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Follicular fluid levels of vascular endothelial growth factor and its receptors and pregnancy outcome of women participating in intracytoplasmic sperm injection cycles

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Summary

Purpose: The intracytoplasmic sperm injection (ICSI) outcome is depended mainly on oocyte quality. Cytokines and their receptors play a critical role in oocyte maturation, fertilization, and embryo implantation. The purpose of the study was to study the levels of vascular endothelial growth factors (VEGFA, VEGFR1, VEGFA) in follicular fluids (FF) women participating in ICSI-in vitro fertilization (IVF) cycles in relation to cycle's outcome. *Material and methods:* One hundred and fifty three samples of 70 women participating in ICSI cycles were classified in three infertility groups: male factor, female factor, and low responders. For controlled ovarian stimulation in male and female factor group, the long agonist protocol with leuprolide and recombinant follicle stimulating hormone (FSH) was employed, while the antagonist cetrorelix was used in low responders. Cytokines levels were evaluated with enzyme-linked immunosorbent assay (ELISA) . *Results:* In a total of 153 samples, the overall pregnancy rate was 51.6%., the higher one observed in female factor group (59% vs 37.5% and 28.6% in male a factor and low responders group, $p = 0.013$. VEGFR2 differed statistically significantly between the two groups, being higher in the pregnancy group [median (IQR): 5,630 (4,870 - 6,651) vs 4938 (4,068 - 6,020) in the non-pregnancy group, $p = 0.003$]. There were significant correlations between VEGF receptors, differentiated depending on infertility groups. *Conclusions:* The VEGFA/VEGFR2 system is important in human reproduction and the association pattern between VEGFA receptors may serve as a marker for ICSI outcome. Examination for spermatozoa functional defects may increase pregnancy rate in male factor group.

Key words: Infertility; VEGFA; VEGF receptors; ICSI IVF; Pregnancy.

Introduction

The success of embryos implantation in women undergoing in vitro fertilization (IVF) is depended mainly on oocyte quality [1-3]. Numerous local factors promote or inhibit follicular development and affect reproductive outcome [4-7]. Cytokines play a critical role in oocyte maturation, fertilization, and embryo implantation. Among them, the vascular endothelial growth factors (VEGFs) regulate vascular development during embryogenesis and blood vessel formation [8]. There are five VEGF family members, which are dimeric glycoproteins of approximately 40 kDa: VEGFA, B, C, D, and placenta growth factor (PLGF). They exert their actions after binding to specific VEGF receptors (VEGFRs) and co-receptors. The receptors are tyrosine kinases (RTKs), known as VEGF receptor-1, -2 and -3 (VEGFR1–3), while co-receptors (lacking established VEGF-induced catalytic function) include heparin sulphate proteoglycans (HSPGs) and neuropilins. The VEGFRs have an extracellular portion consisting of seven immunoglobulin-like domains, a single transmembrane spanning region and an intracellular portion containing a split RTK domain.VEGFA, B, and PLGF bind to VEGFR1, VEGFA, and E bind to VEGFR2, and VEGFC and D bind to VEGFR3. Thus, VEGF-A binds to VEGFR-1 and VEGFR-2. The latter seems to participate in almost all of the known cellular responses to VEGF [9]. VEGFR-1 is thought to modulate VEGFR-2 signaling.VEGFA has drawn special attention as a particular feature of the VEGFA ligand is the dramatic upregulation of its expression levels under hypoxic conditions [10].

The expression of VEGFRs (especially VEGFR1 and VEGFR2) is also induced by hypoxia and from a teleological view this expression contributes to augmentation of oxygen supply to oocytes and implanted embryos. Indeed, oocytes are particularly sensitive to hypoxic dam-

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age [11]. Sufficient oxygen supply is crucial for embryo development procedures and adequate vascular network is necessary for adequate blood supply, which in turn is translated into oxygen and nutrients supply [12, 13].

Controlled ovarian hyperstimulation (COH) is used in IVF cycles in order to increase the number of mature oocytes and to maximize conception chances. Improving pregnancy rates is associated with investigation of all possible factors affecting embryo implantation and development and VEGFA among other cytokines has been the target of intense research in the last decade. The research has focused mainly on follicular fluids (FF), as serum concentrations of these factors are low and interesting findings has been presented: VEGF levels were found to be significantly correlated with grade of perifollicular vascularity and the presence of the VEGF-A receptors, particularly in the granulosa cells, suggests that VEGF-A might be involved in proliferation initiation of primordial follicles or play a role in human preantral follicles [14,15]. VEGF levels increase during the first trimester, but FF-VEGF levels have been found significantly elevated in non-pregnant women as compared to pregnant women [3].

The various cytokines involved in fertilization and the rather contradictory reported results ask for further investigation, in attempt to study pregnancy outcomes in women undergoing IVF. Significant questions remain regarding the role of VEGFRs as only recently experimental data have suggested that the VEGF/VEGFR-2 pathway plays a key role in the maintenance of early pregnancy, the temporal and spatial relationships of VEGFA, and its receptor expression in a luteal endometrium, as well as the significance of the research finds for the various groups of women undergoing IVF [16,17].

The present study attempts to shift attention to VEGFRs, rather than VEGF itself, as recent findings suggest interaction between VEGFRs subtypes. The aim of this study was to study the levels of VEGFA, VEGFR1, VEGFA, and leptin in FF of three groups of women participating in intracytoplasmic sperm injection (ICS)-IVF cycles: male factor infertility women, female factor, and low responders. The authors also studied pregnancy outcome in relation to the above cytokines and receptors' FF concentrations.

Materials and Methods

The studied sample consisted of 70 women who participated in ICSI cycles. Women had never undergone any IVF technique before. One hundred and fifty three samples were collected and they were classified into three infertility categories depending on infertility cause, according to existing literature [1,18]: a) male factor group (N=56), b) female factor group (women with reproductive disorders, except from polycystic ovarian syndrome (PCOS), N=83), and c) low responders (N=14).

The long agonist protocol for COH in male and female infertility factor group was used as previously described [19]. Briefly, ovarian hyperstimulation performed by administration of recombinant follicle stimulating hormone (FSH) after pituitary suppression with leuprolide started in the midluteal phase of the preceding cycle. In low responders, the antagonist cetrorelix was administered, according to a standard protocol described elsewhere [20]. 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 300-500 pg/ml per follicle, ovulation was induced with 10,000 IU of human chorionic gonadotropin (hCG). Transvaginal oocyte aspiration with ultrasound guidance was performed under general anesthesia 36 hours later. 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 minutes at 1,500 rpm and the supernatants were stored at –75 °C for further analysis.

Two hours after follicular aspiration, the cumulus oophorus and corona radiata were removed mechanically under dissecting microscope, with simultaneous incubation in ICSI cumulase solution (recombinant human hyaluronidase, 80 U/ml for 60 seconds. The incubation in ICSI cumulase was followed by repeated aspirations into denuding pipettes. Only metaphase II (MII) oocytes were used for ICSI that was performed two to three hours later using an inverted microscope with micromanipulators. The injected oocytes were cultured in universal IVF medium, at 37°C, in a humidified atmosphere with 5 % CO_2. After 18 hours 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 (ISM1 culture medium) and cultured for 24-30 hours.

Embryos were further cultured in ISM2 medium beyond second day of fertilization, until embryo transfer and blastocyst development, around $5^{th}/6^{th}$ day after fertilization. The luteal phase was supported daily with 600 mg natural progesterone administrated vaginally. Pregnancies were defined by the presence of hCG >150 IU/ml, around 15^{th} day after implantation and verified by positive fetal heart beats.

Measurements of cytokine concentrations

In every FF sample, commercial enzyme immunoassay (ELISA) kits were used to measure the concentrations of free VEGFA, VEGFRs, and total leptin and leptin receptor. All measurements were carried out in duplicate and according to the manufacturers' instructions.

Statistics

Descriptive and analytic statistics was performed. Data are presented as median and InterQuartileRange (IQR) or mean ±standard deviation (SD), depending on normality of distribution. Normality was checked with Shapiro-Wilk test. Mann-Whitney –U test was used for comparison of two independent samples, if distribution was not normal, whereas in normal distributions t-test was used instead. Rates were compared using x2 test with Yates correction for two-by-two tables, and Spearman correlation was applied for bivariate correlations. A logistic regression model was applied for outcome prediction. Statistical significance was set at $p < 0.05$, while Bonferroni correction test was used for multiple comparisons. All tests were two–tailed.

Table 1. — *Age, BMI, and pregnancy rates in the study groups.*

Infertility categories*	Age Mean ± SD	BMI Mean ± SD	Outcome (Pregnancy rates)# Yes N	Yes %	No N	No %
Male factor N=56	31.20±2.38	25.25±4.86	21	37.5	35	62.5
Female factor ** N=83	34.73±4.21	25.78±6.42	49	59.0	34	41.0
Low responders N=14	40.09±2.52	27.39±4.12	4	28.6	10	71.4
Sig.	F = 59.825, $p < 0.001$*	F = 0.778, $p = 0.461$	$X^2 = 8.756$, $p = 0.013$**			

*male factor <female factor<low responders; **female factor: statistically significant greater rates of pregnancy compared with mf1 & mf3
overall pregnancy rate: 51.6%

Table 2. — *Differences in cytokines, their receptors, and estradiol depending on pregnancy outcome,*

Pregnancy			VEGFR1	VEGFA	Estradiol	VEGFR2
Yes	N=74					
	Percentiles	25	1998.00	1827.53	170700.00	4870.79
		50	4600.50	2142.25	206200.00	5630.00
		75	10082.50	3445.75	938737.70	6651.25
No	N=79					
	Percentiles	25	1762.00	1957.90	158800.00	4068.50
		50	4173.13	2542.38	452722.00	4938.77
		75	8440.00	3801.18	1075900.00	6020.00
Sig. (Mann – Whitney-U test)			p=0.137	p=0.125	p=0.514	p=0.003

Table 3. — *Logistic regression model for pregnancy outcome.*

Parameter	B	Hypothesis Test Wald Chi-Square	df	Sig.	Exp(B)	95% Wald Confidence Interval for Exp (B) Lower	Upper
VEGFR2	0.000	5.861	1	0.015	1.000	1.000	1.001
Age	-0.006	0.023	1	0.879	0.994	0.916	1.078
[Fertility group: male factor/low responders]	-1.217	10.502	1	0.001	0.296	0.142	0.618
[Fertility group: female factor]	0				1		

Dependent variable: pregnancy

Table 4. — *Correlations between cytokines and estradiol levels according to infertility factor group.*

Fertility groups			VEGFR1	VEGFR2	Estradiol
Male factor (N=56)	VEGFA	Rho	0.114	0.462	0.000
		p	0.404	<0.001	0.999
	VEGFR1	Rho		0.379	-0.285
		p		0.004	0.034
	VEGFR2	Rho			-0.106
		p			0.435
Female Factor (N=83)	VEGFA	Rho	0.072	-0.246	0.103
		p	0.518	0.025	0.354
	VEGFR1	Rho		-0.253	0.053
		p		0.021	0.635
	VEGFR2	Rho			-0.208
		p			0.059
Low responders (N=14)	VEGFA	Rho	0.338	-0.029	0.434
		p	0.238	0.922	0.121
	VEGFR1	Rho		0.209	-0.077
		p		0.474	0.793
	VEGFR2	Rho			-0.515
		p			0.060

Results

Regarding infertility categories, 56 samples were classified in male factor group, 83 in female factor, and 14 in low responders. Women's age differed statistically significantly between groups, the youngest age (31.20 ± 2.38 years) observed in male factor group and the oldest (40.09 ± 2.52 years) in low responders. Women were overall overweight, as mean body mass index (BMI) values exert 25.00 in all three categories. In a total of 153 samples, the overall pregnancy rate was 51.6%, the higher one observed in female factor group (59% vs 37.5% and 28.6% in male factor and low responders group, $p = 0.013$ (Table 1). When male factor and low responder categories were merged, differences between female factor group and the remaining subjects regarding pregnancy was more obvious: 35.7% vs 59.0%, $p = 0.007$ (data not shown). When pregnancy and non-pregnancy groups (irrelevant of infertility cause) were comparatively studied, only VEGFR2 differed statistically significantly between the two groups, being higher in the pregnancy group [median (IQR): 5,630 (4,870 - 6,651) vs 4,938 (4,068 - 6,020) in the non-pregnancy group, $p = 0.003$] (Table 2). As infertility group and VEGFR2 were associated with pregnancy, a logistic regression model adjusted for age was applied to study the above factors as predictors of pregnancy (yes/no). In this model subjects were classified in two infertility categories (female factor group against all others). The infertility group emerged as independent predictor of pregnancy, whereas VEGFR2 failed to have a significant component to the equation, due to its zero coefficient. The female factor subjects had approximately 3.4 times greater probability to have a pregnancy (1 / 0.296) (Table 3). Spearman correlations within the three infertility groups revealed *positive* correlations between VEGFR1 - VEGFR2 (p=0.004), and VEGFR2-VEGFA ($p < 0.001$),

while *negative* correlation was observed between estradiol and VEGFR1 ($p = 0.034$). In the female factor group, *negative* correlations between VEGFR1-VEGFR2 ($p = 0.021$) and VEGFR2-VEGFA ($p = 0.025$) were observed. In the low responders and the female factor group, E2 was marginally statistically significantly related to VEGFR2 ($p = 0.059$ and $p = 0.060$, respectively, Table 4).

Discussion

The findings of the present study underline the importance of FF study in human reproduction. Infertility due to female factor seem to be an independent predictor of pregnancy in women under ICSI. The results also call for attention in the case of low responders and male factor group, as these groups exhibit a similar pregnancy outcome, despite possibly different underlying mechanisms, implying that serious–and probably difficult to detect–functional defects may be present in spermatozoa and oocytes.

The three groups of the present study exhibit distinct characteristics regarding the concentrations VEGFA, its receptors, and leptin/leptin receptor. Moreover, pattern of associations is significantly differentiated between male and female factor group, being quite opposite in the case of VEGFA receptors. In male factor group, concentrations of VEGFR1 and VEGFR2 were positively associated to one another: VEGFR1 was negatively associated with E2 and VEGFR2 was positively associated with VEGFA. In female factor group, VEGFR1 and VEGFR2 were *negatively* associated to one another, while VEGFR2 were *negatively* associated to VEGFA. The pattern of associations in the male factor group reflects previously reported data suggesting that estradiol decreased secreted VEGFR1 and increased secreted VEGFA [4, 21-23]. The strong association between VEGFR1 and VEGFR2 indicates that these two receptors may work hand in hand as the formation of heterodimers and interaction between the two receptors has been documented [23, 24]. The marginally negative correlation between E2 and VEGFR-2 in the female factor and low responders may reflect interaction between the two receptors and indicate common pathophysiological pathways. This association may suggest that VEGFR2 is the most important receptor determining embryo development, representing an endogenous female defect. Previous data support the significance of the VEGFA/VEGFR2 system for pregnancy maintenance in animal studies and VEGFR-2 appears to be the most important receptor in VEGF-induced mitogenesis and permeability [18, 25]. The associations between VEGFA/VEGFR2/VEGFR1 might indicate adaptation to hypoxia, the receptors being upregulated in order to bind as many as possible VEGF molecules.

Regarding pregnancy rates in the present study, these are comparable with previous findings. More specific, in the case of male factor infertility, ICSI pregnancy rates range from 15% (germ cell hypoplasia) to 37% (non-obstructive azoospermia) [26, 27]. In the case of female factors, pregnancy rates ranged from 6% to 49%, depending on woman age, the highest rate achieved in women < 34 years (1). Despite the apparent integrity of VEGFA/VEGFR1/VEGFR2 system in the male factor group, pregnancy rates were lower in this group when compared with the female group. This discrepancy could be attributed to functional defects in morphologically integral spermatozoa. DNA fragmentation examination is not routinely used. Indeed, the proportion of sperm with DNA fragmentation appears to be potentially useful as a predictor of ICSI outcome, whereas embryo quality based on morphological criteria, appeared unaffected by DNA fragmentation, while appropriate semen preparation decreases sperm fragmentation levels [28, 29]. Several tests are available to measure sperm DNA fragmentation levels and the routine assessment of DNA fragmentation is strongly recommended in order to improve ICSI outcomes, at least in the male factor group.

Limitations

The small sample size of the low responders group may have obscured some features of this particular infertility subgroup, while the role of the receptors in repetitive ICSI attempts was not studied. Moreover, the role of VEGFR3 and other cytokines was not investigated. Further research should broaden the array of cytokines studied and focus on different subpopulations of women under IVF, as different cytokines profile may well account for the differences observed in fertility rates in these groups.

Conclusion

The inclusion of women with female factor infertility (except from PCOS) provided a useful insight in the possible mechanisms related to female infertility and underline the importance of VEGFA/VEGFR2 system in human reproduction. Instead of VEGFA itself, its receptors and especially their association pattern may serve as a marker for differentiating infertility groups, while infertility group may be a predictor of ICSI outcome. The inclusion of larger cohorts may further elucidate the findings of the present study. Moreover, focusing on functional defects of spermatozoa may lead to better sperm preparation and higher pregnancy rates in male factor groups. Enhancing VEGFR2 production in women with reproductive disorders might also be a future promising therapeutic perspective.

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