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Follicular Fluid Levels of Vascular Endothelial Growth Factor and Leptin Are Associated with Pregnancy Outcome of Normal Women Participating in Intracytoplasmic Sperm Injection Cycles

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Summary

Cytokines play a critical and multifarious role in follicular maturation. Consequently, they may influence the pregnancy outcome in cycles of assisted reproduction. The aim of this study was to measure the levels of tumor necrosis factor-α (TNFα), vascular endothelial growth factor (VEGF) and leptin in serum and follicular fluids (FFs) from women undergoing controlled ovarian hyperstimulation (COH) for intracytoplasmic sperm injection cycles (ICSI). We tried to investigate their interrelationships and to evaluate them as predictive markers for the cycle's outcome. Seventeen women participated in this study. Male factor infertility was the only indication for ICSI cycles. For COH, the long agonist protocol with triptorelin and recombinant FSH was employed. Cytokines levels were evaluated by ELISA. Serum cytokine levels did not differ between pregnant and non-pregnant women. FF-VEGF levels were significantly elevated in non-pregnant women (722.2±1093.2 pg/ml) as compared to pregnant women (290.3±259.8 pg/ml). Leptin concentrations were also significantly higher in FFs of non-pregnant women (682.6±625.1 ng/ml) than those of pregnant women (231.6±286.5 ng/ml). There were significant positive correlations between FF-leptin and age, as well as between FF-leptin and FF-VEGF concentrations. It was concluded that elevated FF-leptin and VEGF levels are associated with failure of conception in IVF cycles and may serve as markers in clinical practice.

Key words

Follicular fluid IVF • Leptin • Tumor necrosis factor-α • Vascular endothelial growth factor

Introduction

Follicular development is a complex process regulated by numerous local factors participating and interacting under the influence of gonadotropins and ovarian steroids. Among these factors, various cytokines

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are involved in many stages of this process and consequently their combined actions determine the final result to a large extent. During the last decade, cytokines have indeed become the target of intensive research as their actions represent a key point in the elucidation of the physiology of the female reproductive system under normal conditions or in response to external ovarian stimulation. Controlled ovarian hyperstimulation is broadly used in *in vitro* fertilization (IVF) cycles in order to increase the number of mature oocytes, to avoid the premature ovulation and to eventually maximize the chance of conception. The hitherto unresolved efforts to improve the pregnancy rates of IVF cycles further stimulated the investigation of cytokines. Thus, various cytokines, in particular the vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNFα) and leptin have been investigated for their influence on oocyte maturation, fertilization and embryo implantation, but the reported results are usually contradictory (Adashi *et al.* 1990, Roby and Terranova, 1990, Barak *et al.* 1992, Wang *et al.* 1992, Putowski and Kotarski, 1995, Cianci *et al.* 1996, Friedman *et al.* 1997, 1998, Loret de Mola *et al.* 1998, Moncayo *et al.* 1998, Barroso *et al.* 1999, Hazzard *et al.* 1999, Johnson *et al.* 1999, Kitawaki *et al.* 1999, Lee *et al.* 2000, Mantzoros *et al.* 2000, Aboul Enien *et al.* 2001, Benifla *et al.* 2001, Brannian *et al.* 2001, Caprio *et al.* 2001, Malamitsi-Puchner *et al.* 2001, Mendoza *et al.* 1999, 2002, Ryan *et al.* 2002, Tsai *et al.* 2002).

In the present study, the levels of VEGF, TNFα, and leptin in serum and follicular fluids (FF) of normal women participating in IVF cycles were studied and evaluated as possible predictive markers for the cycles' outcome. The women participating in the study were stimulated according to the long agonist protocol that is the most common in the preparation of IVF cycles.

Methods

Subjects

The studied group included 17 women without any apparent abnormality of their reproductive system as defined by their medical history, the clinical examinations and common hormonal tests. They participated in ICSI cycles exclusively due to male factor infertility. Their age was 32.3±5.0 years. Low responders as well as women with basal FSH >10 IU were excluded.

The long agonist protocol for controlled ovarian hyperstimulation was used as previously described (Nikolettos *et al.* 2000). Briefly, ovarian hyperstimulation performed by administration of recombinant FSH (Gonal-F, Serono International S.A., Geneva, Switzerland) after pituitary suppression with triptorelin (Arvekap, Ipsen Pharma Biotech, France) started in the midluteal phase of the preceding cycle. The dosages of gonadotropins were individualized according to serum estradiol (E2) levels and transvaginal ultrasound measurements of the follicles. When at least three follicles had reached a diameter of 17 mm and serum E2 levels were increased to approximately 300-500pg/ml per follicle larger than 17mm, ovulation was induced with 10000 IU of hCG (Pregnyl, N.V. Organon, Oss, Holland). Transvaginal oocyte aspiration with ultrasound guidance was performed under general anesthesia 36 hours later. Before oocyte aspiration, a peripheral blood sample was taken in each patient, serum was extracted and stored at –75°C. During oocyte aspiration, FF samples were collected from distinct, mature follicles and they were placed into sterile tubes. Samples with massive blood contamination or flushing fluid were excluded. FF samples were immediately centrifuged for 15 min at 1500 rpm and the supernatants were stored at –75 °C for further analysis.

Immediately after follicular aspiration, the cumulus oophorus and corona radiata were removed mechanically under dissecting microscope, with simultaneous incubation in 0.5 % hyaluronidase solution (Sigma Co, Deisenhofen, Germany) for 30 seconds. The incubation in hyaluronidase was followed by repeated aspirations into finely drawn Pasteur pipettes. Only metaphase II (MII) oocytes were used for ICSI that was performed 3-4 hours later using an inverted microscope (Nikon 108, Japan) with micromanipulators (Narishige, Japan). The injected oocytes were cultured in Ham F10 medium supplemented with serum umbilical cord, at 37 °C, in a humidified atmosphere with 5 % CO_2. After 18 h of incubation, the injected oocytes were examined for the presence of two or more pronuclei as a sign of fertilization. The normally fertilized oocytes, with two pronuclei and two polar bodies (2PN oocytes), were transferred into a fresh medium and further cultured for 24-30 hours.

Embryo transfers were performed with an appropriate catheter (Sherwood Medical, Tullamore, Ireland). The luteal phase was supported daily with 600 mg natural progesterone administrated vaginally (Utrogestan, Besins-Iscovesco, Paris, France). Pregnancies were defined by the presence of positive fetal heart beats.

Measurements of cytokine concentrations

In every serum and FF sample, commercial

enzyme immunoassay kits (CYTELISA and ACCUCYTE, Cytimmune Sciences Inc., Maryland, USA) were used to measure the concentrations of free VEGF, free TNFα and total leptin. The range of detection was 15.6-1000 pg/ml for TNFα, 20-2500 pg/ml for VEGF and 0.488-500 ng/ml for leptin. The intra- and interassay variations were 8.3 and 10.8 % for TNFα, 8.9 and 11.1 % for VEGF, 7 and 10.2 % for leptin, respectively. An ELISA reader with appropriate filters (A-5022, Anthos Labtec Instruments, Salzburg, Austria) and a microplate washer (MultiWash PLUS 8070-15, TRI Continent, USA) were used. All measurements were carried out in duplicate and according to the manufacturers' instructions.

Statistical analysis

In addition to the measured cytokine levels, the following parameters were also evaluated: age of patients, body mass index (BMI), total amount of administered recombinant FSH, E2 at the day of hCG administration, number of retrieved oocytes, number of MII oocytes and number of 2PN oocytes. Descriptive statistics were drawn for all studied parameters. The normality of all studied parameters was evaluated by two tests: the Kolmogorov-Smirnov test for normality and Shapiro-Wilks W-test. Differences between groups of pregnant and non-pregnant women were evaluated by the t-test or the Mann-Whitney U test. Correlations among the studied parameters were evaluated with Spearman's rank correlation coefficients. A probability level of 0.05 or less was considered as statistically significant.

Results

In a total of seventeen cycles, seven of them resulted in pregnancies. The main characteristics of the successful (group of pregnancy) and unsuccessful cycles (group of no pregnancy) are presented in Table 1. The age and BMI were similar in both groups. Similar amounts of gonadotropins were administered in successful and unsuccessful cycles. The successful cycles had almost the same E2 levels on the day of hCG administration as the unsuccessful ones. In the group of pregnancy significantly less oocytes were retrieved than those in the group of no pregnancy. However, there were no significant differences between the two groups regarding the number of MII oocytes or 2PN oocytes.

Table 1. Differences between the successful (group of pregnancy) and unsuccessful (group of no pregnancy) ICSI cycles regarding various clinical parameters, cytokine levels in serum (on the day of oocyte aspiration) and cytokine levels in follicular fluids (FF).

	Pregnancy	**No pregnancy**	**P=**
Age (years)	33.09±4.56	31.62±5.35	0.34
BMI (kg/m^2)	23.55±2.16	24.43±1.7	0.35
Gonadotropins (IU)	3248.08±726.1	3237.5±836.06	0.5
E2 (pg/ml)	1822.54±865.99	1709±704.21	0.55
No of oocytes	9.86±5.87	15.5±5.99	0.04
No of MII oocytes	7.14±2.97	11.1±4.72	0.06
No of 2PN oocytes	5.43±2.51	7.6±3.78	0.22
Serum TNFα (pg/ml)	134,2±97.12	117.12±113.39	0.31
Serum VEGF (pg/ml)	69.93±36.1	71.22±50.3	0.12
Serum leptin (ng/ml)	29.47±9.12	33.28±6.54	0.2
FF TNFα (pg/ml)	275.53±230.61	300.51±401.12	0.54
FF VEGF (pg/ml)	290.32±259.77	722.24±1093.22	0.04
FF leptin (ng/ml)	231.57±286.53	682.55±625.05	0.01

Values are given as mean ± S.D. E2 – estradiol, FF – follicular fluid

Serum levels of the studied cytokines were not significantly different between the pregnant and non-pregnant cycles. Follicular fluid TNFα levels, although lower in the group of pregnancy, were not significantly different between the two groups. By contrast, FF VEGF levels were significantly lower in FFs

from the group of pregnancy than those from the group of no pregnancy. Follicular fluid leptin levels were also significantly lower in the successful cycles than in the unsuccessful ones.

The correlations between cycle characteristics and cytokine levels are presented in Table 2. Serum leptin levels positively correlated with BMI (Spearman R=0.62, p=0.009) as it had been expected. There were no significant correlations between serum and FF levels of the three cytokines. FF VEGF was negatively correlated with E2 (Spearman R=–0.68, p=0.003) and positively with FF leptin (Spearman r=0.41, p=0.02). Follicular fluid leptin was also positively correlated with age (Spearman r=0.41, p=0.018). There were no other significant correlations among the levels of the three cytokines or between the levels of the cytokines and the cycle parameters.

Table 2. Correlations between TNFα, VEGF, leptin, BMI, total amount of administrated gonadotropins and peak estradiol (E2) levels.

	BMI	**Conado -tropins**	**E2**	**Serum TNFα**	**Serum VEGF**	**Serum leptin**	**FF TNFα**	**FF VEGF**	**FF leptin**
Age	–0.40	0.47**	–0.22	–0.17	0.87	0.63	0.05	0.27	0.41*
BMI		–0.19	0.60	–0.01	0.10	0.62**	0.87	–0.87	0.87
Gonadotropins			–0.34	0.09	0.50	0.39	0.13	0.36	0.17
E2				–0.66	0.75	0.75	0.11	–0.68**	–0.33
Serum TNFα					–0.02	–0.60	–0.40	0.02	0.80
Serum VEGF						–0.10	–0.59	–0.50	–0.50
Serum leptin							–0.54	–0.60	0.57
FF TNFα								0.34	0.33
FF VEGF									0.41*

Spearman r values are presented. FF: follicular fluid levels. *: p<0.05, **: p<0.01.

Discussion

The present study involved cases for which male factor infertility was the only indication for IVF treatment. This means that all women were normal, i.e. without any apparent reproductive disorder. In this way, we wanted to avoid any possible bias on the results, as various infertility disorders may be associated with altered cytokine levels. Furthermore, the use of ICSI bypassed male factor infertility, we can thus presume that the successful outcome was mainly dependent on the quality of oocytes and consequently on those factors that influence the oocyte during its follicular development. During the follicular phase, each oocyte is developed into a follicle that consists of a distinct and particular microenvironment with many systemic and local interrelated factors that influence, directly or indirectly, the oocyte. VEGF, TNFα and leptin belong to factors that possibly influence oocyte quality and subsequently pregnancy outcome.

The cytokine levels in the serum, on the day of oocyte aspiration, did not reveal significant differences between the groups of pregnant and no pregnant cycles. No significant correlation was found between serum cytokine levels and clinical parameters of the cycles. These findings suggest that TNFα, VEGF and leptin serum concentrations, on the day of oocyte aspiration, have no prognostic value for IVF outcome. The serum levels of the three studied cytokines were not significantly correlated with the corresponding FF levels. Moreover, FF levels of VEGF and leptin were always notably higher than corresponding serum levels. These findings indicate the importance of local regulation in follicular levels of VEGF and leptin.

The results of the present study confirmed the presence of detectable levels of TNFα in most but not all FFs. Three samples of different women had no detectable levels of FF TNFα and similar findings have also been reported by other investigators (Barak *et al.*, 1992, Punnonen *et al.* 1992, Cianci *et al.* 1996, Aboul Enien *et al.*, 2001). It is worth to note that TNFα was detected in the serum samples of these three women. On the other hand, in these follicles with undetectable levels of TNFα, the concentrations of the other two cytokines were close

to the mean values. In addition, a wide variation in FF concentrations of TNFα from different follicles of the same patients has been found in other studies (Barak *et al.* 1992, Punnonen *et al.* 1992, Mendoza *et al.* 1999, Aboul Enien *et al.* 2001). This variation can be rather explained by interfollicular asynchrony, but it remains to be shown whether and how the presence or absence of TNFα concentrations in FF reflects the status of follicular function. In this study, there was no significant correlation between FF TNFα and the other studied parameters, in particular the fertilization rate and conception. It is worth noting that controversial opinions have been expressed for the association of TNFα with the quality of oocytes and their fertilizability. Lee *et al.* (2000) found higher levels of TNFα in follicles with poor quality oocytes, whereas Mendoza *et al.* (1999) reported higher concentrations in follicles contained oocytes that were successfully fertilized. In the present study, although TNFα levels in follicles of non-pregnant women were higher than in those of pregnant, there was no significant correlation between TNFα and pregnancy outcome. Similarly, other studies have failed to find such a correlation (Barak *et al.* 1992, Lee *et al.* 2000, Aboul Enien *et al.* 2001, Mendoza *et al.* 2002).

The concentrations of FF VEGF in non-pregnant group were significantly elevated compared with those of pregnant group. FF VEGF levels demonstrated a negative correlation with peak E2 levels indicating an influence of sex steroids on VEGF production in follicular compartment. These findings corroborate the work of Friedman *et al.* (1998) who showed that elevated concentrations of VEGF in FF are significantly associated with diminished pregnancy potential in IVF cycles and significantly correlated with serum E2 levels. Furthermore, Friedman *et al.* (1998) suggested VEGF as a marker of follicular hypoxia.

In this study, FF leptin was also significantly associated with the pregnancy outcome of ICSI cycles. Non-pregnant women had threefold higher FF total leptin levels than pregnant and this difference was statistically significant. This finding confirms the results of a previous study showing that lower concentrations of leptin in FFs were significantly associated with positive outcome of IVF cycles (Mantzoros *et al.* 2000). Moreover, two recent studies have also reported that low serum leptin levels (at the time of hCG administration) or baseline serum leptin/BMI ratio may be predictive of positive IVF pregnancy outcome (Brannian *et al.* 2001, Tsai *et al.* 2002). However, the mechanism by which leptin affects pregnancy remains unclear. It is known that leptin can influence reproduction acting centrally at the hypothalamus-pituitary level, but also directly in the ovaries and endometrium (Caprio *et al.* 2001, Baratta, 2002, Moschos *et al.* 2002). The existence of leptin receptors in ovarian tissues and endometrium confirms the importance of leptin as a local regulator of ovarian and endometrial function (Karlsson *et al.* 1997, González *et al.* 2000). Since controlled ovarian hyperstimulation bypasses the pituitary, the explanation for the impact of high FF leptin concentrations on pregnancy outcome may be based upon its direct actions on the ovary. A number of in-vitro studies have shown that leptin has a negative effect on ovarian steroid output (Spicer and Francisco, 1997, Zachow and Magoffin 1997, Karlsson *et al.* 1997, Agarwal *et al.* 1999, Tsai *et al.* 2002). If this is true, it may suggests that low E2 levels in the follicles may impair the normal follicular development resulting in oocytes of poor quality with a low implantation potential. In the present study, a significant correlation between FF leptin and FF VEGF was also found. A correlation between the two cytokines was also reported by Barroso *et al.* (1999) who provided evidence that both VEGF and leptin may be markers of hypoxic conditions in the follicular compartment and consequently of suboptimal oocyte and embryo quality. This is possibly an adequate explanation for the negative impact of the elevated levels of the two cytokines on the pregnancy outcome.

A considerable variation was found in the FF level of the three cytokines in both studied groups. Such variation has also been found in other studies (Barak *et al.* 1992, Punnonen *et al.* 1992, Anasti *et al.* 1998, Friedman *et al.* 1998, Barroso *et al.* 1999, Mendoza *et al.* 1999, Aboul Enien *et al.* 2001, Benifla *et al.* 2001, Malamitsi-Puchner *et al.* 2001) and may be explained by the interfollicular asynchrony. Nevertheless, this variation in cytokines concentrations, namely of VEGF and leptin, makes the establishment of cut-off values for the prediction of pregnancy outcome difficult.

According to the results of the present study, VEGF and leptin concentrations in FFs are negatively associated with pregnancy outcome of ICSI cycles. FF leptin levels are positively correlated with those of VEGF, whereas FF VEGF levels are negatively correlated with peak E2 serum levels. Serum levels of VEGF, leptin and TNFα, as well as FF TNFα levels are not associated with pregnancy outcome and they are not significantly correlated with the FF levels of the other two cytokines, the peak E2 serum levels or any of the

other studied parameters. It is worth noting that the studied population was strictly selected, including women without any apparent reproductive disorders that might bias the results, and that all of them underwent controlled ovarian stimulation according to the long agonist protocol. In conclusion, VEGF and leptin concentrations in FFs may serve as reliable predictive markers for ICSI outcome in women with no apparent reproductive disorders. However, we believe that further research is necessary in order to confirm these findings and to set appropriate algorithms for clinical practice. Further research is also needed to elucidate the mechanisms in which the two cytokines negatively affect the pregnancy outcome of IVF cycles.

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