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Original article

Follicular fluid vascular endothelial growth factor is associated with type of infertility and interferon alpha correlates with endometrial thickness in natural cycle *in vitro* fertilization

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ABSTRACT

The aim of this study was to analyse the presence of vascular endothelial growth factor (VEGF) and interferon alpha (IFN-α) in the follicular fluid (FF) and their possible influence, as pro-angiogenic or anti-angiogenic factors, on *in vitro* fertilization outcome. The concentrations of VEGF and IFN-α were correlated with oocyte and embryo quality, concentrations of hormones in the serum, perifollicular blood flow and endometrial thickness. VEGF was detected in all FF samples (median 706.6 pg/ml, range 182.9–6638 pg/ml). IFN-α was detected in 60% of the samples (median 6.5 pg/ml, range 0–79.4 pg/ml), while in 40% of the samples its levels were below the test detection limit. VEGF and IFN-α concentrations did not correlate with the cause of infertility, concentrations of FSH, LH, E2 and prolactin, oocyte or embryo quality. Significantly higher concentrations of VEGF have been found in women with primary compared with secondary infertility ($p = 0.011$, Mann Whitney test). The concentrations of VEGF and IFN-α did not correlate with the resistance index (RI) on days of hCG administration, follicular aspiration and embryo transfer. However, the concentrations of IFN-α correlated with endometrial thickness on the day of embryo transfer (Spearman correlation coefficient $\rho = 0.4107$; $P < 0.05$) but not on days of hCG administration and follicular aspiration. The mechanism of VEGF association with the previous ability of having a child needs to be clarified in future studies. The results of this study indicate a possible role of IFN-α in pathways of endometrial remodelling.

1. Introduction

Human follicular fluid (FF) contains a variety of biological active products (hormones, growth factors, cytokines) known to affect follicular growth, oocyte viability and developmental potential and fertilization. Follicular fluid components are produced by both granulosa and theca cells, as well as resident and infiltrating leukocytes [1–3]. Cytokines detected in the FF seem to derive from intrafollicular local production rather than from the peripheral blood entering the follicles through theca interna [4].

Follicular development as well as the formation and regression of the corpus luteum are accompanied with changes in the capillary network in the ovary. This process enables ovarian cells to obtain the oxygen, nutrients and precursors necessary to synthesize and release different hormones essential for maintenance of the ovarian function.

The VEGF, originally named vascular endothelial permeability factor, is a glycoprotein of molecular weight of approximately 45 kDa [5]. The VEGF family includes several members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PIGF), and two VEGF-like proteins.

The main function of VEGF is to regulate angiogenesis and vasculogenesis by stimulating endothelial cells proliferation, migration and survival. The role of VEGF in the ovary is to lead an angiogenic process which is critical for the follicular growth. However, Lam and Haines [6] have stated that VEGF plays more than a simple angiogenic role in the ovary as it is involved in a number of key events in the course of ovarian cycle including follicular growth, ovulation, corpus luteum development, and ovarian steroidogenesis. The authors have suggested that

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VEGF expression is under the regulation of gonadotropins or ovarian sex hormones. The main source of VEGF in the FF during the pre-ovulatory stage are granulosa cells [4].

Type I interferons: IFN-α, IFN-β and IFN-ω, are most frequently found in humans. They have antiproliferative, apoptotic and anti-angiogenic effects and they modulate the immune response, specifically by activating dendritic cells, cytolytic T and NK cells. Preclinical data suggest that IFN-α anti-angiogenic activity may be associated with the regulation of endothelial cell motility [7] and survival [8], and with the inhibition of VEGF transcription [9].

The aim of this study was to investigate the role of VEGF and IFN-α in human FF as possible prognostic markers for assisted reproduction outcomes by correlating the concentrations of these biological response modifiers with hormones levels, oocyte/embryo quality, perifollicular blood flow, endometrial thickness or pregnancy.

2. Materials and methods

2.1. Patients' characteristics

The study included 40 married couples who entered the natural cycle (NC) IVF/ET program at the Department of Obstetrics and Gynaecology, Sveti Duh Hospital, Zagreb, Croatia. The median age of female patients was 35 (range from 22 to 44).

The causes of infertility in the patients included tubal factor (n = 14), male factor (n = 12), unexplained infertility (n = 2) and mixed cause of infertility (n = 12).

The results of routine haematological, serological, microbiological and molecular tests (differential blood count, sedimentation, routine serological assays for blood donors, presence of anaerobic and aerobic bacteria, Human Papillomaviruses, *Chlamydia trachomatis* as well as *Ureaplasma and Mycoplasma* in cervical swabs) did not reveal acute or chronic infections in our patients. Endometriosis and pelvic inflammatory disease were excluded.

Written informed consent to use the FF samples was obtained during oocyte recovery by all patients. The Ethics Committee of the Hospital approved this study.

2.2. Blood collection and FSH, LH, E2, PRL analysis

Blood sample was collected on natural cycle Day 3 and centrifuged at 600 × *g* for 10 min. Serum was stored at −20 °C until quantification by electrochemiluminescence immunoassay (ECLIA) with the use of Elecsys 1010 (Roche Diagnostics, Minneapolis, MN, USA). The following kits were used: FSH reagent kit 1.775.863, CalSet FSH 1.775.880; LH reagent kit 1.732.234, CalSet LH 1.732.269; E2 reagent kit 1.776.002, 1.776.037 CalSet Estradiol; PRL reagent kit 1.775.952; 1.775.987 CalSet Prolactin. The reference values (median, range) for follicular phase are: FSH 6.9 IU/l (3.5–12.5); LH 5.9 IU/l, (2.4–12.6); E2 53.9 pg/ml, (24.5–195); PRL 218 mIU/l, (72–511).

2.3. Follicular fluid collection and cytokine assays

The study group included patients who underwent IVF procedure in the natural ovarian cycle and had one preovulatory follicle. After follicular aspiration and oocyte isolation, FF was taken and centrifuged at 600x g for 10 min, at room temperature. Supernatant was stored at −80 °C. Quantification of VEGF and IFN-α in the FF was performed at the Department of Immunological and Molecular Diagnostics, University Hospital for Infectious Diseases "Dr. Fran Mihaljević", Zagreb, Croatia, according to the manufacturer's instructions. The concentrations of VEGF in FF were determined by the commercial Human VEGF Immunoassay kit (Bio Source International Inc., Camarillo, CA, USA). The minimal and maximal detectable amount for VEGF was < 0.5 pg/ml and 1500 pg/ml, respectively. The concentrations of IFN-α in FF were determined by the commercial enzyme immunoassay Quantikine Human IFN-α Immunoassay (R&D Systems, Minneapolis, MN, USA). The minimal and maximal detectable amount for IFN-α was < 10 pg/ml and 500 pg/ml, respectively.

2.4. Ovarian blood flow measurement and ultrasound assessment of the endometrium

Forty patients in the natural cycle underwent transvaginal ultrasound follow up of follicular development from natural cycle Day 8. Ultrasound device (Aloka, Tokyo, Japan) was equipped with a 5-MHZ frequency transvaginal transducer that was used for follicle measurement and blood flow assessment by color and pulsed wave Doppler. When the follicle reached 18 mm in diameter, ovulation was triggered by the administration of 5000 IU human chorionic gonadotropin - hCG (Choragon, Ferring Pharmaceuticals Ltd, GmbH, Kiel, Germany).

Blood flow of the dominant follicle, the same one from which the FF was obtained, was examined for the presence of color signals indicating vascularization, while the blood flow assessment was expressed as resistance index (RI). RI was defined as (PSV-EDV)/PSV (peak systolic velocity, PSV; end-diastolic velocity, EDV) and higher RI value indicated lower blood flow. The spatial peak temporal average intensity for B-mode and Doppler imaging was below 95 mW/cm^2. Data of RI were recorded on the day of administration hCG, on the day of follicular aspiration and on the day of embryo transfer.

The B-mode ultrasound was used to evaluate thickness and echogenicity of the endometrium on the day of hCG administration, on the day of follicle aspiration and on the day of embryo transfer. The endometrium was depicted in the sagittal plane and measured as a bilayer thickness from the proximal myometrial-endometrial junction to the distal myometrial-endometrial junction.

2.5. In vitro fertilization – embryo transfer procedure

After recovery, the oocyte was washed free from the FF. Oocyte maturity (i.e. quality) was assessed after mechanical dissection of cumulus oophorus till corona radiata. The oocyte was pre-incubated for 4 h in the Quinn's Advantage Fertilization medium with 5.0 mg/ml HSA (SAGE In Vitro Fertilization, Inc., Trumbull, CT, USA) at 37 °C in 5.8% CO_2 and humidified air.

Semen samples were collected on the day of follicle aspiration. The semen samples were centrifuged at 300x g in the Quinn's Sperm Washing Medium (SAGE In Vitro Fertilization) and subsequently processed by the swim-up method in the Quinn's Advantage Fertilization medium with 5.0 mg/ml HSA (SAGE In Vitro Fertilization). Each oocyte was inseminated with 40×10^3 to 80×10^3 of motile sperm. Fertilization, as the presence of pronuclei, was checked about 20 h after insemination and zygotes were placed into the Quinn's Advantage Cleavage medium with 5.0 mg/ml HSA (SAGE In Vitro Fertilization). If oocytes did not contain pronuclei, the fertilization was checked once more after another 24 h.

Embryos were graded according to the Istambul consensus [10]. On culture Day 3, after ultrasound evaluation of the uterus and ovaries, only a single embryo has been transferred. Luteal phase support has been accomplished with the micronized progesterone (Utrogestan; Laboratories Piette International S.A., Brussels, Belgium) 600 mg/day starting from the day after oocyte retrieval.

Biochemical pregnancy test was performed two weeks after follicular aspiration, and clinical confirmation of pregnancy was performed by sonography after 6 weeks of gestation. Clinical pregnancy with foetal cardiac activity was described as a viable intrauterine pregnancy. Clinical pregnancy rate was expressed as the number of clinical pregnancy per embryo transfer. The term IVF outcome referred to the live birth rate per embryo transfer.