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ASSISTED REPRODUCTION TECHNOLOGIES

Perifollicular blood flow and its relationship with endometrial vascularity, follicular fluid EG-VEGF, IGF-1, and inhibin-a levels and IVF outcomes

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Abstract
Purpose The aim of this study is to investigate the association of perifollicular blood flow (PFBF) with follicular fluid EG-VEGF, inhibin-a, and insulin-like growth factor-1 (IGF-1) concentrations, endometrial vascularity, and IVF outcomes.
Methods Forty women with tubal factor infertility were included in a prospective cohort study. Each woman underwent IVF/ICSI procedure. Individual follicles of ≥16 mm ($n = 156$) were evaluated by power Doppler analysis and categorized as well-vascularized follicles (WVFs) or poorly vascularized follicles (PVFs). WVFs referred to those with perifollicular vascularity of 51–100 %. Each follicular fluid (FF) was individually aspirated and FF/serum EG-VEGF, inhibin-a, and FF IGF-1 levels were evaluated. Zones III–IV endometrial vascularity was classified as a well-vascularized endometrium (WVE). The presence of a WVE and mature oocytes, in addition to the embryo quality and clinical pregnancy rate (CPR), were recorded for each follicle. The main outcome measures were FF serum EG-VEGF, inhibin-a, IGF-1 levels, and WVE and IVF outcome per PFBF.
Results For WVFs, the level of FF EG-VEGF ($p = 0.008$), oocyte quality ($p = 0.001$), embryo quality ($p = 0.002$), a WVE ($p = 0.001$), and CPR ($p = 0.04$) increased significantly. The pregnant group was characterized by increased numbers of WVFs ($p = 0.044$), a WVE ($p = 0.022$), and increased levels of FF IGF-1 ($p = 0.001$) and serum EG-VEGF ($p = 0.03$). FF IGF-1 >50 ng/mL (AUC 0.72) had 75 % sensitivity and 64 % specificity for predicting CPR.
Conclusions WVFs yield high-quality oocytes and embryos, a WVE, increased FF EG-VEGF levels, and increased CPRs.

Keywords Follicular vascularity · Endometrial vascularity · Follicular blood flow · Endometrial blood flow · IGF-1 · EG-VEGF · Inhibin-a

Capsule The vascularity of a follicle may be a valuable marker of oocyte selection and good-quality embryos, with implantation potential.

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Introduction

Folliculogenesis defines the progress of a primordial follicle to a mature follicle. It is a complex and well-organized process, which includes dynamic and endocrine changes. The antral follicle contains the outermost thecal layers, which contain vasculature and steroidogenic cells and synthesize and secrete androgen. The inner granulosa cells aromatize androgen to produce estrogen. They also produce other protein hormones and secrete proteoglycan to produce an osmotic gradient and fluid-filled cavity [1]. The resulting capillary network mediates the transport of oxygen, nutrients, and precursor substances [1, 2]. Vascularization is the primary essential step in follicular growth, and the follicular microenvironment is an essential factor in oocyte growth [3, 4].

A variety of parameters, including hypoxia, aging, paracrine factors, and autocrine factors, modulate angiogenesis [4, 5]. Vascular endothelial growth factor (VEGF) is a major mitogen in folliculogenesis and plays an important role

in triggering angiogenesis and regulating vascular permeability [5, 6]. VEGF is expressed in granulosa lutein cells and in theca cells of the ovaries and endometrium [5–8]. Studies have revealed the expression of endocrine gland (EG)-derived VEGF (EG-VEGF) in the ovarian stroma [9]. Although EG-VEGF is structurally different from VEGF, they have similar functions [9, 10]. VEGF is secreted from granulosa lutein cells. EG-VEGF is secreted from theca lutein cells [10]. VEGF acts as a rate-limiting step in capillary network formation in the corpus luteum, and EG-VEGF further stimulates angiogenesis in the midluteal phase and stabilizes angiogenesis [10], and both VEGF and EG-VEGF seem to act synergistically [9–13].

Prior studies demonstrated a strong relation between perifollicular blood flow (PFBF) and embryo quality and pregnancy [14–20]. PFBF was shown to be correlated with follicular oxygenation and VEGF levels [6, 13]. There have been few studies of the relationship between EG-VEGF and PFBF [11, 12]. Endometrial vascularity is thought to be related to endometrial receptivity [10]. However, there is no agreement on the role of endometrial vascularity in IVF outcomes [21–25].

The role of a number of cytokines, either alone or in combination, in reproductive health has been studied [5]. Insulin-like growth factor-1 (IGF-1) is an intraovarian regulator of follicle function. In granulosa cells, IGF-1, together with gonadotrophins, promotes hormone secretion and follicular growth and prevent apoptosis of mature follicles. Although several studies have investigated IGF-1 in follicular fluid, most of these studies have involved animal models or in vitro cell culture techniques in humans and exogenous rather than endogenous IGF-1 [26, 27]. There is no consensus on the role of IGF-1 in human follicular fluid [27–33].

Follicular granulosa cells secrete two different types of inhibins, inbibin-a and inhibin-b, belonging to the transforming growth factor beta family. These inhibins have diverse actions, and their concentrations vary throughout the menstrual cycle. The level of inhibin-b increases from the luteal phase to the follicular, reaching maximum levels in the midfollicular phase. Inhibin-b reflects granulosa cell activity and follicular development. The level of inhibin-a increases in the late follicular phase [5]. Inhibin-a is secreted by mature follicles and reflects follicular maturity [5]. The role of inhibin-a in follicular fluid and its association with PFBF is not clear [34–37].

The selection of high-quality oocytes and therefore embryos is a key factor in the success of IVF. Various oocyte selection methods have been proposed [5]. However, the results are not always satisfactory, and there is a need for noninvasive methods of oocyte selection. Angiogenesis and the follicular fluid microenvironment are the two most important components of oocyte quality and maturity. Previous studies documented that perifollicular perfusion influenced oocyte competence, embryo viability, and implantation potential [14–20]. Studies confirmed that embryos from fertilized oocytes obtained from well-vascularized follicles (WVFs) yielded higher pregnancy rates than oocytes obtained from poorly vascularized follicles [21, 26]. Therefore, it is reasonable to presume that WVFs provide a suitable follicular fluid milieu and high-quality oocytes, with increased implantation potential. This study investigated PFBF and its association with follicular fluid concentrations of EG-VEGF, inhibin-a, and IGF-1, as well as with endometrial vascularity and IVF outcomes.

Materials and method

This prospective study was performed in the Assisted Reproduction Unit of Kocaeli University School of Medicine from April 2012 to December 2014. The local ethics committee approved the study (KADB-F19-R00, 2012). All the subjects were informed about the details of the study, and both written and verbal informed consent were obtained.

Subjects

Normoresponder patients ≤35 years with tubal factor infertility who underwent IVF/ICSI (in vitro fertilization/intracytoplasmic sperm injection) procedures were included in the study. All the patients had normal ovarian function (a follicle-stimulating hormone [FSH] level of <12 mIU/mL, antimullerian hormone [AMH] level of 1–5 ng/mL, and antral follicle count [AFC] of 5–15) and regular spontaneous ovulatory menstrual cycles.

The exclusion criteria were women with male factor infertility, prior ovarian surgery, endometriosis, polycystic ovarian syndrome, obesity (a body mass index [BMI] of >30 kg/m^2), unexplained infertility, poor ovarian reserves (FSH >12 mIU/mL, AMH <1 ng/mL, or AFC <5), poor ovarian response (<3 oocyte retrieved), smoking, abuse of alcohol, neoplasms, autoimmune diseases, concurrent medical illness, or medication use within the last 3 months.

Laboratory evaluation

Blood samples were taken at day 3 of menstrual cycle for basal hormonal evaluations. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2), and AMH were evaluated on random days. A Gen II microELISA (Beckman Coulter®, Kraemer Blvd. Brea, CA 92821 USA) was used in the AMH measurements, with high sensitivity (0.017 ng/mL). This method has 5 % intra-assay variability and 8 % inter-assay variability. An immunoassay system (Siemens Advia Centaur XP®, Ireland) and Advia Centaur

kit (Australia) were used to measure FSH, LH, E2, and progesterone levels.

The oocyte retrieval (OR) day follicular fluid and serum evaluations of inhibin-a, EG-VEGF, and IGF-1 were performed with commercially available ELISA kits. The sensitivity of inhibin-a assay (Human inhibin-a ELISA kit, Eastbiopharm®, Hangzhou, China) is 2.51 pg/mL. The EG-VEGF assay (Alisei-rodim®, Rome, Italy) (Human EG-VEGF ELISA kit, Aviscera Bioscience®, CA, USA) has a sensitivity of the test of 3.9 pg/mL. The intra- and inter-assay coefficients of variation of the EG-VEGF kit are 6 and 10 %, respectively. The sensitivity of the IGF-1 assay (Human IGF-1 ELISA kit, IDS®, France) is 8.8 ng/mL.

Ultrasonography

Each patient underwent serial ultrasonographic examinations during the controlled ovarian hyperstimulation (COH) protocol. To prevent interobserver variation, the same ultrasonographer (ED) performed the ultrasonographic scans using a 6.5 MHz transvaginal transducer with Doppler facility (Sonoace X8, Samsung Medison Co. Ltd., South Korea). There was no significant intraobserver variation in the repeated measurements ($p > 0.05$).

Perifollicular blood flow

PFBF was evaluated by power Doppler, with the highest longitudinal plane of the ovaries used in all the measurements. Perifollicular vascularization around the dominant follicles was evaluated by power Doppler analysis and graded as described previously [14]. Vascularization around follicles of >15 mm was assessed by the percentage of blood flow. Follicles with PFBF of ≤50 % were defined as poorly vascularized follicles (PVFs). Follicles with 51–100 % vascularity were defined as well-vascularized follicles (WVFs).

Endometrium

The endometrial thickness and morphology were evaluated at the longitudinal axis of the uterus. The maximum thickness of the endometrium, including both layers, was measured. Endometrium morphology was reported as hyperechoic, isoechoic, or triple line. Power Doppler of the thickest part of the endometrium was used to evaluate the vascular distribution, with the different colored zones graded as described previously [38]. Zone I referred to vascularization in the subendometrial region. Zone II denoted vascularization in the outer hyperechoic zone, and Zone III referred to vascularization in the inner hypoechoic zone. Zone IV defined vascularization reaching the endometrial cavity. Patients with zones III–IV were classified as having a well-vascularized endometrium (WVE).

COH protocol and OR

All the subjects underwent a flexible gonadotropin releasing hormone (GnRH) antagonist COH protocol using (HP-hMG, Menopur® Ferring Pharmaceuticals, Saint-Prex, Switzerland) and GnRH antagonist (Cetrorelix® 0.25 mg; Serono, Switzerland). During the COH protocol, serial measurements of serum levels of E2, LH, and progesterone were obtained from the patients, and they were monitored via ultrasonographic examinations. The recombinant choriogonadotropin alpha (rhCG, Ovitrelle® 250 μg; Serono, Switzerland) was administered if one or more follicles (≥16 mm size) developed during the COH protocol. OR was performed 36 h after the injection of rhCG.

Follicular fluid collection, ICSI, and embryo transfer procedure

A total of 156 follicles were assessed by power Doppler ultrasonography, and the follicular fluid was aspirated. The serum samples of the patients ($n = 40$) and the fluid from the follicles ($n = 156$) on the day of oocyte retrieval were assessed for inhibin-a, EG-VEGF, and IGF-1. All follicles with a mean diameter of ≥16 mm were aspirated separately, using different needles each time to avoid contamination. Samples with blood contamination were discarded. The centrifuged (Kubota®-2420 centrifuge, Tokyo, Japan) samples of follicular fluid were stored at −80 °C (Sanyo® MDF-U7386S, Moriguchi, Osaka, Japan).

We performed ICSI if duration of infertility was long, no prior live birth, prior IVF failure, and to minimize possible risk of fertilization failure. Two expert embryologists (blind to the study) assessed the oocyte maturity on the day of oocyte retrieval. MII oocytes without any anomalies were accepted as the best-quality oocytes. Following the microinjection process, cleavage embryos with seven or eight cells on day 3 after OR that contained <20 % anucleate fragments and no apparent morphological abnormalities were classified as good quality. Blastocyst-stage embryos were graded according to Gardner's grading [39]. Embryo transfer (ET) was performed guided under ultrasound, and excess embryos were frozen. The embryos were transferred on day 3 (cleavage stage) or day 5 (blastocyst stage). Only one embryo was transferred due to government policy. β-hCG levels were detected by blood test 12 days after transfer. The term clinical pregnancy referred to a viable intrauterine pregnancy, with fetal cardiac activity after 6 weeks of gestation. Ongoing pregnancy was defined as a vital pregnancy, confirmed by ultrasonography after 12 weeks of gestation.

Main outcome measures

The follicular milieu (FF serum EG-VEGF, inhibin-a, IGF-1 levels), endometrial blood flow (EBF), and IVF outcomes (oocyte/embryo quality and clinical pregnancy rate [CPR]) were compared with regard to PFBF.

Statistical analysis

The Statistical Package for Social Sciences for Windows 18.0 program (SPSS Inc., Chicago, IL, USA) was used. Data were analyzed using descriptive statistical methods (mean, standard deviation, and frequency). All data were evaluated with their 95 % confidence intervals (CIs). The Student's t test, χ^2 test, Mann–Whitney test, and Fisher's exact test were used in the statistical analysis, as appropriate, according to the distribution of the data. The relationships between the data were evaluated using Pearson's correlations. Wilcoxon's signed-ranks test was used for intraobserver reliability. A receiver operating characteristics (ROC) analysis was used to determine the predictive value of IGF-1 levels in CPR.

Results

Forty-four women aged between 23 and 35 (mean age of 29.3 ± 4.3) were enrolled in this study. Of the 44 patients, 4 were excluded from the study due to poor resolution of the Doppler images (perifollicular or subendometrial). The study was completed with 40 patients and 156 follicles. Seventeen (42.5 %) singleton clinical pregnancies occurred. Of these, three (7.5 %) were aborted at 6 to 7 weeks of gestation.

The subjects were categorized into two groups, according to their conceptus/nonconceptus and follicular status (WVFs or PVFs). Sixty-eight of the follicles were graded as WVFs, and 88 were graded as PVFs. Table 1 shows the basal characteristics of the patients. The ages, gravidity, BMI, duration of infertility, AFC, day 3 hormonal evaluations and gonadotrophin doses, and durations of the pregnant and nonpregnant groups were similar ($p < 0.05$). The fertilization rates were significantly higher in the pregnant group ($p < 0.05$).

Table 2 presents the comparison of the WVFs and PVFs. WVFs were associated with significantly increased oocyte quality, embryo quality, and increased CPRs ($p < 0.05$). Despite a high fertilization rate in the WVFs, this increase did not reach statistical significance. FF IGF-1, FF/serum inhibin-a, and serum EG-VEGF levels were similar in the WVF and PVF groups ($p > 0.05$). WVFs had increased FF EG-VEGF levels and WVE compared to the PVFs. The WVFs yielded good-quality oocytes and embryos and increased endometrial vascularization and CPRs ($p < 0.05$).

The endometrial thickness (on hCG day and OR day) was similar in the pregnant and nonpregnant groups ($p > 0.05$). Triple-line endometrium morphology ratio was similar both in the pregnant and nonpregnant groups and WVF and PVF groups ($p > 0.05$). Although the EBF grading was similar on OR days, the vascularity of the endometrium of the pregnant and nonpregnant groups was significantly different on hCG day ($p < 0.05$). The following were significantly increased in women with a WVE compared with those with a PVE: FF IGF-1 levels (54.6 ± 9.0 vs. 45 ± 11.4 $p = 0.001$), ratio of WVFs (56.2 vs. 20.8 % $p = 0.02$), and CPR (68.7 vs. 26.9 %, $p = 0.009$). The FF serum EG-VEGF, FF serum inhibin-a levels, and embryo quality were similar in the WVE and PVE groups ($p > 0.05$).

Table 3 shows the comparison of the pregnant and nonpregnant group. The pregnant group had significantly increased FF IGF-1 ($p = 0.001$) and serum EG-VEGF ($p = 0.03$) compared to the nonpregnant group, in addition to increased percentages of WVFs and WVE. FF EG-VEGF and FF/serum inhibin-a levels were similar between the pregnant and nonpregnant groups ($p > 0.05$). FF IGF-1 was positively correlated with serum EG-VEGF ($r = 0.342$, $p = 0.02$) and WVE on hCG day ($r = 0.351$ $p = 0.02$). There was no significant association between FF IGF-1 and FF EG-VEGF, FF/serum inhibin-a, PFBF grading, and oocyte and embryo quality ($p > 0.05$). The ROC analysis revealed a significant association between FF IGF-1 and CPRs. FF IGF-1 of ≥50 ng/mL (AUC 0.72, $p = 0.01$, CI 0.5–0.8) had 75 % sensitivity and 64 % specificity for predicting CPR.

Table 4 shows the perinatal outcomes of WVF and PVF. The percentages of operative delivery, birth weight, Apgar scores, infant sex, preterm labor, preterm premature rupture of membranes, abortion, gestational diabetes, and preeclampsia were similar in the two groups. All patients underwent cesarean section operation due to obstetric indications. The cesarean section indications were acute fetal distress ($n = 5$), cephalopelvic disproportion ($n = 4$), and arrested labor ($n = 2$).

Discussion

Perifollicular perfusion is important in oocyte competence, embryo viability, and implantation potential [1, 6]. This study was based on the hypothesis that the follicular milieu and endometrial vascularity of WVFs may be better than those of PVFs, thereby yielding good-quality embryos, with implantation potential and increased pregnancy rates. Briefly, this study demonstrated that WVFs yielded high percentages of good-quality embryos, a WVE, high FF EG-VEGF levels, and high CPRs. Pregnant women had an increased percentage of WVFs, a WVE, and elevated FF IGF-1 and serum EG-VEGF levels but similar FF EG-VEGF and FF serum inhibin-a levels.

Table 1 Comparison of the basal characteristics of the pregnant and nonpregnant groups

	Pregnant ($n = 17$)	Nonpregnant ($n = 26$)	P value
Age	29.1 ± 4.6	30.4 ± 4.3	0.381
Duration of infertility (years)	6.4 ± 4.7	7.8 ± 4.5	0.302
Gravidity	0.2 ± 0.5	0.2 ± 0.5	0.799
BMI (kg/m^2)	25.9 ± 5.1	24.5 ± 4.0	0.448
Antral follicle count	8.3 ± 3.5	8.6 ± 3.2	0.840
FSH (mIU/ml)	5.9 ± 2.4	7.0 ± 1.9	0.165
LH (mIU/ml)	3.8 ± 2.9	5.0 ± 2.3	0.196
E_2 (pg/ml)	41.6 ± 35.7	48.9 ± 52.2	0.669
AMH (ng/ml)	2.3 ± 1.8	3.3 ± 3.3	0.456
Prolactin (ng/ml)	10.4 ± 5.8	14.0 ± 8.2	0.193
hCG day E_2 (pg/ml)	1707 ± 123.3	1248 ± 96.0	0.386
No. of oocyte retrieved	7.8 ± 4.0	6.9 ± 4.0	0.479
MII oocytes	5.7 ± 2.9	4.9 ± 3.0	0.371
Fertilization rate	78.9 ± 24	53.8 ± 28.6	0.03
Gonadotrophin dose	2311 ± 1294	2904 ± 1502	0.331
Gonadotrophin days	10 ± 1.7	10.6 ± 1.7	0.378

Poor vascularity of follicles and the endometrium are some of the factors implicated in poor oocyte quality and low pregnancy outcomes [14–20]. A previous study reported that the degree of follicular vascularization was not size-dependent and that follicles with full vascularization were associated with increased pregnancy rates [14]. Since that study [14], many studies have examined PFBF grading [15–20]. Power Doppler studies demonstrated a fivefold increase in CPRs when embryos were obtained from WVFs [17]. In the present study, WVFs were associated with more good-quality oocytes (73.5 vs. 29.5 %) and embryos (70.5 vs. 29.5 %) and higher CPRs (64.3 vs. 30.7 %) than PVFs. Perifollicular vascularization plays a critical role in folliculogenesis, and oxygenation appears to have an essential role in the completion of meiosis, maturity of oocytes, and cleavage of embryos [6, 40]. Research has suggested that diminished PFBF led to intra-follicular hypoxia and follicles with a low oxygen level resulted in greater numbers of chromosomal abnormalities [6]. The intra-follicular oxygen content was shown to be related to oocyte

Table 2 Comparison of well-vascularized follicles (WVFs) and poorly vascularized follicles (PVFs)

	WVF ($n = 68$)	PVF ($n = 88$)	P value
IVF outcome			
Embryo quality	48/68 (70.5 %)	26/88 (29.5 %)	0.002
Oocyte quality	50/68 (73.5 %)	26/88 (29.5 %)	0.001
Fertilization rate	74.9 ± 26.0	59.3 ± 29.9	0.254
CPR	64.3 %	30.7 %	0.04
Endometrium			
WVE	45/68 (66.1 %)	24/88 (27.29 %)	0.001
hCG day endometrial thickness (mm)	9.2 ± 2.1	9.3 ± 2.2	0.962
OR day endometrial thickness (mm)	9.3 ± 1.3	9.9 ± 2.7	0.586
Triple pattern	48/68 (70.5 %)	56/88 (63.6 %)	0.314
Laboratory evaluation			
Follicular fluid			
FF EG VEGF pg/ml	1905 ± 243.8	1586 ± 705.2	0.008
FF inhibin-a pg/ml	728 ± 498.1	738 ± 490.5	0.929
FF IGF-1 ng/ml	50.7 ± 11.9	45 ± 11.4	0.512
Serum			
Serum EG VEGF pg/ml	72.3 ± 38.2	57.3 ± 30	0.063
Serum inhibin-a pg/ml	290 ± 271.2	314 ± 319.6	0.738

Table 3 Comparison of pregnant and nonpregnant cycles

	Pregnant	Nonpregnant	*P* value
Perifollicular and endometrial blood flow			
WVFs	9/17 (52.9 %)	5/23 (21.7 %)	0.044
WVE	11/17 (64.7 %)	5/23 (21.7 %)	0.022
Endometrium			
hCG day endometrial thickness (mm)	9.1 ± 2.2	9.7 ± 2.4	0.466
OR day endometrial thickness (mm)	9.3 ± 1.0	10.0 ± 2.9	0.594
Triple pattern	11/17 (64.7 %)	15/23 (65.2 %)	0.987
Laboratory evaluation			
Follicular fluid			
FF EG VEGF pg/ml	1639 ± 669.6	1795 ± 467.4	0.216
FF inhibin-a pg/ml	781 ± 478.0	440 ± 499.5	0.712
FF IGF-1 ng/ml	73.4 ± 46.4	45.9 ± 11.1	0.001
Serum			
Serum EG VEGF pg/ml	73.4 ± 46.4	54.9 ± 18.2	0.03
Serum inhibin-a pg/ml	330 ± 365.7	321 ± 295.3	0.901

quality and maturity, and follicular vascularity was found to be key factor in the follicular milieu [5, 6, 13].

EG-VEGF, also known as prokineticin [9], is an EG-specific vasoactive substance [10]. The expression of EG-VEGF in ovarian tissue was previously reported to be correlated with VEGF [11, 12]. In this study, follicular fluid EG-VEGF levels increased significantly in the WVFs (1905 ± 243.8 vs. 1586 ± 705.2, $p = 0.008$), but the FF EG-VEGF levels were similar in the conceptus/nonconceptus cycles ($p > 0.05$). A previous study of EG-VEGF levels in follicular fluid and IVF outcomes found a negative correlation between EG-VEGF, VEGF, and oocyte maturity but a positive correlation with embryo quality [11]. The same study reported increased serum EG-VEGF levels in pregnant cases. The present study found no direct correlation between perifollicular grading and the FF EG-VEGF level, but WVFs had higher FF EG-VEGF levels. In common with earlier findings [11], this study observed increased serum EG-VEGF levels in the pregnant group compared to the nonpregnant group (73.4 ± 46.4 vs. 54.9 ± 18.2, $p = 0.03$). Previous studies reported increased expression of EG-VEGF in peri-implantation endometrium and decidua [10, 12]. In this study, serum EG-VEGF levels were measured on the oocyte retrieval day but not on the hCG day or in the midluteal phase. Therefore, future studies with serial measurements of serum EG-VEGF levels throughout the cycle are needed.

Table 4 Comparison of perinatal outcomes of pregnancies originating from well-vascularized follicles (WVFs) and poorly vascularized follicles (PVFs)

	WVF ($n = 9$)	PVF ($n = 8$)	*P* values
Take home baby	7	7	0.599
Abortion	2	1	0.599
Preterm labor	3	2	0.598
Gestational diabetes	3	1	0.312
Preeclampsia	0	1	0.274
PPROM	2	0	0.155
Birth weeks	36.0 ± 3.2	36.4 ± 3.4	0.698
Apgar 1	7.2 ± 1.2	7.1 ± 1.6	0.860
Apgar 2	8.5 ± 0.9	8.4 ± 1.2	0.818
Birth weight	3015 ± 727	2904 ± 759	0.785
Newborn sex (male/female)	3/4	3/4	1.00
Cesarean operation	5/7	6/7	0.821

Several studies have investigated the role of the follicular fluid microenvironment in oocyte quality and IVF outcomes [5]. Previous research reported that the addition of IGF-1 to in vitro culture medium prevented apoptosis [26]. Most studies have employed in vitro cell culture techniques, with exogenous supplementation of IGF [6, 27]. The role of FF IGF-1 in CPRs and embryo quality is not well-established, with conflicting reports [27–33]. Some studies reported increased FF IGF-1 levels in conceptus cycles [27, 31]. In the present study, FF IGF-1 was not significantly associated with and FF EG-VEGF, FF serum inhibin-a, or PFBF grading ($p > 0.05$). Although the levels of FF IGF-1 were significantly increased in women with a WVE, the levels were similar in WVFs and PVFs ($p > 0.05$). Serum FF IGF-1 levels were positively correlated with serum EG-VEGF levels ($r = 0.342$, $p = 0.02$) and a WVE ($r = 0.351$, $p = 0.02$). In a previous study, IGF-1 mediated the influence of the endometrial milieu on embryos [33]. Further studies are needed to clarify the relationship between serum FF IGF-1 levels and endometrial vascularity. A cut-off level of 58 ng/mL for FF IGF-1 was reported to predict

pregnancy [27]. In the current study, FF IGF-1 above 50 ng/mL (AUC 0.72) had 75 % sensitivity and 64 % specificity for predicting the CPR. These results suggested that FF IGF-1 may be a biochemical marker of pregnancy.

A number of studies have examined FF inhibin-a levels and IVF outcomes [34–37]. However, there are no prior studies of the association of FF/serum inhibin-a with PFBF and EG-VEGF levels. Other than a study by Öcal et al. [37], the majority of studies of FF IGF-1 found that follicular fluid inhibin-a levels were not associated with embryo quality and pregnancy rates [34–36]. In the present study, the serum FF IGF-1 and serum inhibin-a levels of well-perfused and poorly perfused follicles were similar. Furthermore, FF serum inhibin-a on OPU day was not associated with PFBF, endometrial vascularization, EG-VEGF, and IGF-1 levels. These results, which support the findings of previous studies [5], suggest that FF or serum inhibin-a levels on OR day reflect granulosa cell function rather than embryo quality or pregnancy.

Successful implantation and placentation depend on angiogenesis. Previous studies found that the absence of subendometrial and intraendometrial vascularity was associated with nonpregnant cycles and suggested a grading system for endometrial vascularity [38, 41]. There have been many studies of the endometrial vasculature [20–24, 41–43]. Some of these found no relation with endometrial vascularity and pregnancy [23, 42], whereas others found a relation with clinical pregnancy [20, 21, 24, 38, 42]. In this study, we measured the vascularity of the endometrium on the hCG day and OR days. The results showed that endometrial vascularization was similar on the oocyte retrieval day in pregnant and nonpregnant women. In addition to vascularization, we compared the endometrial morphology (triple line) and thickness in conceptus/nonconceptus cycles. Similar to prior studies, neither morphology nor endometrial thickness changed in conceptus/nonconceptus or WVF/PVF cycles [25]. However, both the endometrial vascularity (hCG day) and percentage of WVE increased significantly in the pregnant group.

There is no consensus on the optimum timing of Doppler imaging during IVF procedures. Some studies performed Doppler imaging on the oocyte retrieval day or embryo transfer day [21, 24, 42], whereas others, including the current study, performed it on the hCG day [20, 38, 43]. In this study, as shown by power Doppler imaging, the following increased significantly in women with a WVE compared to those with PWE: FF IGF-1 levels (54.6 ± 9.0 vs. 45 ± 11.4, $p = 0.001$), ratio of WVFs (56.2 vs. 20.8 %, $p = 0.02$), and CPRs (68.7 vs. 26.9 %, $p = 0.009$). Patients with WVE on the hCG day had increased ratio of WVFs, FF IGF-1 levels, and CPRs.

Follicular fluid is a dynamic milieu containing a variety of cytokines and hormones. The major obstacle in follicular fluid studies is monofollicular design [6]. In this study, the FF of each individual follicle was assessed using power Doppler studies. This is the first study to simultaneously examine PFBF and EBF, together with serum/FF EG-VEGF, IGF-1, and inhibin-a levels. The findings of the present study support the hypothesis that perifollicular vascularization has an influence on the follicular milieu, embryo competence, endometrial vascularization, and CPRs. The perinatal outcomes of embryos originating from WVFs were similar to those from PVFs. However, the study sample was small, and further large-scale studies of PFBF and implantation and pregnancy complications are needed. EG-VEGF seemed to be related to follicular perfusion and serum levels in pregnancy, but these preliminary data should be confirmed in other studies.

In conclusion, well-vascularized follicles are associated with increased FF EG-VEGF, good-quality oocytes and embryos, a well-vascularized endometrium, and increased pregnancy rates. Power Doppler assessments of PFBF and EBF should be an integral part of all IVF/ICSI procedures. Perifollicular blood flow and follicular fluid IGF-1 and serum EG-VEGF might be independent markers for pregnancy outcomes.

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