

Article

Differential expression of follicular fluid cytokines: relationship to subsequent pregnancy in IVF cycles



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Abstract

The objective of this study was to investigate the role of cytokines in the peri-ovulatory follicular fluid (FF) during IVF cycles. FF from 112 women was obtained during oocyte retrieval for IVF. The concentrations of five cytokines [interleukin (IL)-1 β , IL-6, IL-12, IL-13, and tumour necrosis factor alpha (TNF α)] were measured in FF and their concentrations compared among women who became pregnant and those who did not. Thirty-one endometriosis patients, 15 idiopathic infertility, 21 tubal factor infertility, 15 ovarian factor infertility, and 30 patients with male factor infertility were included. Interleukin-13 and TNF α were absent in the peri-ovulatory FF of all patients. Fifty-two patients achieved pregnancy, whereas 60 did not. Both pregnant and non-pregnant groups were comparable for age, parity, ovarian stimulation parameters, fertilization rates, and embryo freezing rates. Concentrations of FF IL-1 β were not significantly different between pregnant and non-pregnant cycles. Concentrations of FF IL-6 were significantly higher in pregnant compared with non-pregnant cycles ($P = 0.0005$). Concentrations of FF IL-12 were significantly lower in pregnant compared with non-pregnant cycles ($P = 0.0002$). Higher concentrations of IL-12 and lower concentrations of IL-6 in the peri-ovulatory FF are associated with a negative outcome in IVF treatment. Interleukins-12 and IL-6 are biological markers that appear to affect IVF outcome.

Keywords: cytokines, follicular fluid, pregnancy

Introduction

While the number and activity of polymorphonuclear leukocytes in fresh whole blood do not change significantly during the natural menstrual cycle, their number increases and their migration activity decreases during ovarian stimulation (Giuliani *et al.*, 2004).

The migration of leukocytes during ovulation is limited to the peri-ovulatory follicles and corpus luteum (CL), suggesting hormonal control of leukocyte migration to these tissues. The pre-ovulatory follicular fluid (FF) possesses neutrophil chemotactic activity that is higher in conception than in non-conception cycles (Herriot *et al.*, 1986). It has been shown that monocyte chemotactic protein

1 (MCP-1) concentrations in women with endometriosis are significantly lower compared with a control group with tubal infertility (Xu *et al.*, 2006).

Individual cytokines in human ovarian FF play an important role in the follicular microenvironment and can be used as predictors for IVF outcome. Mean concentrations of interleukin (IL)-1 were lower in treatment attempts leading to a clinical pregnancy compared with those in which no pregnancy was established (Mendoza *et al.*, 2002; Baka and Malamitsi-Puchner, 2006). Detectable serum concentrations of IL-1 β were associated

with a higher implantation rate in IVF-embryo transfer patients (Karagouni *et al.*, 1998). Leal *et al.* (2006) found differences in the concentrations of IL-1 β in FF associated with a lack of pregnancy after excluding patients with endometriosis (Leal *et al.*, 2006). IL-6 is a regulator of inflammation and immunity, which may represent a physiological link between the endocrine and the immune systems. IL-6 is produced by rat and bovine granulosa cells *in vitro* (Gorospe *et al.*, 1992; Gorospe and Spangelo, 1993; Alpizar and Spicer, 1994). IL-6 can influence steroidogenesis and granulosa cell steroidogenesis (Adashi, 1990).

IL-6 and IL-1 may act on early and late stages of normal B lymphocyte differentiation, and their synergistic effect is potentiated by glucocorticosteroids (Emilie *et al.*, 1988). Immunohistochemical studies on human ovaries show IL-6 secretion in corpora lutea and theca cells, and its bioactivity has been detected in FF of IVF patients (Baes *et al.*, 1987; Spangelo *et al.*, 1989). No significant difference between FF IL-6 concentrations of low- and high-responder intracytoplasmic sperm injection (ICSI) patients was detected (Hammadeh *et al.*, 2003).

IL-12 is produced by B-cells, phagocytic cells, and other antigen-presenting cells (Sartori *et al.*, 1997). The promotion of production of a potent natural killer (NK) activator, interferon- γ (IFN- γ) by IL-12 is responsible for regulating abortion in mice (Hayakawa *et al.*, 1999). The correlation between FF and/or serum concentrations of IL-12 and negative IVF outcome ranged from studies showing a strong association to studies showing no association at all (Gazvani *et al.*, 2000).

So far as is known, none of the studies in this regard has established a connection between FF cytokines and oocyte recruitment and maturity. The objective of this study was to investigate the role of FF cytokines in relation to oocyte recruitment and maturity as well as IVF outcome.

Materials and methods

The study was approved by the Institutional Review Board (IRB) of the Cleveland Clinic. Follicular fluid was collected from 112 patients undergoing 112 consecutive IVF cycles over a 12-month period. Fifty patients underwent conventional IVF cycles and 62 patients underwent ICSI. There were 31 endometriosis patients, 15 idiopathic infertility, 21 tubal factor infertility, 15 ovulatory factor infertility, and 30 with male factor infertility. Patients with blood contaminated FF were excluded. Those who were included were divided into two groups: those who achieved pregnancy and those who did not.

Ovarian stimulation

The long down-regulation protocol (Lupron; Tap Pharmaceutical Inc., Deerfield, IL, USA) was used in all patients as a daily subcutaneous (s.c.) dose of 10 IU (0.5 mg) started on cycle day 21. Once serum oestradiol concentrations were suppressed to ≤ 50 pg/ml, the dose was reduced to 5 IU (0.25 mg) and continued until the day of human chorionic gonadotrophin (HCG) administration. Ovarian stimulation with recombinant FSH (Gonal-f; Serono, Randolph, MA, USA), urinary FSH (Metrodin, Serono) or human menopausal gonadotrophin (Humegon, Organon, West Orange, NJ, USA) was begun following pituitary down-regulation. The standard initial dose was 300 IU starting on day 3 of the

treatment cycle. The selection of the ovarian stimulation regimen was individualized based on the results of the ovarian reserve screening.

The ovarian response was monitored by serial serum oestradiol concentrations and transvaginal ultrasonograms beginning on day 5 of stimulation until the day of HCG administration. Based on these results, the FSH dose and subsequent monitoring was individualized. Ovarian stimulation was continued until at least two follicles reached a mean diameter of ≥ 18 mm, at which time HCG (Profasi; Serono) 10,000 IU s.c. was administered, 36 h before oocyte recovery.

Oocyte retrieval

Oocytes were collected by transvaginal ultrasound-guided needle aspiration of the follicles. The procedure was performed under deep conscious sedation. The retrieved oocytes were rinsed, graded and placed in HEPES buffered human tubal fluid (HTF) (Irvine Scientific, Santa Ana, CA, USA) at 37°C under 5% CO₂, 5% O₂ and 90% N₂.

Sperm collection and preparation

Semen samples were obtained by masturbation concurrently with oocyte retrieval following 3–5 days of abstinence. Spermatozoa were prepared by a density gradient centrifugation technique. Samples were loaded onto a single 90% Isolate (Irvine Scientific) layer and centrifuged at 300 g for 20 min at room temperature. The resulting pellet was washed by centrifugation for an additional 7 min and re-suspended in HTF media supplemented with 5% synthetic serum substitute (SSS; Irvine Scientific) at room temperature until the time of IVF or ICSI. In conventional IVF, 150–200 $\times 10^3$ spermatozoa were added to each culture dish containing four or five oocytes. A single morphologically normal appearing spermatozoon was selected for ICSI.

Gamete and embryo culture

After cumulus dissection and washing, the oocytes were placed in 1 ml of HTF supplemented with 5% SSS. Fertilization was confirmed 14–16 h post-insemination by the presence of two pronuclei (2PN) and extrusion of the second polar body. Normally fertilized oocytes were cultured in groups of four or five in 1 ml of HTF with SSS until the early afternoon of day 3. Following a five drop rinse in blastocyst medium (Irvine Scientific), they were then placed in 1 ml of in the same medium. A second change over to fresh blastocyst medium was done on the morning of day 5 after embryo evaluation and before embryo transfer. Embryo transfers were scheduled between 12:00 and 14:00 hours, 3 or 5 days after oocyte retrieval.

Embryo evaluation

Day 3 and day 5 embryos were evaluated with an Olympus X70 inverted microscope ($\times 600$, Olympus America, Melville, NY, USA), equipped with Hoffman Modulation Optics (Narishige, Tokyo, Japan). Cell number, degree (%) and pattern of fragmentation were recorded on day 3. The degree of fragmentation was defined as the embryonic volume occupied by enucleated cytoplasmic fragment.

Development on day 5 was recorded as follows. A distinct inner cell mass (ICM), organized as a compacted mass of cells, or a cohesive layer of numerous tightly packed cells in the trophectoderm (TE) were considered normal. Highly irregular ICM and/or TE cells on days 5 and 6 were considered abnormal and arrested.

Preparation of the follicular fluid

Follicular fluid was aspirated from the follicles on the day of oocyte retrieval. The cellular constituents of the FF were removed by centrifugation at 300 g for 20 min. Follicular fluid supernatants were then collected and stored in aliquots at -70°C until the cytokine concentrations were determined.

Detection of cytokines in follicular fluid

Commercially available, cytokine-specific, enzyme-linked immunosorbent assays (ELISA; R and D Systems Inc., Minneapolis, MN, USA) were used to measure the concentrations of IL-1 β (kit number DLB50), IL-12 (whole, kit number D1200), IL-6 (kit number D6050), IL-13 (kit number D1300), and TNF α (kit number DTA00C) in the FF. The frozen samples were thawed and analysed. Samples from each patient group were always measured in parallel and in duplicate to avoid inter-assay variance. The samples were pooled per patient. The sensitivities of the IL-1 β , IL-12, IL-6, IL-13, and TNF α ELISA were 1, 0.7, 5, 32, and 4.4 pg/ml respectively with standard curve ranges of 3.9–250, 3.12–300, 7.8–500, 62.5–4000, and 15.6–1000 pg/ml respectively. The intra-assay and inter-assay coefficients of variation were: for IL-1 β , 2.8–8.5 and 4.1–8.4 pg/ml; for IL-12, 1.1–1.5 and 3.3–7.1 pg/ml; for IL-6, 2.0–4.2 and 3.3–6.4 pg/ml; for IL-13, 2.4–6.3 and 3.8–6.5 pg/ml; and for TNF α , 4.2–5.2 and 4.6–7.4 pg/ml.

Statistical analysis

The demographic variables and FF measurements were compared across the patient groups with Kruskal–Wallis tests. Pairwise comparisons between the groups were performed with the Wilcoxon's rank sum test. Results of these analyses are reported using the median and interquartile range (IQR: 25th to 75th percentile). Statistical computations were performed with SAS version 8.1 (SAS Institute, Cary, NC, USA), and statistical significance was assessed using two-tailed tests and an alpha level of $P < 0.05$.

Results

Demographic variables and assessment of cycle outcome

Fifty-two patients (46%) achieved pregnancy, whereas 60 (54%) did not. The demographic features of the study population and the indications for assisted reproduction are presented in **Table 1**. A total of 112 patients underwent 112 cycles of IVF throughout the duration of the study. The patients' ages, parity and percentage of primary versus secondary infertility were similar between groups of pregnant and non-pregnant cycles.

There were no significant differences in days of stimulation, number of FSH ampoules or amount of FSH used (IU) between the groups (**Table 2**). Serum concentrations of day 3 FSH, day 7 LH, day 3, day 7, and day 10 oestradiol (pg/ml) as well as oestradiol on day of HCG administration (pg/ml) were similar between pregnant and non-pregnant cycles. The number of mature follicles

Table 1. Demographic features and indications for assisted reproduction technologies in both groups.

Parameter	Non-pregnant cycles	Pregnant cycles
No. of patients	60	52
No. of cycles	60	52
Mean age (years) \pm SD	34.17 \pm 4.25	33.6 \pm 3.56
Mean parity \pm SD	0.4 \pm 0.5	0.3 \pm 0.5
No. with secondary infertility (%)	26 (43)	27 (52)
No. with primary infertility (%)	34 (57)	25 (48)
Infertility factor <i>n</i> (%)		
Male	17 (28)	13 (25)
Tubal	10 (17)	11 (21)
Ovarian	8 (13)	7 (13)
Endometriosis	18 (30)	13 (25)
Unexplained	7 (12)	8 (15)

There were no statistically significant differences between the two groups.

Table 2. IVF cycle parameters and follicular fluid cytokines.

Parameter	Non-pregnant cycles (n = 60)	Pregnant cycles (n = 52)
Days of stimulation	9.55 ± 1.79	9.57 ± 1.53
FSH used (IU)	2851.05 ± 880.58	2647.2 ± 855.38
Day 3 FSH (IU/ml)	9.43 ± 2.45	8.23 ± 1.34
Day 3 oestradiol (pg/ml)	39.18 ± 19.26	30.63 ± 15.93
Oestradiol on day of HCG administration (pg/ml)	1879.8 ± 977.5	1955.9 ± 981.6
No. of mature follicles on day of HCG administration	12.96 ± 6.7	13.8 ± 6.4
No. of oocytes retrieved	11.8 ± 7.1	12.27 ± 6.88
Fertilization rate	0.64 ± 0.2	0.66 ± 0.21
No. of frozen 2PN-stage embryos	0.97 ± 2.32	1.72 ± 3.73
No. of arrested embryos	1.68 ± 2.32	2.36 ± 2.38
No. of frozen blastocyst	0.704 ± 1.53	1.23 ± 1.9
No. of embryos transferred	2.85 ± 0.95	2.87 ± 0.76
IL-1β (pg/ml)	0.50 ± 0.65	0.65 ± 0.9
TNFα (pg/ml)	7.83 ± 10.26	23.10 ± 29.74
IL-12 (pg/ml)	24.45 ± 20.15 ^a	3.38 ± 10.6 ^a
IL-6 (pg/ml)	6.364 ± 6.203 ^b	17.378 ± 14.303 ^b

Values are mean ± SD.

Values with the same superscript letter are significantly different: ^a*P* = 0.0002; ^b*P* = 0.0005. There were no other significant differences between the two groups.

HCG = human chorionic gonadotrophin; IL = interleukin; NS = not statistically significant; PN = pronuclear; TNFα = tumour necrosis factor α.

on day of HCG administration and oocytes retrieved were also similar between the two groups. The number of oocytes injected in the ICSI group was apparently higher among the non-pregnant group, but not significantly.

There were no significant differences in fertilization rate, number of arrested embryos, frozen 2PN stage embryos, frozen blastocysts and embryos transferred between the groups (**Table 2**).

Follicular fluid cytokines

Interleukin-13 and TNFα were not detected in the peri-ovulatory FF of any patient. Concentrations of IL-12 (pg/ml) were significantly lower among pregnant cycles than in the non-pregnant cycle group (*P* = 0.0002). Concentrations of FF IL-6 were significantly higher in pregnant compared with non-pregnant cycles (*P* = 0.0005). Although concentrations of FF interleukin-1β (pg/ml) and tumour necrosis factor TNFα (pg/ml) were higher among the pregnant group, these differences were not significant (**Table 2**).

Discussion

The role of FF interleukins as biological markers for IVF outcome is gaining increasing attention in the literature. Herriot *et al.* found neutrophil chemotactic activity in pre-ovulatory FF to be higher in conception than in non-conception cycles (Herriot *et al.*, 1986). IL-12 is involved in induction and maintenance of the immune response (Lamont and Adorini, 1996). Vujisic *et al.* suggested a role of IL-12 in human reproduction, and confirmed the presence of the p40 subunit of IL-12 in human FF

from women undergoing natural cycle IVF and embryo transfer (Vujisic *et al.*, 2004).

The present study found significantly (*P* = 0.0002) higher concentrations of IL-12 in the peri-ovulatory FF of women who failed to become pregnant with IVF, consistent with the results reported by Gazvani *et al.* (2000). These workers attributed this to follicle immaturity, as well as negative effects of IL-12 on folliculogenesis, oocyte quality, and/or implantation. IL-12 is important in the initiation of a T helper (Th)-1 response that is detrimental to implantation and, indirectly, in down-regulation of a Th-2 response that is associated with successful pregnancy. The anti-angiogenic properties of IL-12 and its down-regulating effects on Th-2 response may be the underlying cause of impaired implantation (Gazvani *et al.*, 2000). Ledee-Bataille *et al.* (2004) suggested that IL-12 is an abortifacient because it induces production of IFN-γ, a potent NK activator (Ledee-Bataille *et al.*, 2004).

The present study also noted that IL-6 was significantly (*P* = 0.0005) higher in the pregnant compared with the non-pregnant cycles. It has been suggested that IL-6 significantly inhibited HCG-induced progesterone secretion of granulosa lutein cells which could compromise endometrial receptivity (Kawasaki *et al.*, 2003). It is concluded that higher concentrations of IL-12 and lower concentrations of IL-6 in the peri-ovulatory FF appear to be associated with a negative outcome in IVF treatment. Both cytokines are powerful biological markers that may influence IVF outcome through an effect on oocyte quality and/or implantation.

Acknowledgements

Research support provided by the Research Programs Committee, Cleveland Clinic Foundation.

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Presented in part at the 60th Annual Meeting of the American Society for Reproductive Medicine, 2004, October 16–20, Philadelphia, PA, USA.

Received 23 April 2007; refereed 14 May 2007; accepted 21 May 2007.