Human Reproduction, Vol.32, No.4 pp. 820–831, 2017
Advanced Access publication on February 15, 2017 doi:10.1093/humrep/dex005

human reproduction

ORIGINAL ARTICLE *Infertility*

Gonadotrophin stimulation in IVF alters the immune cell profile in follicular fluid and the cytokine concentrations in follicular fluid and serum

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Submitted on May 31, 2016; resubmitted on December 28, 2016; accepted on February 9, 2017

STUDY QUESTION: Are the immune cell profiles and the cytokine concentrations in follicular fluid (FF) and serum at the preovulatory stage different in conventional exogenous gonadotrophin stimulated IVF (c-IVF) compared with natural cycle IVF (NC-IVF)?

SUMMARY ANSWER: The cell counts of CD45+ leucocytes and T cell subpopulations and the cytokine concentrations in FF and serum are different in c-IVF compared to NC-IVF.

WHAT IS KNOWN ALREADY: FF-derived cells are heterogeneous. Immune cells are involved in intra-ovarian processes and cytokines are required for normal follicular development. Gonadotrophins stimulate the regulatory intrafollicular system and influence the local distribution of immune cells and the intrafollicular release of cytokines. Administration of exogenous gonadotrophins may have a significant effect on this local regulatory system, which then in turn could influence oocyte quality.

STUDY DESIGN, SIZE, DURATION: The study included 105 patients, 69 undergoing c-IVF and 36 undergoing NC-IVF. c-IVF was performed by exogenous ovarian stimulation with hMG and GnRH antagonists.

PARTICIPANTS/MATERIALS, SETTING, METHODS: FF samples were collected from the first dominant follicle in c-IVF without pooling and from single leading preovulatory follicles in NC-IVF. Three different approaches were used to analyze FF samples: (i) microscopic investigation of CD45+ leucocytes, (ii) fluorescence-activated cell sorting to determine CD19+ B cells and CD3+ T cells including T cell subpopulations (CD4+, CD8+), and (iii) evaluation of tumour necrosis factor-alpha (TNF-α), interferon-gamma (INF-γ), interleukins (IL)−2, −6, −8, −10 and vascular endothelial growth factor (VEGF) levels in matched FF and serum samples using the Bio-Plex® platform.

MAIN RESULTS AND THE ROLE OF CHANCE: FF obtained from c-IVF contained proportionally more CD45+ leucocytes ($P = 0.0384$), but fewer CD8+ cytotoxic T cells than FF from NC-IVF. CD3+ T lymphocytes were the most common type of lymphocytes, and the number thereof was comparable in the two study groups. In c-IVF, serum VEGF levels were higher ($P = 0.007$) than in NC-IVF while FF contained marginally decreased concentrations of IL-8 in c-IVF in comparison to NC-IVF. The cytokine concentration gradient between FF and serum in c-IVF was 10-fold for IL-8 and 8-fold for VEGF and thereby markedly lower than in NC-IVF, where the differences were 32-fold and 30-fold, respectively. Strong positive correlations were determined between FF- IL-10 and FF- VEGF in c-IVF ($r = 0.85$, $P < 0.0001$) and in NC-IVF ($r = 0.81$, $P < 0.0001$).

LARGE SCALE DATA: N/A.

LIMITATION, REASONS FOR CAUTION: The ovulation of NC-IVF follicles was induced by the exogenous administration of hCG, which means that the environment did not fully correspond to the physiological situation.

WIDER IMPLICATIONS OF THE FINDINGS: The differences in the immune profile and the cytokine concentrations in c-IVF and NC-IVF follicles support the hypothesis that conventional ovarian stimulation affects indirectly and heterogeneously the intrafollicular milieu, and thereby possibly affects the oocyte quality and the IVF outcome. However, further studies are needed to confirm our findings and to refine stimulation protocols in the context of optimizing the intrafollicular environment during oocyte maturation.

STUDY FUNDING/COMPETING INTEREST(S): The study was supported by a research grant from IBSA Institut Biochimique SA and MSD Merck Sharp & Dohme GmbH. The authors are clinically involved in low dose mono-follicular stimulation and IVF-therapies, using gonadotrophins from all gonadotrophins distributors on the Swiss market, including Institut Biochimique SA and MSD Merck Sharp & Dohme GmbH.

Key words: natural cycle / ovarian stimulation / *in vitro* fertilization / leucocytes / cytokines

Introduction

Follicular fluid (FF) is a serum transudate, which contains metabolism products by granulosa and theca cells (McNatty, 1978; Gosden *et al.*, 1988) and provides the micro-environment of the grown oocyte. This environment is influenced by endocrine signalling (Bristol and Woodruff, 2004) and by the type of gonadotrophin the follicle is exposed to during the follicular phase (Kovacs *et al.*, 2008). FF-derived cells isolated from follicle aspirates at oocyte retrieval from IVF patients have been described as a heterogeneous cell population (Fedorcsak *et al.*, 2007) which includes immune cells in addition to steroidogenic cells, ovarian structural cells and epithelial cells (Enien *et al.*, 1998; Smith *et al.*, 2005). The immune cells are involved in intra-ovarian reproductive processes, including ovulation (Vinatier *et al.*, 1995) and formation of corpus luteum (CL) and its regression (Wu *et al.*, 2004; Galvao *et al.*, 2012).

Several studies have indicated that immune cell-derived cytokines and chemokines play an important regulatory role in ovarian function (Tabibzadeh, 1994; Qiao and Feng, 2011), particularly for follicular growth, ovulation and luteinization (Field *et al.*, 2014). For instance, tumour necrosis factor-alpha (TNF-α) plays a role in folliculogenesis, influences oocyte quality and induces progesterone synthesis in differentiated ovarian cells (Wang *et al.*, 1992; Zolti *et al.*, 1992; Lee *et al.*, 2000; Bornstein *et al.*, 2004), and interferon-gamma (INF-γ) and interleukin (IL)-2 have an impact on embryo cleavage (Lédée *et al.*, 2008), whereas IL-6 provides a regulatory effect on cumulus cell function and contributes to increase oocyte quality (Liu *et al.*, 2009). Both IL-6 and IL-8 regulate ovarian steroid production and the process of inflammation during ovulation (van der Hoek *et al.*, 1998; Büscher *et al.*, 1999). In addition, it has been shown that IL-10, an anti-inflammatory cytokine produced by macrophages and B/T cells subsets (Couper *et al.*, 2008), is detectable in human preimplantation embryo culture media (Ozörnek *et al.*, 1995), but its predictive value on the IVF outcome is not fully clear (Vujisić and Zidovec, 2004). Moreover, IL-10 and vascular endothelial growth factor (VEGF) levels promote angiogenesis and CL formation (Brännström *et al.*, 1999; Gomez *et al.*, 2003, 2010).

Most IVF cycles are performed using exogenous medications for growth of multiple follicles. In the recent years, a renewed interest in natural cycle IVF (NC-IVF) has arisen. This alternative treatment to the conventional IVF (c-IVF) is based on monitoring the spontaneous cycle, and retrieving a single oocyte during LH-peak (Nargund *et al.*, 2007). The use of exogenous ovarian stimulation might have an effect on the follicular milieu in a different way than in NC-IVF and thereby an impact on oocyte maturation and developmental competence. Previous studies have been focused on the hormonal follicular milieu and on the molecular characterization of dominant preovulatory follicles and compared them with unstimulated IVF. A significant change in FF hormones such as testosterone, androstendion, estradiol and LH in c-IVF has been earlier demonstrated (De los Santos *et al.*, 2012). Our group has reported a significant alteration in the hormonal follicular milieu especially for anti-Mullerian hormone (AMH) protein release (von Wolff *et al.*, 2014a) and for AMH mRNA expression in cultures of isolated granulosa cells from c-IVF when compared with NC-IVF cells (Kollmann *et al.*, 2015). However, how the exogenous hormone stimulation influences the intrafollicular change in the cellular and humoral composition, is not yet fully clarified.

The aim of our study was to assess the composition of immune cells and cytokines in the follicular environment from conventional stimulated cycles (c-IVF) and to compare them with those of similar-sized follicles obtained from NC-IVF. In our study, we were interested in the distribution of immune cells in the FF as well as in the differences in cytokines, chemokine and angiogenic factors released at the preovulatory stage in FF and in the patient's serum, by focusing on Th1 and Th2 cytokines produced by CD4+, CD8+ T cells.

Material and Methods

Sample collection and patient data

For this study, a total of 105 FF samples, classified as clear based on gross and microscopic appearance, were collected from patients undergoing oocyte retrieval at the University Hospital of Berne. Samples containing blood were excluded from the study. Samples were divided into patients who underwent c-IVF (n = 69) and patients who had NC-IVF (n = 36). Patients' demographics and cycle characteristics are given in Table I. There was no significant difference in either age or the aetiology of infertility between the two groups. The number of samples used for each analysis is presented in the study flow chart (Fig. 1). Our exclusion criteria and the variation in FF volume from a single follicle limited the sample number in the study. Informed written consent was obtained from the patients and the study was approved by the local ethical committee (IRB, no. 12–023, Inselspital Berne).

IVF procedure and sample collection

c-IVF patients were stimulated with 150–300 IU per day of highly purified human menopausal gonadotrophin (Menopur HP®, Ferring AG, Baar, Switzerland) which was initiated between Days 3 and 5 of the menstrual cycle.

Administration of GnRH antagonist (Orgalutran®, Ganirelix 0.25 mg, MSD Merck Sharp & Dohme GmbH, Lucerne, Switzerland) was started between Days 6 and 7 of the menstrual cycle and continued until ovulation induction. After adequate follicular growth, ovulation was induced with 10 000 IU of urinary hCG (Pregnyl®, MSD Merck Sharp & Dohme GmbH, Lucerne, Switzerland) and oocyte retrieval was performed 36 h later under general anaesthesia. The FF aspirated from the first, largest follicle was collected separately.

Women undergoing NC-IVF did not receive any medical intervention except for the administration of hCG (Pregnyl®, MSD Merck Sharp & Dohme GmbH, Lucerne, Switzerland) to induce ovulation. When the diameter of the follicle reached 18 mm 5000–10 000 IU of hCG was administered and oocyte retrieval was performed 36 h later under sterile conditions and without anaesthesia. After oocyte retrieval and the isolation of the cumulus oophorus complex, the total volume of the aspirate was collected in a 15-ml polystyrene tube (BD Falcon).

The FF was centrifuged at 440 × *g* for 10 min. The pellet was retained for cytospin and fluorescent-activated cell sorting (FACS) analyses after being suspended in 1 ml supernatant of FF. The supernatant was clarified by a further centrifugation step (1800 × *g*, 10 min) and stored at −70°C for cytokine measurements. Moreover, a venous blood sample was drawn on the same day for the assessment of cytokines in the serum.

Table I Patients' demographics, mean age, causes of infertility and cycle characteristics.

Variables	Group A NC-IVF	Group B c-IVF	*P*
FF samples Total	36	69	
Age (years)	35.6 ± 5.1	35.5 ± 4.5	NS
Range	21–43	23–43	
Aetiology of infertility (*n*)			
Tubal factor	6/36	3/69	
Male factor	12/36	37/69	
Idiopathic	18/36	27/69	
Pre-chemotherapy	0/36	2/69	
Total hMG dose (IU)	0	2312 ± 892.4	<0.0001***
No. of retrieved oocytes	1	8.1 ± 4.0	<0.0001***

Note: Values are mean ± SD; NS, not significant.

The total hMG used and the number of retrieved oocytes were higher in stimulated IVF compared with natural IVF cycle.

Immunohistochemistry

Immunohistochemistry staining for CD45+ leucocytes in the dominant preovulatory follicle was performed on cytospin slide preparations. A 100-µl sample of cell suspension was centrifuged at 24 × *g* for 2 min and fixed onto Superfrost Plus Menzel-slides (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA). The slides were air-dried and fixed for 10 min with 4% paraformaldehyde solution. After fixation, the slides were flushed three times with PBST (Phosphate Buffered Saline + Tween 20 0.1%, Sigma-Aldrich), air-dried and stored at 4°C until staining. To avoid unspecific binding of primary antibody, slides were blocked with 10% normal goat serum in PBS (Phosphate Buffered Saline, Sigma-Aldrich) for 30 min at room temperature, then flushed three times with PBST. To quench endogenous peroxidase, slides were exposed to 3% hydrogen peroxide (H_2O_2) in aquadest for 30 min before rinsing again three times with PBST. The slides were exposed to the primary antibody, a monoclonal mouse anti-human CD45 IgG1 (1:400 dilution with PBS), for 2 h at room temperature, followed by addition of HRP-polymer and detection substance, AEC kit (SuperPicTure TM Polymer, Detection kit, Invitrogen, CAT. No 87–9963), added stepwise according to the manufacturer's instructions. The cell nuclei were stained with haematoxylin for 2 min.

Quantitative evaluation

The distribution of CD45+ cells in the immune-stained slides was evaluated in four to eight randomly chosen fields and the mean of these values was calculated as described elsewhere (Jasper *et al.*, 2000; Wu *et al.*, 2007). Results are expressed as percentage area of AEC positive stain, normalized to total stained area (the area of haematoxylin counterstain plus AEC stain).

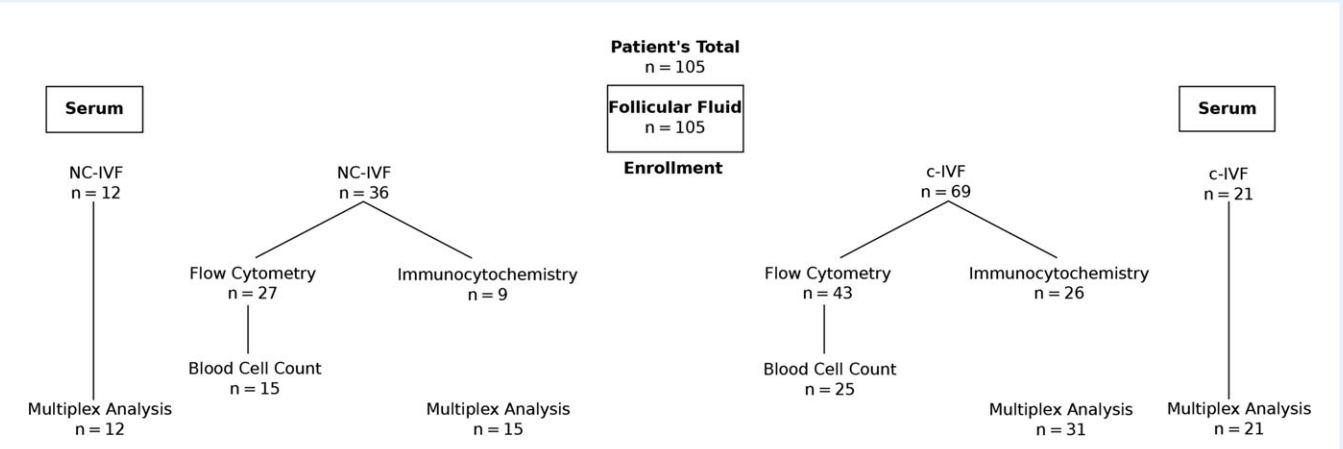

Figure 1 Study flow chart of patient enrolment and samples used for analysis. The follicular fluids (FFs) (total *n* = 105, natural cycle IVF [NC-IVF] *n* = 36, c-IVF *n* = 69) were analyzed by flow cytometry and immunohistochemistry. Some samples used for flow cytometry were randomly selected and the blood cell count was determined. Furthermore, a random selection of supernatants was analyzed by multiplexed analysis of cytokines and chemokines. The numbers of analyzed samples for each type of assay and each study group are shown (*n*). In addition, 12 and 21 serum samples after NC-IVF and c-IVF, respectively, were analyzed by multiplexed analysis.

Blood count

Haemogram, a basic laboratory test for red and white cell counts was run on FF samples obtained from c-IVF ($n = 25$) and from NC-IVF ($n = 15$). The test was done for white blood cell counts and to ensure that the selected FF samples were not contaminated with peripheral red blood cells before running a flow cytometric analysis.

Flow cytometry

A flow cytometric analysis was run on FF samples from c-IVF ($n = 43$) and NC-IVF ($n = 27$). The relative counts of CD19+ B lymphocytes, CD16+/CD56+ natural killer (NK) cells, and CD3+ T lymphocytes and their subsets of CD4+ and CD8+ T lymphocytes were identified and determined using the BD Multitest Reagent Kit (BD Biosciences, Allschwil, Switzerland CAT No. 342416 and 342417, respectively). The FACS analysis was performed on FACSCanto II. Data were analyzed using FACSDiva software version 6.1.3 (BD Biosciences).

Multiplex analysis system for cytokine and chemokine concentrations

IFN-γ, IL-2, IL-6, IL-8, IL-10, TNF-α and VEGF were measured in randomly selected FF samples from c-IVF ($n = 31$), NC-IVF ($n = 15$) patients and in 21, 12 serum samples, respectively, using Luminex xMAP multiplexing technology on the Bio-Plex® platform (Bio-Rad Laboratories) as previously described (Bersinger *et al.*, 2014). Briefly, the samples were diluted (1/4 for serum and 1/2 for FF) and run in duplicate. The processing of data was set according to the manufacturer's instructions. The data were analyzed using the Bio-Plex Manager software, version 6.1.

Statistical analysis

Non-parametric Mann–Whitney *U*-tests were used to investigate statistical differences following the immunohistochemical and flow cytometric assessments of leucocytes, lymphocytes and lymphocytes subsets distribution in the FF of the two study groups. If not otherwise specified, data were expressed as mean ± (SEM). The data of cytokine and chemokine concentrations were also compared for statistical difference with the non-parametric Mann–Whitney *U*-tests and expressed as median [range]. The *P*-value was adjusted using the Holm method for multiple testing (Holm, 1979). Regression analyses were calculated using the Spearman rank correlation method. A *P*-value of <0.05 was considered as statistically significant.

Results

The percentage of CD45+ leucocytes is significantly higher in FF from c-IVF than from NC-IVF

The distribution data for leucocytes in preovulatory FF from the two study groups are summarized in Table II.

The absolute number of leucocytes did not show a significant difference between the two study groups (13846 ± 3042 for c-IVF vs 12067 ± 2812 for NC-IVF). Importantly, however, analysis of the composition of the cell types by immunohistochemistry revealed that the relative percentage of CD45+ leucocytes was significant higher in the FF from c-IVF group than from the NC-IVF group (28.87 ± 2.39 vs 14.39 ± 3.80, $P = 0.0384$) (Table II).

The percentage of CD8+ T lymphocytes is decreased in FF from c-IVF

Since we observed a higher percentage of CD45+ cells in c-IVF compared to NC-IVF, we assessed the lymphocyte subpopulations at the preovulatory stage in FF from both study groups by flow cytometry. Figure 2 shows that the relative quantity of CD45+ leucocytes was higher in c-IVF than in NC-IVF, confirming our immunohistochemistry data. Furthermore, the flow cytometry data demonstrate that CD3+ T lymphocytes were the most common lymphocyte subset in c-IVF and NC-IVF since CD19+ B lymphocytes and CD16+/CD56+ NK cell were hardly detectable (Fig. 2A and B). Although the percentage of CD3+ T lymphocytes was comparable between the treatment groups (75.65 ± 3.15 in c-IVF vs 78.87 ± 2.13 in NC-IVF), the percentage of CD8+ cytotoxic T lymphocytes was (non-significantly) lower in c-IVF than in NC-IVF (17.63 ± 1.85 vs 27.65 ± 3.54, $P = 0.154$). This difference, however, did not yield a change in CD4/CD8 ratio (Fig. 2C and Table II).

The cytokine concentration in serum obtained from c-IVF patients is higher than in NC-IVF

Since we observed a lower percentage of CD8+ cytotoxic T lymphocytes in c-IVF than in NC-IVF, we have analyzed the level of IL-10 that plays an important role in enhancing the growth of CD8+ T cells (Chen and Zlotnik, 1991). Furthermore, we have included IL-6, IL-8, IFN-γ, TNF-α, IL-2 and VEGF in our study.

IL-2 and TNF-alpha were under the detection limit in most samples and IFN-γ was even absent in sera obtained from NC-IVF patients (Table III and data not shown). In contrast, IL-8, IL-10 and VEGF were

Table II Follicular fluid (FF) distribution of leukocytes as assessed by blood cell count, immunohistochemistry and flow cytometry analysis.

Cell type	NC-IVF	c-IVF	*P* value
[a]Leucocytes	12067 ± 2812	13846 ± 3042	NS
[b]CD45	14.39 ± 3.80	28.87 ± 2.39	0.0384*
[c]CD19	ND	ND	
[c]CD16/CD56	ND	ND	
[c]CD3	78.85 ± 2.13	75.65 ± 3.15	NS
[c]CD4	59.21 ± 3.70	66.83 ± 3.36	NS
[c]CD8	27.65 ± 3.54	17.63 ± 1.85	0.154
[c]CD4/CD8	6.11 ± 2.34	6.61 ± 1.24	NS

[a]Leucocytes/ ml was determined using the haemogram.
[b]Immunohistochemical analysis: data are calculated as percent area of positive stain, normalized to total stained area (the area of haematoxylin counterstain plus positive AEC stain area).
[c]Flow cytometry analysis: data are shown as percentage of CD3+ within CD45+ and CD4+, CD8+ within CD3+ cells. Data are expressed as mean ± (SEM).
Statistically differences of intrafollicular immune cells distribution at preovulatory stage between the two study groups were determined with non-parametric Mann–Whitney *U*-test and adjusted *P*-value using the Holm method for multiple testing.
*$P < 0.05$. NS, not significant; ND, not detectable.

detectable in the majority of serum samples of both c-IVF and NC-IVF where VEGF but not IL-8 and IL-10 was significantly higher in c-IVF than in NC-IVF patients (Table III, Fig. 3A). Remarkably, IL-6 was detectable in most c-IVF sera and with higher levels but was detectable in only one-third of NC-IVF sera.

In contrast to serum samples, in FF samples all analyzed cytokines (IL-6, IL-8, IL-10, VEGF, IL-2, IFN-γ and TNF-α) were detectable in most patients of the two study groups. Furthermore, we have shown that all tested mediators were comparable between c-IVF and NC-IVF, except IL-8 which was marginally lower in c-IVF (Table III, Fig. 3B). No significant correlation was observed between the concentrations of FF IL-8, IL-6, IL-10 and VEGF in the c-IVF group and either the total administrated gonadotrophins (hMG) or the number of retrieved oocytes (results not shown).

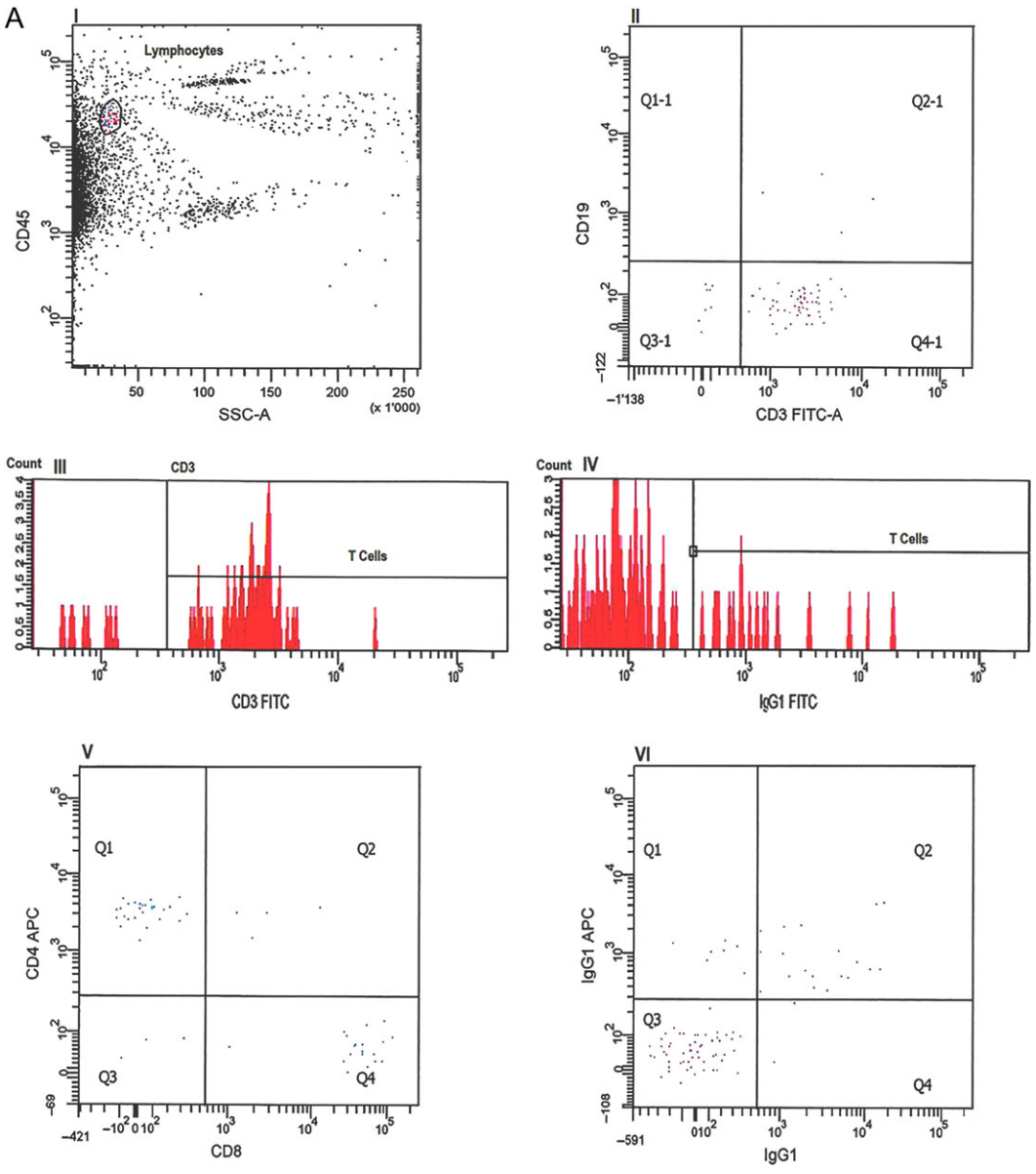

Figure 2 Flow cytometric analysis performed on single FF. Immune cells in the FF collected from patients undergoing NC-IVF (**A**) ($n = 27$) and c-IVF (**B**) ($n = 43$) were dispersed, labelled with a series of fluorochrome-conjugated leucocyte markers and analyzed using flow cytometry. (A, B) Gating strategy to determine relative quantity of lymphocytes subpopulations: a primary gate was set around CD45-positive/SSC-low leucocytes (I). Within CD45-positive/SSC-low leucocytes, CD19+ B cells and CD3+ T cells were determined (II). To evaluate percentage of CD4+ and CD8+ cells (V), a gate was set around CD3+ T cells (III). Isotype controls for anti-CD4 APC and anti-CD8 PE and anti-CD3 FITC are shown in IV and VI, respectively. (**C**) Data show median of percentage of CD3+ T cells within CD45+ cells and of CD4+ and CD8+ cells within CD3+ T lymphocytes of NC-IVF (●) and c-IVF (Δ). The percentage of FF- CD8+ in c-IVF was lower compared with NC-IVF. However, the difference was not significant ($P = 0.154$) as calculated by Holm method for multiple testing.

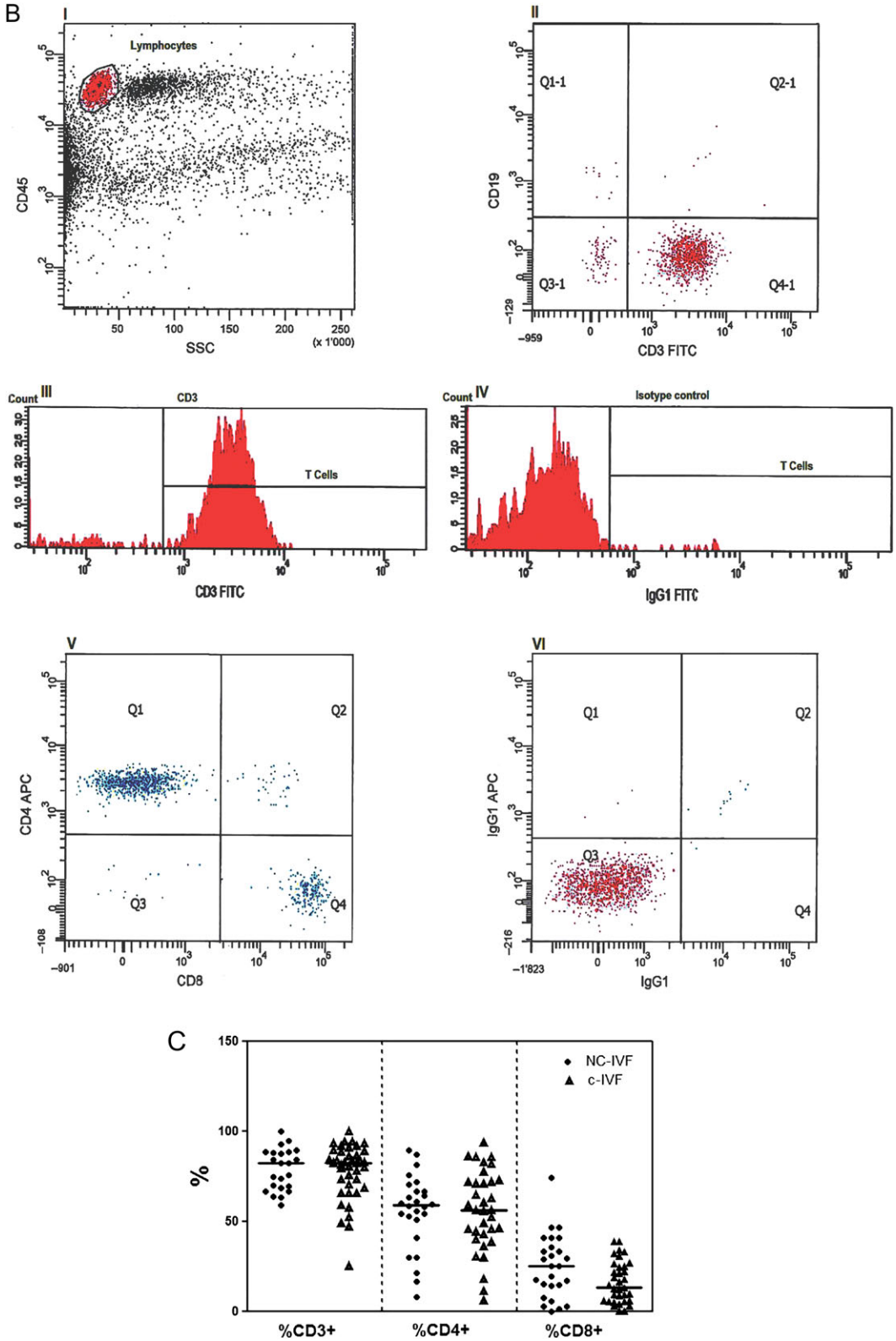

Figure 2 *Continued*

Table III Cytokine concentrations in sera and FF of the study groups.

pg/ml	Serum		*P* value	FF		*P* value
	NC-IVF	c-IVF		NC-IVF	c-IVF	
IL-2	0.5 [0.5–31.6] (2/9)	0.5 [0.5–25.1] (4/16)	1	2.9 [0.5–6.1] (11/12)	1.9 [0.5–6.9] (17/23)	0.597
IL-6	1.0 [0.4–31.5] (4/12)	1.5 [1.0–263.3] (13/21)	0.205	4.8 [1.3–12.8] (15/15)	6.9 [1.6–18.0] (31/31)	0.700
IL-8	2.6 [1.0–35.2] (6/12)	7.7 [1.0–5892] (13/21)	1	224.6 [35.6–332.1] (14/14)	133.3 [34.2–361.0] (31/31)	0.077
IL-10	7.5 [1.0–32.1] (10/12)	14.3 [2.0–80.8] (21/21)	0.21	66.7 [35.7–332.1] (14/14)	62.0 [14.0–172.4] (31/31)	0.793
TNF-α	1.0 [0.5–39.7] (2/9)	1.0 [1.0–45.9] (3/16)	1	2.6 [1.0–6.8] (7/12)	1.0 [0.3–6.8] (10/23)	0.546
IFN-γ	ND (0/9)	200 [200.0–817.2] (2/16)		327.3 [200.0–387.8] (11/12)	284.3 [200.0–363.7] (14 /23)	0.605
VEGF	32.1 [5.0–140.7] (8/12)	97.3 [28.1–554.1] (21/21)	0.007***	1110 [41.0–2687] (15/15)	1034 [202.5–4015] (31/31)	1

Values are given as Median [range].
Numbers of samples with detectable cytokine levels/total number of analyzed samples are shown in parentheses (). Statistically differences of cytokines concentrations in sera as well in the FFs between the two groups were analyzed by non-parametric Mann–Whitney *U*-test. Adjusted *P*-value determined by Holm method for multiple testing.
IFN-γ was in all serum samples obtained from NC-IVF cycles not detectable (ND).
IL, interleukin; TNF-α, tumour necrosis factor; IFN-γ, Interferon gamma.

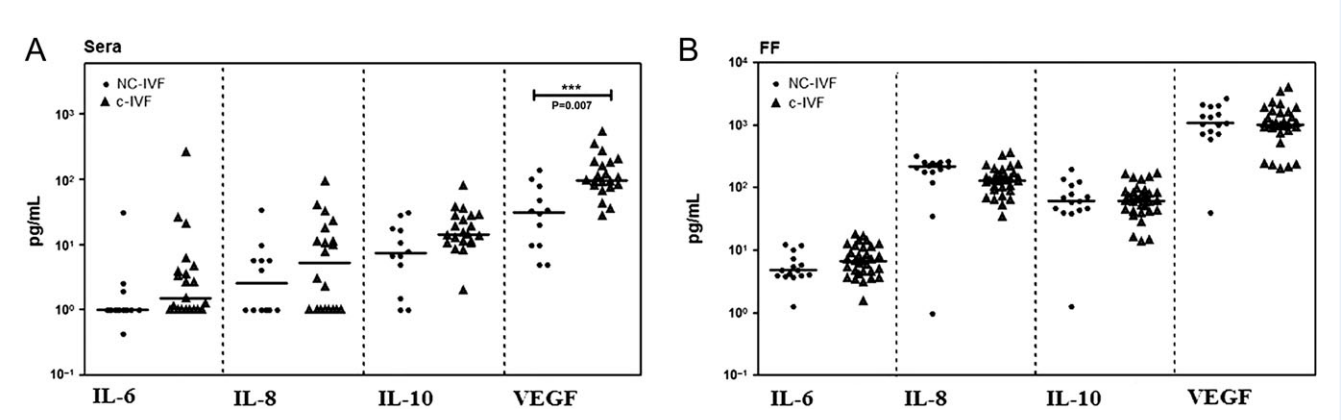

Figure 3 Levels of cytokines in sera and FF. Concentrations of IL-6, IL-8, IL-10 and vascular endothelial growth factor (VEGF) in individual sera (**A**) collected from patients undergoing NC-IVF ($n = 12$) and c-IVF ($n = 21$) at the preovulatory stage and in FF (**B**) from NC-IVF ($n = 15$) and c-IVF ($n = 31$) are shown. The levels of the cytokines were measured using the Bio-Plex® platform. The samples were run in duplicates. The medians are shown as solid horizontal lines. The *P*-values are given in Table III, ***$P < 0.001$.

c-IVF show lower cytokine gradients between FF and serum than NC-IVF

To distinguish the intrafollicular local production for cytokines under exogenous stimulation from systemic release and to compare them with physiological preovulatory events as in natural cycle, we calculated the concentration gradient of IL-6, IL-8, IL-10 and VEGF within FF and serum obtained from both IVF study groups. We observed that the concentrations of those four analyzed cytokines were significantly higher in FFs than in sera of both c-IVF and NC-IVF (Fig. 3). Notably, the cytokine gradients between FF and serum were markedly lower in c-IVF than in NC-IVF. For instance, in c-IVF, the concentration gradient of IL-8 and VEGF was only 10-fold and 8-fold, respectively greater in FF than in serum, whereas in NC-IVF, the levels of IL-8 and VEGF were up to 32-fold and 30-fold increased in FF compared to serum (Fig. 4).

In addition, we investigated whether the percentage of CD8+ or CD4+ T cells correlate with cytokine levels of FF in both study groups or with the hMG total administrated dose and the number of retrieved oocytes in c-IVF. However, no such correlation was found. Nevertheless, correlations between cytokines concentrations in FF obtained from c-IVF groups were stronger than in FF from NC-IVF. A strong correlation ($P < 0.0001$) was measured between IL-10 and IL-2 ($r = 0.75$), IL-10 and IFN-γ ($r = 0.78$), IL-2 and VEGF ($r = 0.83$), IL-10 and VEGF ($r = 0.85$) and IFN-γ and VEGF ($r = 0.73$). In NC-IVF, such a strong correlation was only observed between IL-10 and VEGF ($r = 0.81$).

Discussion

Our study compared the cellular distribution and cytokine profile within FF collected during oocyte retrieval and within serum from c-IVF cycles with those from NC-IVF cycles. The results of the present study show that the percentage of CD45+ leucocytes is significantly higher in FF from c-IVF than in NC-IVF. It also demonstrates that

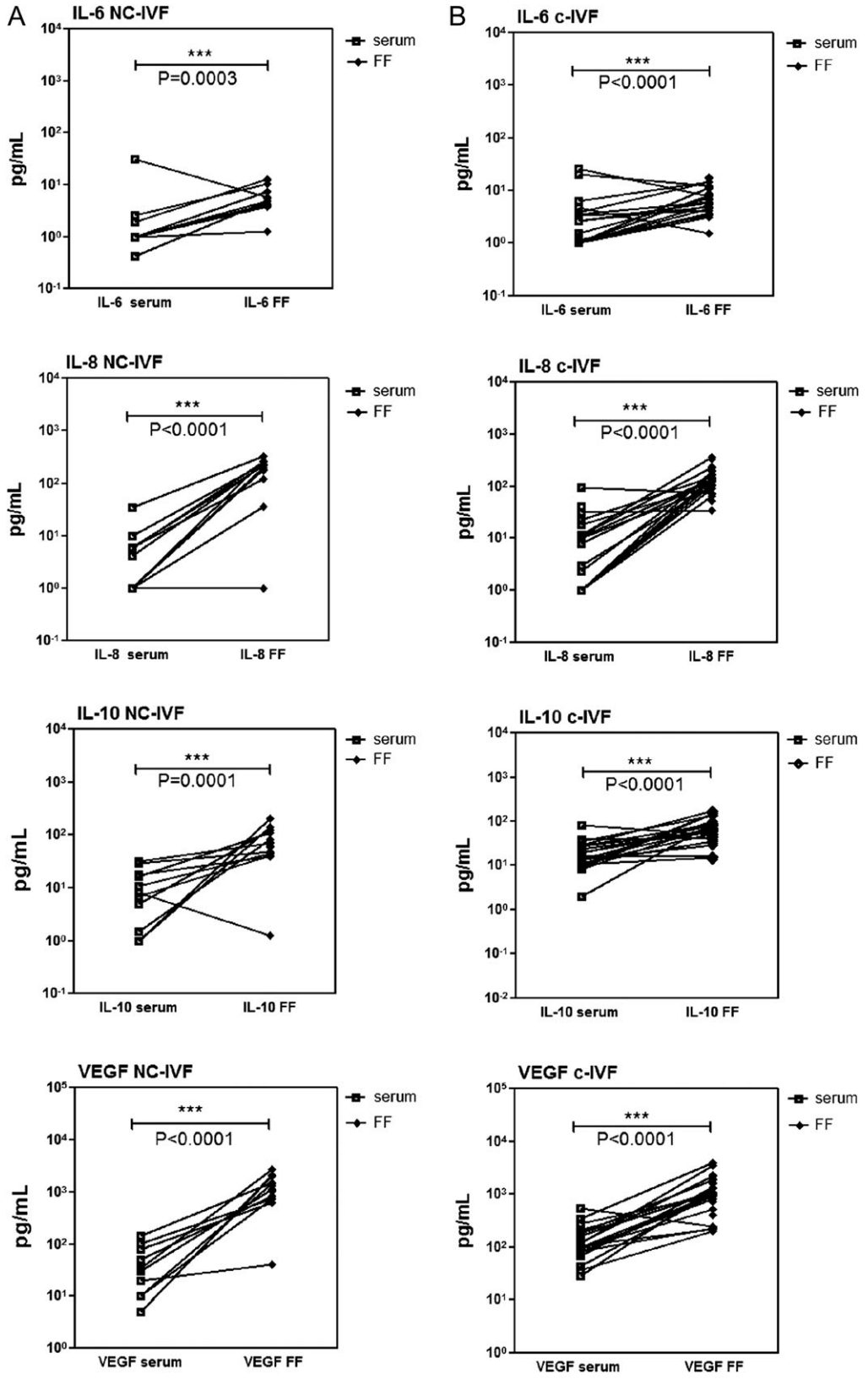

Figure 4 Comparison of concentrations of mediators between FF and sera within the same IVF cycle and individual patients. Data points of IL-6, IL-8, IL-10 and VEGF concentrations in FF (●) and sera (□) obtained from NC-IVF (**A**) and c-IVF (**B**) cycles are shown for individual patients. *P*-values using Mann–Whitney *U*-tests are noted on the graphs, ***$P < 0.001$.

CD3+ T lymphocytes represent the most frequent populations in FF of the preovulatory follicle. Furthermore, we observed that the presence of CD3+ T lymphocytes and the absence of CD19+ B lymphocytes and NK cells in FF did not differ between the stimulated and non-stimulated IVF cycles. These observations are consistent with earlier studies in humans, demonstrating a remarkable rise in macrophages and T lymphocytes in the ovarian stroma and in the theca layer before ovulation, and also in the FF at the time of ovulation (Castilla *et al.*, 1990; Loukides *et al.*, 1990; Brännström *et al.*, 1994). Moreover, studies analyzing the leucocyte and lymphocyte subpopulation in the human ovaries by conventional morphological and immunohistochemical analyses demonstrated that macrophages and T lymphocytes were the most frequent immune cells present in the ovary of reproductive age women, whereas B lymphocytes and NK cells were absent (Brännström *et al.*, 1994; Best *et al.*, 1996). However, the difference in leucocytes subpopulation in FFs of infertile patients in both study groups is difficult to interpret in the context of older studies where the results are less consistent (Lachapelle *et al.*, 1996; Lukassen *et al.*, 2003; Vujisić *et al.*, 2004).

Investigators have suggested that locally produced factors such as chemokines, which are subsequently released in the human FF, have the ability to activate the entrance of leucocytes from the vascular system (Runesson *et al.*, 1996; Wong *et al.*, 2002; Dahm-Kähler *et al.*, 2006). Our study did not include the analysis of chemokines except IL-8 which was lower in the FF from c-IVF compared to NC-IVF and can therefore not be attributed to the increased percentage of CD45+ cells in c-IVF. Hence, further investigations are needed in order to identify the panel of chemokines inducing increased influx of CD45+ leucocytes in c-IVF.

One finding of the present study is the lower percentage of FF CD8+ T lymphocytes in c-IVF compared to NC-IVF. The importance of this finding derives from the crucial contribution of CD8+ T cells to create an immunosuppressive environment for the development of foetal tolerance (Erlebacher, 2013). A study in mice showed an enhanced proliferation rate of CD8+ T cells in the spleen and the uterine draining lymph nodes in pregnant mice during early to mid-gestation (Norton *et al.*, 2010). Studies in humans are limited when stimulated and natural cycles IVF are compared in terms of the implantation rate and the FF CD8+ T cell count. Whether the decreased in FF CD8+ T lymphocytes in c-IVF is mediated directly via the GnRH administration in mid and late follicular phase has not previously been answered. The immunomodulatory effect of hormones such as progesterone, would be another explanation of the decreased CD8+ number in FF from c-IVF. An interplay between the suppressive effect of progesterone on CD8+ T cells in maintaining of pregnancy has been earlier suggested by Szekeres-Bartho *et al.* (1989). We observed in our previously published study a marked higher serum-progesterone concentration in c-IVF samples when compared to NC-IVF (von Wolff *et al.*, 2014a). Thus, a decreased number of CD8+ T cells in FF of the c-IVF group due to elevated serum progesterone levels or indirectly linked to the ovarian stimulation protocol might lead to a lack of mechanisms that prevent embryo rejection and this might explain the higher implantation rate in NC-IVF when compared to c-IVF cycle (Gordon *et al.*, 2013).

In a comparison of FF cytokines concentrations between the two study groups, we found lower IL-8 levels in FF from c-IVF than from NC-IVF. IL-8 plays an important role in follicular growth (Goto *et al.*, 1997). Additionally they regulate progesterone synthesis in luteinized granulosa cells (Shimizu *et al.*, 2012). LH/hCG induce IL-8 production in ovarian stromal cell cultures (Arici *et al.*, 1996). Our work therefore supports the hypothesis that LH suppression with administration of GnRH during the preovulatory stage results in a decrease of FF IL-8 levels. This decrease may lead to an alteration in follicular growth and CL function.

Moreover, independent of the study group, we detected significant higher concentrations of IL-8, IL-10 and VEGF in FF than in serum of the same patients, which is in line with other studies showing higher levels for IL-8 and for VEGF in FF compared to blood (Arici *et al.*, 1996; Artini *et al.*, 1998; Gao *et al.*, 2012). Therefore, the cytokines detected in FF seem to derive rather from intrafollicular local production than from peripheral blood entering the follicles through theca interna. This is in line with earlier studies demonstrating that granulosa cells produce IL-8 (Runesson *et al.*, 1996; Poleć *et al.*, 2011) and VEGF (Neulen *et al.*, 1998; Pellicer *et al.*, 1999; Chen *et al.*, 2010) during the preovulatory stage. Our data suggest that exogenous gonadotrophins induce the intrafollicular release of cytokines and chemokines, which leads to an increase of endothelial permeability and thereby to an increase of IL-6, IL-10, IFN-γ and VEGF levels in sera.

Correlation analyses revealed that the concentration of IL-10 correlated positively with IL-2 and IFN-γ in FF of c-IVF. IL-10 is usually regarded as immunosuppressive cytokine based on his function in downregulating cytokine and chemokine production of Th1 cells and macrophages (Fiorentino *et al.*, 1991) and in suppressing T lymphocytes (De Waal *et al.*, 1991). Furthermore, IL-10-mediated regulation of T cells seems to be important in maintaining tolerance to alloantigen *in vivo* (Hara *et al.*, 2001). This effect is essential in early implantation. However, in other studies IL-10 has been described as a factor for cytotoxic T cell differentiation (Chen and Zlotnik, 1991), that has, in combination with IL-2, the ability to act as pro-inflammatory cytokine through stimulating the expression of Th1 cytokines such as IFN-γ and IL-2 (Santin *et al.*, 2000). Whether our observed positive correlation of IL-10 and IL-2/ IFN-γ relates to pro-inflammatory effect of IL-10 needs further investigations.

The results of the current study support the concept that gonadotrophins affect the intrafollicular milieu as some immune cells and cytokines concentrations were different in c-IVF compared with NC-IVF. According to the literature (Gordon *et al.*, 2013; von Wolff *et al.*, 2014b), NC-IVF generates embryos with a higher implantation potential than does c-IVF. The reason for the increased quality of NC-IVF oocytes can be attributed to differences in the intrafollicular endocrine milieu when compared with c-IVF (von Wolff *et al.*, 2014a), or linked to the immunological changes demonstrated in this study. We are thus assuming that the effect of conventional exogenous stimulation regime is only an indirect and heterogeneous one, linked to the quality of the obtained oocytes and to the reduced implantation rates in c-IVF.

The minor limitations of the study result from the fact that we did not consider a possible minimal peripheral blood contamination at the time of oocyte retrieval as a change factor as FF is a composite of blood exudate. The implantation potential of the embryos in relation to the analyzed FF samples could not be analyzed because the different oocytes in c-IVF are not treated individually in the laboratory. Additionally, the small sample size restricted the statistical power.

Our data support the hypothesis that exogenous gonadotrophins induce the intrafollicular release of cytokines and chemokines such as VEGF, a key regulator of follicular angiogenesis (Trau *et al.*, 2016), which mediates follicular angiogenesis and stimulate the endothelial and vascular permeability. This could result in an influx of cytokines into blood vessels and lead to a decrease in cytokine concentration gradients between FF and serum in c-IVF compared with NC-IVF. Moreover, the data suggest that administration of GnRH in the final stage of follicular growth as in c-IVF group might cause a decrease in FF CD8+ T lymphocytes and in FF-IL-8 levels compared with NC-IVF.

Further studies with more samples should be performed to confirm our findings and to elucidate the relationship between ovarian stimulation protocols, oocyte/embryo quality, clinical pregnancy rate and the intrafollicular factors such as immune cells and cytokines.

Acknowledgements

The authors thank Ms. Anne Vaucher and the Center of Laboratory Medicine of Berne University Hospital, Inselspital and Mr. R. Pavlovic for their coordination and technical support.

Authors' roles

Z.K. participated in the design of the study, the analysis and interpretation of data and wrote the manuscript. S.S. participated in the cell staining and helped to draft the manuscript. M.F. helped to interpret the data and to draft the manuscript. N.A.B. helped in choosing the cytokines to be tested and in the design of the multiplex analysis. M.V.W. was responsible for the financing of the study, organized the shipment of samples, helped in study coordination and revised the manuscript. All authors read and approved the final manuscript.

Funding

The study was supported by a research grant from IBSA Institut Biochimique SA and MSD Merck Sharp & Dohme GmbH.

Conflict of interest

The authors are clinically involved in low dose mono-follicular stimulation and IVF-therapies, using gonadotrophins from all gonadotrophin distributors on the Swiss market, including Institut Biochimique SA and MSD Merck Sharp & Dohme GmbH.

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