

**Cytokine profile in cases with premature elevation of progesterone serum
concentrations during ovarian stimulation**

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Short title: Cytokine profile and premature elevation of progesterone

Summary

The aim of this study was to investigate the concentrations of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), leptin, tumor necrosis factor- α , interleukin (IL)-1 β and IL-6, in cycles with a premature rise of serum progesterone (P). 25 intracytoplasmic sperm injection cycles (ICSI) cycles with (group 1) and 25 ICSI cycles without a premature elevation of P (group 2) were included. The cut-off value of serum P on the day of human chorionic gonadotropin (hCG) was 0.9ng/ml. The indication for ICSI was male factor infertility exclusively. On the day of hCG, serum IL-6, VEGF and bFGF were significantly higher in group 1 (7.7 ± 24.5 pg/ml, 290.2 ± 161.4 pg/ml and 15.7 ± 8.2 ng/ml respectively) than in group 2 (1.7 ± 0.7 pg/ml, 175.2 ± 92.1 pg/ml, and 9 ± 1.6 ng/ml respectively). On the day of follicular puncture, serum cytokine concentrations were similar between the two groups. IL-6 intrafollicular concentrations were higher in group 1 (14.7 ± 20.7 pg/ml) than in group 2 (9 ± 9.3 pg/ml; $p=0.031$). There were no differences regarding the ICSI outcome. Patients with serum P >0.9 ng/ml, have elevated serum concentrations of IL-6, VEGF, and bFGF, as well as elevated intrafollicular concentrations of IL-6. The outcome of ICSI cycles is not associated with premature elevation of P when the cut-off value is set at 0.9ng/ml.

Key words: premature elevation of progesterone, VEGF, bFGF, leptin, TNF α , IL-1 β , IL-6.

Introduction

Premature elevation of progesterone (P) serum concentrations is a frequent event during controlled ovarian stimulation (COS). It has been related with premature luteinization raising concerns on the impact of prematurely elevated P on the outcome of in-vitro fertilization (IVF) cycles.

Premature luteinization is a highly controversial clinical entity. Although, it is generally accepted that, during COS, a subtle rise of serum progesterone (P) on the day of human chorionic gonadotropin (hCG) administration is unenviable, there is no consensus regarding either the exact definition for premature luteinization or the clinical impact of the P rise. Usually, premature luteinization is defined according to serum concentration of P on the day

of hCG administration, although another definition based on the P/estradiol ratio has been also suggested (Givens et al, 1994; Younis et al, 1998, 2001). The cut-off value of serum P used to define premature luteinization is highly controversial, ranging from 0,8-2 ng/ml (Schoolcraft et al, 1991; Silverberg et al, 1991; Fanchin et al, 1993; Hofmann et al, 1993; Legro et al, 1993; Bustillo et al, 1995; Yovel et al, 1995; Hofmann et al, 1996; Shulman et al, 1996; Ubaldi et al, 1997; Chetkowski et al, 1997; Bosch et al, 2003).

Several investigators have reported reduced pregnancy rates in in-vitro fertilization (IVF) cycles with a premature rise of serum P (Schoolcraft et al, 1991; Silverberg et al, 1991; Fanchin et al, 1993; Yovel et al, 1995; Shulman et al, 1996; Younis et al, 1998, 2001; Bosch et al, 2003), whereas others have shown that a premature rise of serum P has no adverse effect on oocyte and embryo quality (Hofmann et al, 1993; Legro et al, 1993; Givens et al, 1994; Bustillo et al, 1995; Hofmann et al, 1996; Ubaldi et al, 1996; Chetkowski et al, 1997).

From a physiological point of view, a premature rise of P may either contribute to an unfavorable follicular milieu having adverse effects on the oocyte maturation and hence embryo quality, or adversely effect the endometrial receptivity.

Previous studies have evaluated the steroid concentrations, in relation to IVF outcome, in blood or follicular fluids from cycles with premature rise of serum P. Taken into consideration, that cytokines have a major part in ovarian functions, the evaluation of cytokines' concentrations in cycles with a premature rise of serum P is of considerable importance.

The present study was focused on six cytokines due to their importance in follicular function, oocyte maturation and ovulation, as indicated by previous studies.

Vascular endothelial growth factor (VEGF) is the key mediator of angiogenesis in the ovary and the association of its expression with the proliferation of ovarian blood vessels is well established (Ferrara 2004). VEGF production is enhanced during the late follicular and luteal phase (Shweiki et al, 1993; Ravindranath et al, 1992; Unkila-Kallio et al, 2000).

Basic fibroblast growth factor (FGFb) is known as a factor exerting anti-apoptotic effects in ovarian cells of different species (Trollice et al, 1997; Hosokawa et al, 1998; Reynolds and Redmer, 1998; Peluso and Papallardo, 1999; Lynch et al, 2000).

Leptin, is thought to participate in the regulation of ovarian steroidogenesis while at the same time sex steroid hormones, especially P, regulate the circulating levels of leptin (Messinis et al, 1999; Caprio et al, 2001). Furthermore, a body of evidence indicates that leptin influences the outcome of in-vitro fertilization attempts possibly by influencing the quality of oocytes (Mantzoros et al, 2000; Brannian et al, 2001; Tsai et al, 2002; Nikolettos et al, 2004; Asimakopoulos et al, 2005).

Several in-vitro and in-vivo studies have shown that proinflammatory cytokines, namely interleukin (IL)-1 β , IL-6 and tumor necrosis factor α (TNF α) are implicated in reproductive functions (Zolti et al, 1990; Wang et al, 1992; Best and Hill, 1995; Büscher et al, 1999; Mendoza et al, 1999; Johnson et al, 1999; Lee et al, 2000). It is also known that estrogens inhibit IL-1 β , IL-6 and TNF α expression, while P and androgens inhibit IL-1 β production by mononuclear cells (Hu et al, 1988; Pacifici et al, 1989). At the same time, IL-1 β and TNF α have been proposed as modulators of luteinized granulosa cells function (Adashi et al, 1990; Fukuoka et al, 1992).

The purpose of this study was to test the hypothesis that premature elevation of P is accompanied with a modified cytokine profile. VEGF, FGFb, leptin and three proinflammatory cytokines: TNF α , IL-1 β and IL-6 were evaluated both in serum and follicular fluids (ff) from women with and without a premature elevation of P. Additionally, P, luteinizing hormone (LH) and testosterone in serum samples, as well as estradiol (E2) in both serum and ff samples were measured.

Materials and methods

Patients' selection

Fifty intracytoplasmic sperm injection (ICSI) cycles treated in the Department of Obstetrics/Gynecology, University Clinic of Schleswig-Holstein, Lübeck, were included in

the study. Twenty-five of them had a premature rise of P (group 1). The cut-off value for P was set at 0,9 ng/ml on the day of hCG administration according to previous reports (Schoolcraft et al, 1991; Silverberg et al, 1991; Fanchin et al, 1993; Givens et al, 1994; Bustillo et al, 1995; Abuzeid and Sasy, 1996; Hofmann et al, 1996; Shuman et al, 1996; Ubaldi et al, 1997). A control group of another twenty-five cycles, with serum P \leq 0,9 ng/ml, was selected as frequency-matched sample (group 2) to meet the distribution of age in group 1. All women had normal reproductive function and basal follicle stimulating hormone (FSH) levels < 10 mIU/ml. The cause of infertility was male factor, in all cases. The age of women was between 28 and 38 years. The patients gave verbal consent and did not receive any monetary compensation for participating in the study. Only one cycle of each woman was included in the study.

Ovarian stimulation – ICSI – Embryo transfers

COS followed the multidose GnRH antagonist protocol (“Lübeck protocol”) (Diedrich and Felberbaum, 1998; Felberbaum et al., 2000). Briefly, the stimulation was made with recombinant FSH (recFSH) (Gonal-F®, Serono International S.A., Geneva, Switzerland). The total dose of recFSH was individualized according to serum estradiol (E2) levels and transvaginal ultrasound measurements of the developing follicles. The pituitary suppression was made with the use of cetrorelix (Cetrotide®, ASTA Medica AG, Frankfurt/Main, Germany and Serono International S.A., Geneva, Switzerland). In all cases, the induction of ovulation was made with 10000 IU hCG (Choragon®, Ferring Arzneimittel GmbH, Kiel, Germany), when there was at least one follicle with a diameter of ≥ 20 mm measured by transvaginal ultrasound or an E2 concentration ≥ 1200 pg/ml. Transvaginal oocyte retrieval assisted by ultrasound monitoring was performed 36 hours later.

Following oocyte retrieval, ICSI was performed with freshly ejaculated spermatozoa. Up to three two pronuclear (2PN) oocytes, in each cycle, were selected for further development and embryo transfer, while the rest were cryopreserved. One to three embryos were transferred in each cycle, in accordance with the German Law. Before the embryo transfers, a cumulative embryo score (CES) was calculated, paying attention to the degree of

fragmentation and regularity of blastomeres, as described elsewhere (Asimakopoulos et al, 2002). The presence of positive foetal heartbeats was indicative of clinical pregnancies.

Samples collection and measurements

Blood serum was collected on the day of hCG administration and on the day of oocyte retrieval. FF samples were collected on the day of oocyte retrieval. From each patient, FF samples were collected among the first one to three mature follicles containing metaphase II oocytes; they were pooled and placed into sterile tubes. All FF samples were immediately centrifuged for 15 min at 1500 rpm and aliquots of the supernatants were stored. FF samples with obvious blood contamination or mixed with flushing fluids were excluded. All materials were stored at -20°C until further analysis.

In serum and FF samples, the levels of the total fractions of the following cytokines were measured: IL-1 β , IL-6, TNF α , bFGF, VEGF and leptin. E2 was measured in serum and follicular fluids. P, luteinizing hormone (LH) and testosterone levels were measured in serum samples.

In serum samples, E2, progesterone, LH and testosterone were measured with Elecsys immunoanalyzer (Roche Diagnostics, Mannheim, Germany) having the following intra- and inter- assay variation (AV): <5% and <10% for E2, <3% and <5% for progesterone, <5% and <7% for testosterone, respectively. All the other measurements were made with commercial enzyme immunoassay kits as follows. E2: DSL-10-4300 Active Estradiol EIA (DSL, Webster, Texas, USA), intra-AV: 3.3-4.8%, inter-AV: 6.5-8.2%, minimum detectable dose (MDD): 7pg/ml. IL-1 β : Quantikine DLB50 (R&D Systems Inc, Minneapolis, USA), intra-AV: 2.8-8.5%, inter-AV: 4.1-8.4%, MDD: 1pg/ml. IL-6: Quantikine D6050 (R&D Systems Inc, Minneapolis, USA), intra-AV: 1.6-4.2%, inter-AV: 3.3-6.4%, MDD: 0.7pg/ml. TNF α : Quantikine DTA00C (R&D Systems Inc, Minneapolis, USA), intra-AV: 4.2-5.2%, inter-AV: 4.6-7.4%, MDD: 0.5pg/ml. VEGF: Quantikine DVE00 (R&D Systems Inc, Minneapolis, USA), intra-AV: 4.5-6.7%, inter-AV: 6.2-8.8%, MDD: 5pg/ml. FGFb: ChemiKine CYT142 (Chemicon Intern. Inc., USA), intra-AV: 8.2%, inter-AV: 10.1%, MDD: 0.488 ng/ml. Leptin:

Quantikine DLP00 (R&D Systems Inc, Minneapolis, USA), intra-AV: 3-3.3%, inter-AV: 3.5-5.4%, MDD: 7.8pg/ml.

As the above kits are validated for serum samples but not for FFs, before running FF samples, we checked the recovery rate of at least three spiked FF samples and the linearity of the results after multiple dilutions. The results were satisfactory, with recovery rates ranging from 80% to 130%. All samples were measured in duplicate, according to manufacturers' instructions. In cases of very high or low results, the measurements were repeated.

Statistical analysis

The normality of all studied parameters was checked with the Shapiro – Wilks' W test and an additional evaluation of kurtosis and skewness. The statistical analysis included descriptive statistics for both studied groups. The comparisons of clinical data as well as of hormonal and cytokine concentrations in FFs between the groups were performed either with the t-test, or with two non-parametric tests: Mann-Whitney U-test and Kolmogorov-Smirnov. The comparisons of hormonal and cytokine concentrations in serum were performed with two-way mixed ANOVA. Post hoc analysis was performed using Bonferroni's corrections. Rates were compared by the χ^2 test. Correlations were evaluated with the Spearman Rank test. The two-tailed significant level was set at $p < 0.05$. The software we used for statistical analysis was STATISTICA 6.0 (StatSoft Inc., Tulsa, OK, USA). All values are presented as mean \pm standard deviation.

Results

All cycles had successful oocyte retrieval. The metaphase II (MII) oocytes aspirated in the cycles of group 1 were significantly more than those aspirated in the cycles of group 2. The fertilization rate in both groups was similar. In group 1, there were 6 pregnancies, whereas in group 2 there were 8, with the difference not reaching the statistically significant level. The main clinical data of the studied cycles are presented in table I.

The hormonal and cytokine profile in the serum samples of the two groups of patients are presented in table II. There were no elevations of LH above 5 mIU/ml. On the day of hCG as

well as on the day of oocyte aspiration, E2, P and testosterone serum concentrations in group 1 were significantly higher than in group 2. From the day of hCG administration to the day of oocyte retrieval there was a statistically significant elevation of P and a decrease of E2 serum concentrations in both groups. Patients in group 1 presented a greater increase of P levels ($p<0.001$) and a greater reduction of E2 levels ($p<0.05$) compared to patients in group 2.

On the day of hCG, the concentrations of both VEGF and IL-6 in group 1 were significantly higher than in group 2. From the day of hCG to the day of oocyte retrieval, the concentrations of VEGF and IL-6 were significantly increased in both groups. FGFb serum concentrations in group 1 were also significantly higher than in group 2 on the day of hCG.

On the day of oocyte retrieval, there were no statistically significant differences in cytokine serum concentrations between the two groups.

The concentrations of E2 and the studied cytokines in FF samples are presented in table III. Significantly higher IL-6 FF concentrations were found in group 1. The mean value of TNF α concentration was also higher in group 1 due to several samples which had elevated TNF α levels. However, the difference in TNF α concentrations between the two groups was not statistically significant. The concentrations of the rest cytokines were similar in the FF samples of both groups.

Statistical analysis showed that P serum concentrations on the day of hCG were positively correlated with the number of retrieved oocytes, E2, testosterone and FGFb serum concentrations (table IV) as well as intrafollicular IL-6 concentrations ($R=0.34$, $p=0.027$). There was also a positive correlation close to statistical significance, between P and VEGF serum concentrations on the day of hCG administration (table IV). Similarly, the numbers of retrieved oocytes were found positively correlated with E2 concentrations on the day of hCG ($R=0.533$, $p=0.0001$).

Discussion

Several pathophysiological mechanisms have been suggested for the premature elevation of P: the LH activity of exogenous administered human menopausal gonadotropins

(Copperman et al, 1995; Fachin et al, 1995), endogenous LH secretion (Check et al, 1991), high FSH doses (Ubaldi et al, 1997; Bosch et al, 2003) or the pooled secretion of P from multiple mature follicles (Silverberg et al, 1991; Givens et al, 1994). Moreover, Younis et al (1998, 2001) have reported that premature luteinization, defined as $P/E2 > 1$, could be an early manifestation of low ovarian reserve.

In the present study, the stimulation was made with recFSH and at the same time the total dose of recFSH did not differ between the cycles with and without a premature elevation of P. Therefore, we have to reject gonadotropins as a potential cause of this event. Similarly, there were no elevations of the endogenous LH and statistical analysis showed there was no correlation between LH and P values on the day of hCG. On the other hand, there were no signs of low ovarian reserve in our study population, as all women had normal function of their reproductive system, normal basal FSH levels and responded well to COS.

The statistical analysis of our results showed that P serum concentrations were positively correlated with the number of follicles and the number of MII oocytes, which were more in group 1 than in control group. Thus, it is reasonable to suggest that in this study, the premature rise of P in group 1 may be at least partially attributed on the high number of mature follicles and the pooled secretion of P from them.

Along with elevated serum concentrations of P, on the day of hCG, the patients of group 1 presented significantly higher serum concentrations of E2, testosterone, IL6, VEGF and bFGF compared to the control group (group 2).

It is reasonable to suggest that higher amounts of E2 and testosterone were produced in group 1 due to the higher number of follicles in this group compared to group 2. The elevated concentrations of steroid hormones in group 1 can clearly be attributed to the higher number of follicles in this group compared to the group 2. Moreover, statistical analysis showed a strong and significant correlation between E2 and the number of retrieved oocytes.

Previous studies have shown that VEGF production is enhanced during the late follicular phase (Shweiki et al, 1993; Ravindranath et al, 1992; Unkila-Kallio et al, 2000). It could be speculated that neovascularization (induced by VEGF) may increase the supply of

steroidogenic cells with substrates, thus supporting the production of steroid hormones, including P production by granulosa cells. In the present study, a correlation between VEGF and P serum concentrations was found close to statistical significant level that is in agreement with the notion that VEGF facilitates the production of P by granulosa cells.

Several studies have indicated an association between P and bFGF in the ovary. Peluso and Papallardo (1999) reported that P, acting through a putative membrane receptor, stimulates the synthesis of bFGF in small granulosa rat cells; bFGF then activates its receptors within large granulosa cells, initiating a signal transduction pathway that maintains large granulosa cells viability. On the other hand, Grasselli et al (2002) showed that P synthesis by granulosa cells from medium size porcine follicles was enhanced when these cells were incubated with various concentrations of bFGF. The association between P and bFGF is further supported by the strong, positive correlation between the serum concentrations of these two factors that was found in the present study.

Interestingly, the intrafollicular concentrations of VEGF and bFGF, although higher in group 1, were not statistically different than those of group 2. However, it has to be mentioned that our FF samples were derived from mature follicles containing MII oocytes. Actually, we do not know the levels of VEGF, bFGF and of the other cytokines in smaller follicles. Besides, FF samples were drawn after luteinization by hCG administration. Luteinization is known to exert profound changes not only on ovarian steroidogenesis but also on the production and secretion of cytokines by ovarian cells.

The concentrations of IL-6 both in serum, on the day of hCG, as well as in FF samples were also higher in patients with premature elevation of P compared to control ones. The statistical analysis showed a weak correlation between P serum concentrations and IL-6 intrafollicular concentrations, whereas there was no significant correlation between P and IL-6 serum concentrations. A previous work has provided indications that IL-6 participates into ovarian steroidogenesis, while it is known that IL-6 is produced by ovarian granulosa cells of preovulatory follicles (Machelon et al, 1994), being a main mediator of inflammatory responses during ovulation (Büscher et al, 1999). For the time being we can not provide a

solid explanation for the elevation of IL-6 in blood and follicular fluids of women having a premature rise of P. Future studies investigating the influence of P on the IL-6 production and secretion from follicular cells may elucidate this elevation.

The luteinization of the follicles by hCG administration led to an increased secretion of most cytokines, in both groups. 36 hours later, at the time of oocyte retrieval, there were no significant differences in cytokine concentrations between the two groups.

The results of the present study do not provide strong indications for an adverse effect of the premature P rise on the oocyte development and further on the outcome of ICSI cycles: the fertilization rate and the score of the transferred embryos were similar between the two groups, while the pregnancy rate, although lower in group 1, was not statistically different.

In summary, the present study showed that in normal women, following COS due to male factor infertility, patients with P concentrations higher than 0.9 ng/ml, on the day of hCG, have also higher serum concentrations of E2, testosterone, IL-6, VEGF and bFGF compared to patients without a premature rise of P. On the day of oocyte retrieval, patients with a premature rise of P still have elevated serum concentrations of E2 and testosterone. The patients with a premature P rise have also elevated intrafollicular concentrations of IL-6. The elevation of the above hormones and cytokines does not seem to be detrimental to the fertilization, embryo quality and pregnancy outcome of ICSI cycles.

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	Group 1	Group 2	Two sided p-level
Age (years)	32.6±4.56	34.29±3.09	0.253
Body Mass Index	23.21±4.47	24.6±2.8	0.074
Total dose of recFSH (I.U.)	1836±533.81	2002±773.05	0.271
No of follicles	14.2±4.6	10.12±3.93	0.002*
No of retrieved MII oocytes	12.16±4.71	7.8±4.82	0.002**
Fertilization rate (%)	51.83±22.6	56.64±26.65	0.893
No of embryos transferred	2.52±0.59	2.2±0.87	0.271
Cumulative embryo score	21.64±8.99	18.16±10.18	0.206

Table I. Clinical parameters of the studied groups. Values are given as mean±standard deviation. *: statistically significant difference between the two groups as determined by Mann-Whitney U test. **: statistically significant difference between the two groups as determined by t-test.

	Group 1		Group 2	
	On the day of hCG	On the day of oocyte retrieval	On the day of hCG	On the day of oocyte retrieval
Estradiol (pg/ml)	2906.52±1556.34 ^{a, c}	1114.35±415.18 ^{b, c}	1525.92±855.88 ^{a, d}	533.92±258.22 ^{b, d}
LH (mIU/ml)	1.6±1.34		1.88±1.97	
Progesterone (ng/ml)	1.67±1.82 ^{e, f}	19.69±7.48 ^{g, f}	0.62±0.23 ^{e, h}	6.26±3.89 ^{g, h}
Testosterone (ng/ml)	0.74±0.44 ⁱ	0.79±0.39 ^j	0.41±0.15 ⁱ	0.54±0.21 ^j
TNFα (pg/ml)	0.62±0.91	3.44±5.97	0.97±1.31	3.32±4.06
IL1β (pg/ml)	0.4±0.6	0.5±1.1	1.8±6.1	0.3±0.5
IL6 (pg/ml)	7.7±24.5 ^{k, l}	17±31.9 ^l	1.7±0.7 ^{k, m}	9.2±13.1 ^m
VEGF (pg/ml)	290.2±161.4 ^{n, o}	382.3±225.7 ^o	175.2±92.1 ^{n, p}	362.4±173.7 ^p
Leptin (pg/ml)	16034.5±17307.5	17432.7±18507.9	18261.2±14961.3	23516.8±15552.7
bFGF (ng/ml)	15.7±8.2 ^q	9±1.6	8.6±3.1 ^q	12±4.4

Table II. Hormonal and cytokine concentrations (mean±standard deviation) in serum samples of the two studied groups on the day of hCG and on the day of oocyte retrieval. Values with the same superscript letters are significantly different as determined by ANOVA ($p<0.05$).

	Group 1	Group 2	Two sided p-level
Estradiol (pg/ml)	797301±373452.7	861331.4±334403.6	0.594
TNF α (pg/ml)	150.3±584.1	2.9±6.7	0.089
IL1 β (pg/ml)	2±3.7	1.9±3.3	0.46
IL6 (pg/ml)	14.7±20.7	9±9.3	0.031*
VEGF (pg/ml)	3404.2±1214	3124.5±1672.6	0.377
Leptin (pg/ml)	21649±20205.4	23675.8±18283.7	0.534
bFGF (ng/ml)	192±113.5	163.6±90.5	0.36

Table III. Hormonal and cytokine concentrations (mean±standard deviation) in the follicular fluid samples of the studied groups. *: statistically significant difference as determined by Mann-Whitney U test.

	Spearman R	p-level
P & Age	-0.142	0.33
P & total dose of recFSH	-0.12	0.406
P & no of follicles	0.412	0.003*
P & no of MII oocytes	0.372	0.008*
P & fertilization rate	-0.016	0.912
P & cumulative embryo score	0.132	0.36
P & LH on the day of hCG	-0.028	0.848
P & Estradiol	0.559	0.00003*
P & testosterone	0.581	0.0002*
P & TNF α	-0.213	0.213
P & IL1 β	0.154	0.371
P & IL6	0.244	0.158
P & VEGF	0.325	0.057
P & Leptin	-0.098	0.57
P & bFGF	0.538	0.031*

Table IV. Spearman R correlations between Progesterone (P) serum concentrations on the day of hCG, clinical parameters, hormonal and cytokine serum concentrations on the day of hCG.*: statistically significant