS
Gonadotropins used (IU)	2500±967	3278±1265	0.002

The values are given as mean±SD or percent (numbers).
(HCG, human chorionic gonadotropin; E2, estradiole; MII, metaphase II; ET, embryo transfer; NS, not significant, p>0.05).
Student's t-test, Chi-squared test and Fisher's exact test

Interleukins are known to be involved in the immune system and play a role during inflammation. The ovarian follicle is suggested to be a site of inflammatory reactions. Thus various studies were performed to investigate the concentrations of proinflammatory cytokines in the follicular fluid of patients undergoing IVF treatment. The increased follicular fluid levels of some interleukins were noted to probably influence oocyte quality and fecundability by deteriorating the follicular micro-environment.

It has been reported that follicular fluid exerts chemotactic effects on neutrophilic granulocytes and the concentration of this activity is related to the outcome of IVF (12). About 5-15% of the cellular pool of the FF consists of macrophages, and intra-ovarian macrophages are involved in the production of IL-1 β (13,14). IL-1 has been suggested to be one of the regulators of ovarian steroidogenesis (15). Previous studies suggested that serum and FF IL-1 β concentrations were correlated with the pregnancy achievement (4,5). However, the results are controversial. Barrionuevo et al. (11) investigated FF IL-1 β concentrations in relation to fertilization and embryo cleavage rates and revealed no correlation. Karagouni et al. (4) showed significantly higher amounts of FF IL-1 β in the implantation versus non-implantation cycles. In contrast, Leal et al. (5) found FF IL-1 β levels to be significantly higher in non-pregnant women. In the study by Bedaiwy et al. (6), FF IL-1 β concentrations were found to be similar in pregnant and non-pregnant cycles. Similarly, we found no significant difference in FF and serum IL-1 β concentrations between pregnant and non-pregnant patients.

IL-6 is one of the cytokines that can influence granulosa cell steroidogenesis (16). Changes in E2 levels seen during gonadotropin stimulation were found to induce changes in serum IL-6 and TNF- α levels (17). Studies regarding FF IL-6 levels between pregnant and non-pregnant patients following ART are scarce. In a recent study, FF IL-6 concentrations were shown to be significantly higher in pregnant cycles compared to those in non-pregnant ones (6). However, we found similar FF and serum IL-6 concentrations between pregnant and non-pregnant women after IVF. In one study, serum IL-6 levels were found to be significantly higher in PCOS women undergoing IVF compared to those in normally ovulating women undergoing IVF for male factor infertility (18). In some studies, elevated levels of serum IL-6 levels were reported in OHSS cases (19). Conversely, in another study, serum IL-6 levels were found to be correlated negatively with E2 levels (17). FF IL-6 concentrations were also compared between low and high responder patients undergoing ART, and no difference was found (20). In our study, the peak serum E2 levels were similar between the two groups and we did not include the PCOS cases in the study.

IL-8 is one of the potent leukocyte chemotactic cytokines found in the preovulatory follicle. IL-8 was suggested to be an essential part of folliculogenesis. In a previous study, FF IL-8 concentrations were compared between pregnant (n=11) and non-pregnant (n=33) women following IVF and were found to be no different (8). Our results are in accordance with them.

IL-12 is a potent immunomodulatory cytokine involved in inflammatory processes with antiangiogenic effects (21). Gazvani et al. (8) investigated IL-12 levels in FF and reported

that its presence in FF was associated with a negative outcome in IVF treatment. Similarly, in another study FF IL-12 concentrations were found to be significantly lower in pregnant compared with non-pregnant cycles (6). In another study, serum and FF IL-12 levels were studied in relation to the outcome in women undergoing IVF and no association of either serum or FF IL-12 levels with the outcome was demonstrated (5). Another study also reported no significant difference in FF IL-12 concentrations between pregnant and non-pregnant patients (7). Similarly, we observed no significant difference in FF and serum IL-12 concentrations between patients who became pregnant and those who did not following ART.

Regarding FF and serum TNF- α concentrations, very few studies exist in the literature. Bedaiwy et al. (6) in their study compared FF TNF- α concentrations between pregnant and non-pregnant cycles and found no difference. Similarly, Lee et al. (9) revealed no significant difference in FF TNF- α levels of pregnant and non-pregnant patients. However, significantly higher TNF- α concentrations were found in follicles containing poor quality oocytes. In accordance with the above-mentioned studies, similar FF and serum TNF- α concentrations were found in the present study between patients who became pregnant and those who did not following ART.

Nitric oxide (NO), a potent vasodilator, is also produced in the ovary and is involved in folliculogenesis (22). However, the association of FF NO concentrations with pregnancy potential in IVF remains controversial. In a study about FF NO concentrations in IVF cycles, the relationship to embryo grading was sought and it was reported that high NO levels in human follicles may be detrimental (10, 23). Similarly, lower FF NO levels at the time of oocyte retrieval were suggested to be associated with adequate fertilization and embryo cleavage rates (11). However, FF NO concentration was proposed to be of no use as a prognostic marker for the prediction of the pregnancy outcome (24, 25). Concerning the plasma NO levels, its concentration was found to be inversely correlated with uterine artery PI (26). The measurement of impedance to uterine blood flow in IVF cycles has been suggested to contribute to the evaluation of endometrial receptivity (27, 28). The decrease in peripheral impedance in the uterine vascular bed, reflected by a low PI, is a consequence of increased blood flow and tissue perfusion, which may improve uterine receptivity (29). Further studies incorporating Doppler measurements are needed to clarify this point.

Relevant studies in the literature reported various mean values for the serum or FF cytokines. A possible explanation for inconsistency may be related to patients' characteristics and also the timing of sample collection. Furthermore, the discrepancies may be due to technical variations in cytokine measurements, for example, to the different sensitivity of the ELISA system used.

One limitation of our study might be considered as the inclusion of unexplained infertility cases in the study. However, a previous study evaluated FF IL-6 and IL-8 levels according to the etiology of infertility and reported no significant difference (30). In conclusion, the concentrations of follicular fluid and blood serum IL-1 β , IL-6, IL-8, IL-12, TNF- α and NO do not appear to be determinants of the outcome of IVF treatment. Accordingly,

these cytokines and NO cannot be used in the prediction of pregnancy following IVF. Future studies with larger patient numbers are required to understand the regulatory mechanisms in *in vitro* fertilization treatments, which might show the importance of some cytokines as biomarkers for the success of IVF.

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