Cytokine and hormonal profile in blood serum and follicular fluids during ovarian stimulation with the multidose antagonist or the long agonist protocol

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BACKGROUND: The aim of our study was to explore cytokine and hormonal profiles in blood and follicular fluids from normal women stimulated with either the multidose antagonist or the long agonist protocol. METHODS: Fifty-six patients were stimulated with the multidose antagonist protocol and 12 with the long agonist protocol. Interleukin (IL)-1β, IL-6, tumour necrosis factor-α (TNFα), leptin, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), estradiol (E2), progesterone and testosterone levels were measured in serum and follicular fluids by immunoassays. RESULTS: The two treatment groups had similar cytokine concentrations in serum. The intrafollicular concentrations of IL-1β, IL-6, VEGF and leptin were also similar in the two groups. The concentrations of bFGF in follicular fluids from the antagonist group (169.5 ± 113.2 ng/ml) were lower than those from the agonist group (249.7 ± 119.8 ng/ml). bFGF concentrations were correlated with the amount of administered gonadotrophins (R = 0.364, P < 0.01) which was significantly lower in the antagonist group (antagonist group: 2037.7 ± 725.8 IU; agonist group: 2836.4 ± 1163.5 IU). CONCLUSIONS: Normal women stimulated with either the multidose antagonist or the long agonist protocol generally have similar cytokine profiles in serum and follicular fluids. The intrafollicular levels of bFGF tend to be lower in antagonist cycles because of the lower amount of administered gonadotrophins.

Key words: cytokines/estradiol/FGF/ovarian stimulation/progesterone

Introduction

Cytokines, having multifarious functions, are present in every physiological process within the human body. It is unanimously recognized that many cytokines are of crucial importance in reproductive processes such as follicular development, ovulation, fertilization, implantation and embryo development. The role of cytokines in the female reproductive system has been broadly investigated during controlled ovarian stimulation (COS) for IVF attempts. It is clear that under COS, the functions of the female reproductive system are distinctively different than in normal cycles, and it is also generally admitted that the type of COS differentially influences the reproductive functions.

The history of assisted reproduction techniques (ART) during the last 20 years is characterized by the development of new types of COS to maximize the success and minimize the complications. So far, the most common type of COS is the so-called long agonist protocol, while COS with GnRH antagonists is gaining ground during the last 5 years. Both types of COS have been extensively studied and compared in terms of clinical outcome (Albano et al., 2000; Borm and Mannnaerts, 2000; Ludwig et al., 2000; Olivennes et al., 2000; European-Middle East Orgalutran Study Group, 2001; Fluker et al., 2001; Al-Inany and Aboulihel, 2002; Roulier et al., 2003). However, up to now, there have been no studies investigating their possible differences regarding the cytokine levels.

The aim of the present study was to investigate and compare, for the first time, the levels of six cytokines in serum and follicular fluids of women undergoing COS with either the long agonist protocol or the multiple-dose GnRH antagonist protocol. The investigated cytokines included three proinflammatory cytokines—interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNFα)—two growth factors—vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)—and leptin. Additionally, we investigated and compared the levels of steroid hormones—estradiol (E2) and progesterone.
Materials and methods

This prospective study was carried out at the Department of Obstetrics/Gynecology, University Clinic of Schleswig-Holstein, Campus Lübeck (Lübeck, Germany). Sixty-eight patients attending the IVF unit of the department for ICSI cycles were recruited in the period between November 2002 and May 2003, according to the following criteria: (i) absence of any apparent abnormality of their reproductive system, as revealed by their medical history, the clinical examinations and common hormonal tests; the indication for ICSI/embryo transfer was male factor infertility exclusively; (ii) absence of any metabolic and immunological disease; (iii) age of women between 28 and 38 years and (iv) adequate response to COS; low responders as well as patients with basal FSH > 10 mIU/ml were excluded. Patients gave their written informed consent and did not receive any monetary compensation for participating in the study. From each patient, only one cycle was included in the study.

Stimulation protocols, ICSI and embryo transfers

The stimulation was made with recombinant FSH (Gonal-F®; Serono International S.A., Geneva, Switzerland) and hMGs (Menogon®; Ferring Arzneimittel GmbH, Kiel, Germany). The total doses of administered gonadotrophins were individualized according to serum 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.) or triptorelin (Decapeptyl Depot®; Ferring Arzneimittel GmbH). The COS with cetrorelix followed the multidose protocol (Lübeck protocol) and the COS with triptorelin followed the long protocol (Diedrich and Felberbaum, 1998; Ludwig et al., 1999; Felberbaum et al., 2000). Thus, the studied population was divided into two groups: the antagonist group (n = 54) and the agonist group (n = 12). In all cases, the induction of ovulation was made with 10 000 IU hCG (Choragon®; Ferring Arzneimittel GmbH), when the leading follicle reached a diameter of 18–20 mm measured by transvaginal ultrasound and when E₂ levels indicated a satisfactory follicular response. Transvaginal oocyte retrieval assisted by ultrasound monitoring was performed 36 h later.

After ICSI, 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, as described elsewhere (Asimakopoulos et al., 2002), paying attention to the degree of fragmentation and ratio of blastomeres.

The presence of positive fetal heartbeats was indicative of clinical pregnancies.

Sample collection

Blood serum was collected on the day of hCG administration and on the day of oocyte retrieval. Follicular fluid samples were collected on the day of oocyte retrieval. From each patient, follicular fluid samples were collected among the first one to three mature follicles containing metaphase II oocytes; they were pooled and placed into sterile tubes. All follicular fluid samples were immediately centrifuged for 15 min at 480 g and aliquots of the supernatants were stored. Follicular fluid samples with obvious blood contamination or mixed with flushing fluids were excluded. All materials were stored at −20°C until further analysis.

Measurements

In serum and follicular fluid samples, the levels of the following hormones and cytokines were measured: E₂, progesterone, IL-1β, IL-6, TNFα, bFGF, VEGF and leptin. Additionally, testosterone levels were measured in serum samples.

In serum samples, E₂, progesterone and testosterone levels were measured with Elescsy immunoanalyzer (Roche Diagnostics, Mannheim, Germany) having the following intra- and inter-assay variation (AV): <5 and <10% for E₂, <3 and <5% for progesterone and <5 and <7% for testosterone, respectively. All the other measurements were made with commercial enzyme immunoassay kits as follows:

(i) E₂: DSL-10-4300 Active Estradiol EIA (DSL, Webster, TX, USA), intra-AV: 3.3–4.8%, inter-AV: 6.5–8.2% and minimum detectable dose (MDD): 7 pg/ml; (ii) progesterone: DE2200 (R&D Systems, Minneapolis, MN, USA), intra-AV: 4.9–7.65%, inter-AV: 2.7–8.3% and MDD: 8.57 pg/ml; (iii) IL-1β: Quantikine DLB50 (R&D Systems), intra-AV: 2.8–8.5%, inter-AV: 4.1–8.4% and MDD: 1 pg/ml; (iv) IL-6: Quantikine D6050 (R&D Systems), intra-AV: 1.6–4.2%, inter-AV: 3.3–6.4% and MDD: 0.7 pg/ml; (v) TNFα: Quantikine GTA00C (R&D Systems), intra-AV: 4.2–5.2%, inter-AV: 4.6–7.4% and MDD: 0.5 pg/ml; (vi) VEGF: Quantikine DVE080 (R&D Systems), intra-AV: 4.5–6.7%, inter-AV: 6.2–8.8% and MDD: 5 pg/ml; (vii) bFGF: ChemiKine CYT142 Chemicon International, Temecula, CA, USA, intra-AV: 8.2%, inter-AV: 10.1% and MDD: 0.488 ng/ml; and (viii) leptin: Quantikine DLPP00 (R&D Systems), intra-AV: 3–3.3%, inter-AV: 3.5–5.4% and MDD: 7.8 pg/ml.

As the above kits are validated for serum samples but not for follicular fluids, before running follicular fluid samples, we checked the recovery rate of at least three spiked follicular fluid 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. The cytokine and hormonal concentrations are expressed as nanograms or picograms per millilitre of follicular fluid.

Statistical analysis

The normality of all studied parameters was checked with Shapiro-Wilk’s W-test. The statistical evaluation included descriptive statistics for both studied groups. Comparisons between the groups were performed with either the t-test or two non-parametric tests—Mann-Whitney U-test and Kolmogorov-Smirnov. Comparisons between the two times of sampling were made with the application of t-test for dependent samples or the Wilcoxon test for matched pairs. Rates were compared by χ² test. Correlations were evaluated with Spearman’s 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, Tulsa, OK, USA).

Results

Demographic and clinical data of the studied groups are presented in Table I.

The group of agonist received a significantly higher amount of gonadotrophins to achieve satisfactory follicular development [95% confidence interval (CI): 2054.7–3618 versus 1841.5–2233.9 IU]. There were 14 pregnancies in the antagonist group and four pregnancies in the agonist group, with the difference not at a statistically significant level (Yates corrected χ² = 0.1, P = 0.918; Fisher’s exact P = 0.735). There were only five cases of mild ovarian hyperstimulation syndrome (OHSS), three of them in the agonist group (χ² = 5.12, P = 0.024; Fisher’s exact P = 0.055).

Hormonal and cytokine concentrations in blood serum

The concentrations of the three hormones (E₂, progesterone and testosterone), as well as of the six cytokines in blood serum samples, in both studied groups, are presented in Table II.
The present study is the first one comparing the cytokine profiles in serum and follicular fluids in women stimulated with the long agonist or the multidose antagonist protocol. The two studied groups were comparable in terms of several parameters such as age, BMI and the number and quality of transferred embryos. ICSI was applied in all cases, by the same experienced personnel. The inclusion of women with normal reproductive functions and adequate response to ovarian stimulation was imperative, since various infertility disorders seem to be related to elevated cytokine levels, and thus, possible bias in the results was avoided. In the Department of Obstetrics/Gynecology of Lübeck, the antagonist protocols are almost solely used owing to the large experience that has been acquired on the use of GnRH antagonists. In this study, the recruitment period coincided with the transitional period of switching from the long protocol to the total use of antagonist protocols. Consequently, it was difficult to recruit many patients in the agonist group, whereas at the same time, they would fulfill the inclusion criteria. The number of patients in the agonist group constitutes the main limitation of the study. It can be argued that with a larger number of patients in the agonist group, some differences, such as those in the intrafollicular progesterone levels, could have reached the level of statistical significance.

We did not adjust the protein levels in each sample before measuring the levels of the cytokines, as follicular fluid samples contaminated with blood or flushing medium were excluded. Besides, this is the usual method followed by most of the relevant published studies. By expressing intrafollicular levels of cytokines as picograms or nanograms per milligram of total protein content, the comparison of our data with the data published in other studies would have become extremely difficult.

According to the results, there were no significant differences between the two stimulation protocols regarding the concentrations of the studied cytokines in serum, either on the day of hCG administration or on the day of oocyte aspiration. Similarly, the intrafollicular concentrations of TNFα, IL-1β, IL-6, VEGF and leptin did not present significant differences between the two stimulation protocols.

In general, the concentrations of the six cytokines studied here were higher in follicular fluids than in serum samples. This fact underlines the importance of the local regulation of the six cytokines in the follicular compartment. Since cytokines have mostly a paracrine or an autocrine mode of action, we believe that intrafollicular concentrations represent their role in ovarian functions better than blood concentrations. The concentrations of cytokines in the blood may be influenced by extraovarian production sites, and the extraovarian production of cytokines does not always contribute significantly to the concentrations of cytokines in the follicular compartment.

**Discussion**

**Hormonal and cytokine concentrations in follicular fluid**

The concentrations of the studied hormones and cytokines in follicular fluid samples are presented in Table III.

The differences between the two studied groups in the intrafollicular concentrations of proinflammatory cytokines, VEGF and leptin were not statistically significant.

The concentrations of bFGF in follicular fluids from the agonist group were significantly higher (95% CI: 169.2–330 ng/ml) than those from the antagonist group (95% CI: 138–201 ng/ml). At the same time, there was a difference close to the statistically significant level regarding progesterone intrafollicular concentrations which were higher in the agonist (95% CI: 20 848.1–30 081 ng/ml) than in the antagonist group (95% CI: 16 734.9–21 925.7 ng/ml).

Intrafollicular progesterone concentrations were correlated with the total amount of administered gonadotrophins (R = 0.433, P < 0.05). bFGF concentrations were positively correlated with the patients’ age (R = 0.502, P < 0.01) and the amount of gonadotrophins (R = 0.364, P < 0.01).

**Table I. Clinical data of the two studied groups**

<table>
<thead>
<tr>
<th></th>
<th>Antagonist group (n = 56)</th>
<th>Agonist group (n = 12)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.1 ± 2.7 (33)</td>
<td>34.3 ± 3.9 (33)</td>
<td>0.523</td>
</tr>
<tr>
<td>BMI</td>
<td>22.7 ± 3.9 (23.6)</td>
<td>23.7 ± 3.5 (23.1)</td>
<td>0.867</td>
</tr>
<tr>
<td>Total amount of gonadotrophins (IU)</td>
<td>2037.7 ± 725.8 (1800)</td>
<td>2836.4 ± 1163.5 (2475)</td>
<td>0.01</td>
</tr>
<tr>
<td>Number of follicles</td>
<td>12.1 ± 5 (12)</td>
<td>14 ± 6.6 (12)</td>
<td>0.469</td>
</tr>
<tr>
<td>Number of retrieved oocytes</td>
<td>9.7 ± 5.2 (8)</td>
<td>11.3 ± 6.4 (11)</td>
<td>0.498</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>56.3 ± 24.5 (51.6)</td>
<td>64.5 ± 20.4 (60)</td>
<td>0.253</td>
</tr>
<tr>
<td>Number of transferred embryos</td>
<td>2.4 ± 0.7 (2)</td>
<td>2.7 ± 0.5 (3)</td>
<td>0.253</td>
</tr>
<tr>
<td>Cumulative embryo score</td>
<td>22 ± 10.5 (24)</td>
<td>20.8 ± 9.9 (22)</td>
<td>0.917</td>
</tr>
<tr>
<td>Number of pregnancies</td>
<td>14</td>
<td>4</td>
<td>0.918</td>
</tr>
</tbody>
</table>

The values are given as mean ± SD (median). Significant difference (in bold) based on t-test.
Thus, we believe that intrafollicular concentrations of cytokines have a special interest regarding the study of the role of cytokines in ovarian functions.

The intrafollicular levels of bFGF were significantly lower in the antagonist group than in the agonist one. bFGF is known as a growth factor involved in the folliculogenetic process. It has been reported as an initiator of folliculogenesis by inducing primordial follicle development (Nilsson et al., 2001) and as a regulator of ovarian angiogenesis, though not as drastic as VEGF. During the follicular development, FSH induces the expression of functional receptors for bFGF, and this growth factor possibly plays a role in the differentiation and the viability of granulosa cells (Shikone et al., 1992). At the time of ovulation, it seems that bFGF exerts antiapoptotic actions on granulosa cells, thus supporting the formation of corpus luteum (Peluso and Pappalardo, 1999; Lynch et al., 2000). It is also known that bFGF is one of the major luteal angiogenic factors (Reynolds and Redmer, 1998). Thus, it could be presumed that higher bFGF intrafollicular concentrations, at the time of ovulation, may contribute to the formation of a more functional corpus luteum.

The intrafollicular concentrations of progesterone were also found to be lower in GnRH antagonist cycles than in GnRH agonist ones, with the difference being close to statistical significance.

At the same time, the intrafollicular concentrations of both progesterone and bFGF were significantly correlated with the amount of gonadotrophins. Taking into consideration that the patients stimulated with the GnRH antagonist protocol received significantly less gonadotrophins than the patients stimulated with the long protocol, it seems reasonable to suggest that the differences in bFGF and progesterone concentrations between the two groups are at least partly explained by the difference in the amount of administrated gonadotrophins.

The concentrations of the three proinflammatory cytokines (TNFα, IL-1β and IL-6) were low or below the detection limit in both groups. This can be explained by the absence of any apparent pathology in the women included in this study. High levels of proinflammatory cytokines have been reported in cases of infertility due to immunological disorders, endometriosis and polycystic ovarian syndrome (Cianci et al., 1996; Calogero et al., 1998; Pellicer et al., 1998; Bedaiwy et al., 2002; Amato et al., 2003). Samples with undetectable concentrations of proinflammatory cytokines have also been reported by other investigators (Barak et al., 1992; Punnonen et al., 1992; Büscher et al., 1999; Aboul Enien et al., 2001; Asimakopoulos et al., 2005), whereas it has been reported that TNFα in follicular fluids not only has low concentrations but also shows minimal bioactivity (Wang et al., 1992).

In conclusion, normal women following COS with a GnRH antagonist or a GnRH agonist have a generally similar cytokine profile in serum and follicular fluid samples. The intrafollicular levels of bFGF and progesterone tend to be lower in normal women following COS with a GnRH antagonist than in those following the long protocol, possibly due to the lower amount of gonadotrophins administrated in GnRH antagonist cycles.
Hormonal and cytokine concentrations in the follicular fluid samples of the studied groups

![Table III. Hormonal and cytokine concentrations in the follicular fluid samples of the studied groups](image)


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Table III. Hormonal and cytokine concentrations in the follicular fluid samples of the studied groups

<table>
<thead>
<tr>
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<th>Antagonist group (n = 56)</th>
<th>agonist group (n = 12)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 (pg/ml)</td>
<td>781 616.2 ± 422 087 (809 250)</td>
<td>849 363 ± 431 556.2 (720 044.9)</td>
<td>0.829</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>19 330.3 ± 614.64 (18 583.3)</td>
<td>25 464.6 ± 2901.2 (26 712.5)</td>
<td>0.059</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>17.7 ± 38.3 (1.7)</td>
<td>1.2 ± 2.1 (0)</td>
<td>0.163</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>2.4 ± 4.4 (0.6)</td>
<td>3 ± 4.9 (0.8)</td>
<td>0.878</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>13.2 ± 32.2 (7.1)</td>
<td>10.8 ± 10.1 (7.4)</td>
<td>0.598</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>2991.8 ± 1471.2 (2599.9)</td>
<td>3013.8 ± 1266.6 (2539.9)</td>
<td>0.819</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>22 735.6 ± 17 919.5 (17 523.4)</td>
<td>24 263.2 ± 22 599.9 (14 660.3)</td>
<td>0.819</td>
</tr>
<tr>
<td>bFGF (pg/ml)</td>
<td>169.5 ± 113.2 (158.7)</td>
<td>249.7 ± 119.8 (227.4)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

bFGF, basic fibroblast growth factor; E2, estradiol; IL-1β, interleukin-1β; TNFα, tumour necrosis factor-α; VEGF, vascular endothelial growth factor. The values are given as mean ± SD (median). Significant difference (in bold) based on Mann-Whitney U-test.

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References


**Cytokine and hormonal profiles according to stimulation**