



Middle East Fertility Society
Middle East Fertility Society Journal

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ORIGINAL ARTICLE

Comparative evaluation of vascular endothelial growth factor-A expression in pre-ovulatory follicular fluid in normogonadotrophic and endometriotic patients undergoing assisted reproductive techniques

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Received 16 May 2013; accepted 3 June 2013

KEYWORDS

Vascular endothelial growth factor A;
Endometriosis;
Infertility;
ICSI

Abstract Objective: The aim of this study is to compare the differential expression of VEGF-A in pre-ovulatory follicular fluid of both normogonadotrophic and endometriotic patients undergoing ICSI and its relation to ICSI outcome.

Setting: Agial IVF – ICSI center, Alexandria, Egypt.

Methodology: The study was a prospective randomized control trial that included 90 infertile patients who were subdivided into two groups:

Group A: 45 normogonadotrophic patients as patients having tubal factor and unexplained infertility.

Group B: 45 endometriotic patients diagnosed by laparoscopy.

All patients underwent the same ovarian hyperstimulation protocol (the long luteal phase protocol). In all patients the blood was sampled on the day of HCG administration. The isolated sera were frozen and stored at -20°C for later hormone analysis (assay of estradiol and progesterone). The follicular fluid was collected on the day of oocyte retrieval from all the patients. Only aspirates from follicles larger than 17 mm, uncontaminated with blood were included in this study. Follicular

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Peer review under responsibility of Middle East Fertility Society.



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fluid samples were centrifuged for 10 min, and the supernatants were stored at -20°C until hormone measurements were carried out (assay of estradiol, progesterone and androstenedione). VEGF-A assay was performed using the ELISA technique for all the follicular fluid samples and the results were correlated to the ICSI outcome in both groups.

Outcome measures: The primary outcome measures were: (a) The number of mature oocytes per cycle of induction, (b) the grading of embryos obtained, (c) implantation rate, (d) serum level of estradiol and progesterone on the day of HCG administration and (e) follicular fluid level of estradiol, progesterone and androstenedione on the day of oocyte retrieval.

The secondary outcome measure was pregnancy rate which was diagnosed by:

1. Serum B-hCG assay 14 days after embryo transfer.
2. Clinical pregnancy rate.

Results: There was a statistically significant difference between the two groups when comparing the mean value of the follicular fluid VEGF-A concentrations. In group A, the mean value was 529.4 ± 309.7 (pg/ml), while in group B, it was 1388.7 ± 1152 (pg/ml) (p value = 0.0001). There was no statistical significant difference between both groups as regards the outcome of pregnancy. When correlating between ICSI outcome and different studied parameters a positive correlation was detected between the ICSI outcome and endometrial thickness. No correlation could be detected between the ICSI outcome and other studied parameters. No correlations could be detected between VEGF-A expression and different studied laboratory parameters. However, A positive correlation was detected between VEGF-A and ICSI outcomes in both groups, it means that increasing the level of VEGF-A is accompanied with a decrease in the pregnancy rate ($p = 0.003$), a negative correlation was detected between VEGF-A and endometrial thickness, a positive correlation was detected between VEGF-A and metaphase I oocytes, otherwise no other correlations could be detected.

Conclusion: The current study concluded that follicular fluid VEGF-A is correlated negatively with the ICSI outcome.

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1. Introduction

Endometriosis is a steroid hormone-dependant disease and is one of the most common benign gynecological disorders with a frequency varying from 2% to 10% in women with reproductive age and up to 30% in women with sub-fertility. Endometriosis may more likely develop in women who fail to conceive for other reasons and thus be a secondary phenomenon, so it is preferable to call it endometriosis-associated infertility. Endometriosis can affect women from pre-menarche to post-menopause, regardless of race or whether or not the woman has had children. It is more common in women with infertility (20–50%) and women with chronic pelvic pain (about 80%). Although endometriosis growth can be felt during pelvic exam and its symptoms are suggesting, the diagnosis cannot be confirmed without laparoscopy (1).

Similar to tumor metastasis, endometriotic implants require neovascularization to proliferate, invade the extracellular matrix (ECM) and establish an endometriotic lesion. Angiogenesis is an important event for endometrial growth and tissue repair (2). Angiogenesis is the formation of new capillaries from pre-existing vessels, whereas vasculogenesis is the de novo capillary formation from endothelial precursor cells (EPCs) (3).

Angiogenesis is a key process in many aspects of reproduction from the development of reproductive system where angiogenesis plays a pivotal role in the formation of healthy gonads (4), follicular progenesis and ovulation (5), corpus luteum development (6), endometrial proliferation (7), oocyte implantation, placentation (8), organogenesis and develop-

ment (9). Also it is necessary for the secondary reproductive features such as mammary gland development (10).

The regulation of angiogenesis seems to be dependent on the interaction among many growth factors that can act in different moments, some of them stimulating growth, while others mediating endothelial cell reorganization in more complex vascular structure (11). It has been suggested that these growth factors cooperate to coordinate re-inervation and vascularization in diseases such as cancers that involve new tissue growth (12).

Several pro-angiogenic factors are well-known such as fibroblast growth factor-2 (FGF-2), angiotensin-11 (ANG-11), insulin like growth factor-1(IGF-1), epidermal growth factor (EGF), angiopoitin (NAPT), endothelin-1(ET-1) and vascular endothelial growth factor (VEGF). The most important of them in angiogenesis are FGF-2, ANG-11 and VEGF (13). Abnormal angiogenesis may contribute to several endometrial-related pathologies such as menorrhagia, endometrial cancer and endometriosis (14).

The effect of angiogenesis inhibition was initially studied by using a model in which human endometrium was implanted into nude mice (15). Immediately after implantation of cultured human endometrium fragments, two VEGF inhibitors were administered, a truncated soluble inhibitory receptor and an affinity purified anti-body to human VEGF. Both the angiostatic agents were effective in preventing blood vessels' growth and development of the endometriotic explants in this animal model.

It was subsequently demonstrated that the transplantation of human endometrium onto the chicken chorioallantoic membrane leads to a strong angiogenic and to the formation of

endometriosis-like lesions (16). Administration of angiostatic agents e.g. anti-human VEGF antibody, significantly inhibited this angiogenic response and reduced the formation of endometriosis-like lesions.

In animal models anti-VEGF compounds inhibit ectopic endometrial growth (17). Some investigators suggest that angiogenesis in women with endometriosis may be related to pelvic pain (18). The administration of anti-angiogenic drugs has been proved to reduce the establishment, maintenance and progression of endometriotic lesions in different laboratories and animals. However further investigations are required before clinical trials can be planned in humans (19).

Angiogenesis and vascularization of endometriotic lesions are significantly down regulated by simultaneous inhibition by VEGF, fibroblast growth factor and platelet derived growth factor but they are not reduced by antagonizing VEGF alone.

1.1. VEGF-A role in endometriosis

Endometrium undergoes cyclical growth and regression during the menstrual cycle, which depends on the ovarian steroid levels. It is therefore a rich source of angiogenic growth factors. A variety of parameters including oxygen tension, aging and endocrinal or local factors can modulate the expression of angiogenic factors. A decline in local oxygen concentration (hypoxia) is a primary initiator of angiogenesis in normal and pathological tissues (20).

VEGF is an important signaling protein involved in both vasculogenesis and angiogenesis. The normal function of VEGF is to create new blood vessels during embryonic development, new blood vessels' formation after injury as in muscles after exercise, and new blood vessels (collateral circulation) to bypass blocked vessels. Over expression of VEGF can contribute diseases. It is a key mediator of neovascularization in cases of cancer and many other diseases.

VEGF is composed of at least six members (VEGF-A, B, C, D, E, and F), in which VEGF-A is the first member that has been discovered and was just known as VEGF. VEGF-A which is also called the vascular permeability factor, has emerged as the single most important regulator of blood vessels' formation in health and in disease. All members of VEGF family stimulate cellular response by binding to tyrosine kinase receptors (VEGFRs) on the cell surface, causing them to dimerize and become activated through transphosphorylation. The VEGF receptors have an extra cellular portion consisting of seven immunoglobulin-like domains, a single transmembrane spanning region and an intracellular portion containing a split tyrosine-kinase domain. There are two known VEGF-A receptors: VEGFR-1 (Flk-1), and VEGFR-2 (KDR/Flk-1). VEGFR-2 appears to mediate almost all of the known cellular response of VEGF (21) and it is expressed specially in angiogenic endothelial cells and regulates the effects of VEGF-A on the proliferation and migration of these cells (22).

The function of VEGF-1 is less well defined although it is thought to modulate VEGFR-2 binding (which appears to be particularly important during vasculogenesis in the embryo (23,24) VEGFR-1 is expressed in quiescent and proliferative endothelial cells (25) and induced the formation of vessels by VEGF-A. A third receptor has been discovered VEGFR-3, however VEGF-A is not a ligand for this receptor, as VEGFR-3 mediates lymphoangiogenesis in response to VEGF-C and VEGF-D (26).

VEGF-A has become a center of interest due to its essential role in vasculogenesis and angiogenesis (27) in a variety of physiological and pathological processes such as: wound healing, rheumatoid arthritis, cardiac ischemia, tumors, pre-eclampsia and the female reproductive system (28). VEGF-A induces endothelial cell proliferation, migration, differentiation and capillary formation (29). Several studies have reported an increase in VEGF-A levels in endometriosis and it has been suggested that VEGF-A plays a role in the progression of the disease (30). So VEGF-A is considered as a key regulator of angiogenesis which is thought to be involved in the pathogenesis of endometriosis (31).

The earliest evidence of VEGF expression is correlated to blood vessels' growth came from a study published in 1990, showing that VEGF mRNA was expressed at low levels in the avascular granulosa cells in the ovary, where it was up-regulated in the highly vascularized corpus luteum (32). Furthermore the high affinity binding site of VEGF was selectively expressed in endothelial cells in vivo (33). In 1992 it was reported that VEGF mRNA is strongly expressed by the highly vascularized glioblastoma multiform in situ (34,35).

Human VEGF-A gene is organized in eight exons separated by seven introns, and spans approx. 14 Kb (36). VEGF-A covers two families' result from alternate splicing of mRNA from a single exon (exon8) VEGF gene. The two families are referred according to their terminal exon splice sites, the proximal site is denoted as VEGF_{xxx} and the distal splice site is denoted as VEGF_{xxx}b. In addition alternate splicing of exons 6 and 7 alters their heparin-binding affinity and amino acid number which will give human isoforms of at least twelve isoforms of VEGF-A: VEGF 111, VEGF 121, VEGF 121 b, VEGF 145, VEGF 148, VEGF 162, VEGF 165, VEGF 165 b, VEGF 183, VEGF 189, VEGF 189 b, VEGFVEGF 206.(37)

The most widely studied isoform is VEGF 165b (38), but VEGF 121 b (39) and VEGF 189 b (40) have also been identified at the mRNA and protein level (41). VEGF 165 b mRNA was first isolated at 2002 by RT (reverse transcription) PCR of tissue of renal cortex (42). The two families are formed by alternative 3 splice site selecting in the terminal exon (exon 8) (43) to give two different C-terminal sequences. These two families are termed VEGF _{xxx} (pro-angiogenesis) and VEGF _{xxx}b (anti-angiogenic), where xxx denotes the amino acid number. These domains have important functional consequences for the VEGF splice variants, as terminal (exon 8) splice site determines whether the proteins are pro-angiogenic (proximal splice site, expressed during angiogenesis) or anti-angiogenic (distal splice site, expressed in normal tissues). An inclusion or exclusion of exons 6 and 7 mediates interactions with heparin sulfate proteoglycans (HSPGs) and neuropilin co-receptors on the cell surface, enhancing their ability to bind and activate the VEGF receptors (VEGFRs). A distal splicing event results in an open reading form of the same number of nucleotides as the proximally spliced (pro-angiogenic isoforms), however the translated amino acid sequence is different, which relies on implications for the biological properties of the protein.

Discovery of VEGF165b reveals these two families with VEGF _{xxx} being angiogenic and permeability-inducing, while VEGF _{xxx}b is antigenic and its role in permeability regulation is a complex one. VEGF _{xxx}b is also involved in physiological angiogenesis such as mammary development during pregnancy and lactation and fertility control. VEGF 165b is expressed in

many tissues and its expression levels are tightly controlled, as: in the ovary, it is expressed only in the theca externa, but not in the theca interna.

Recently it is reported that over expression of VEGF 165b in mouse ovary results in reduced litter size defects in follicular development and lack of cumulus oocyte complex formation (44,45). Over expression of VEGF165b in areas where angiogenesis is required therefore appears to be detrimental.

However in normal pregnancy circulating VEGF 165b levels rise rapidly after fertilization, with most patients having levels four times more than in the non pregnant levels within the first few weeks of pregnancy (46). The switch from anti to pro angiogenic isoforms of VEGF-A in colonic tumors (47), and proliferative diabetic retinopathy (48,49), suggested that the regulation of the ratio pro- to anti- angiogenic isoforms of VEGF-A by alternating splicing is potentially therapeutic for the angiogenesis associated diseases.

There is a switch between anti-angiogenic VEGF-A isoforms in a variety of disease states (50), little is known about the molecular and cellular pathways that regulate alternative splicing of VEGF-A pre-mRNA in general and of the exon 8a/8b alternative splice site in particular.

Therapies that inhibit angiogenesis have been approved after clinical trials in cancer and age-related macular degeneration (AMD) (51). However the concept of alternate splicing of the VEGF-A gene resulting in anti-angiogenic as well as pro-angiogenic isoforms indicates that it might be insufficient to simply measure the quantitative changes of the VEGF-A molecule in cancer and other diseases, a quantitative characterizations of the balance of each isoform family might be essential to define the optimal therapeutic option and dosing (52).

In 2008 Bergers and Hanahan concluded that anti-VEGF drugs can show therapeutic efficacy in mouse models of cancer and in increasing numbers of human cancers. But the benefit was transitory and was followed by a restoration of tumor growth and progression (53). VEGF is inhibited by thiazolidinediones and this effect on granulosa cells gives the potentials of thiazolidinediones to be used in ovarian hyperstimulation syndrome (54).

In adult tissues, capillary growth rarely occurs and the vascular endothelium represents a stable population of cells with a low mitogenic rate (55). The only exception is the rapid growth and regression that occurs in the female genital organs, associated with equivalent changes in its vascular network (56). The mature ovary shows a highly developed vasculature reflecting its high metabolic rate, which turns this organ into a unique model for studies of angiogenesis regulation during growth, differentiation and regression in adults (57).

1.2. The role of VEGF-A expression in folliculogenesis

The ovarian follicle is the structural and functional unit of the mammalian ovary, which supplies the necessary environment for oocyte growth and maturation (58). It is known that vasculature is not equally distributed among the population of follicles of the adult ovary, since only, theca cell layers present in later follicular stages, have vessels. Quiescent primordial follicles and slow growing preantral follicles do not have a vascular supply of their own, but instead they rely on vessels in surrounding stroma. In 2009 Martali showed that an autonomous vascular supply starts to be evident in preantral follicles with diameter from 110 μm . As a follicular antrum develops the

theca layer acquires a vascular sheath consisting of two capillary networks located in the theca interna and externa, respectively (59).

In the ovary pro-angiogenic factors promote vascular permeability, supporting antral formation and the events that induce follicular rupture (60). Follicular growth during the estrous cycle in cattle is characterized by, two to three follicular waves (61). At each follicular wave a cohort of follicles is initiated to grow, but only the dominant follicles continue to grow and have a great capacity to produce estradiol-17 B estrogen (62). Maintenance of follicular health depends on the presence of angiogenesis and functional vascularization (63). It has been demonstrated that angiogenesis and the development of vascularity may influence the maturation of the pre-ovulatory follicle and the selection of a dominant ovulatory follicle (64).

VEGF-A expression was demonstrated in preantral follicles. The protein VEGF-A has been identified in oocytes of human primordial follicles (65) and human and rat primary follicles. In bovine follicles VEGF-A is weakly expressed during early development and this expression becomes higher in granulosa and theca cells of dominant follicles (66).

Expression of mRNA of VEGF-A in the granulosa and theca as well as the protein of VEGF-A in all follicle compartments and follicular fluid significantly increases according to the stage of follicular development (67,68). In rats VEGF-A expression was occasionally in the early preantral follicles during the late stages of development (69,70).

Kezele et al. identified that the gene encoding for VEGF-A is an important regulator for primordial follicle development in rat. Danaforth et al. showed that VEGF-A increases the number of primary and secondary follicles in rat ovaries. Another study verified that VEGF-A promoted the transition from primary to secondary follicles in bovine (71). It showed that endogenous VEGF-A is essential to rodent primordial follicle survival (72).

The inhibition of VEGF-A activity produces an increase in ovarian apoptosis through an unbalance in the pro- and anti-apoptotic protein rates leading to a great number of atretic follicles (73). Other authors observed that the direct injection of VEGF-A into the ovary increases vasculature, (74) and the number of antral follicles, and inhibit apoptosis (75).

VEGF-A is thought to be important in the development of the oocyte due to increased angiogenesis suggesting that patient with severe endometriosis experienced compromised oocyte maturation (76). This may also explain the observed decrease in ovarian reserve in patients with moderate to severe endometriosis (77), although other causes also may be operational including the oocyte toxicity by peritoneal fluid components. Harlow et al. also demonstrated a decreased level of granulosa cell steroidogenesis in patients with endometriosis (78), presumably contributing to decreased oocyte maturation and therefore ovarian reserve.

It has been shown that angiogenesis is followed by a vasodilatation, a functional adaptation for the occurrence of ovulation as well as by the development of the theca endocrine function. There are evidence that the theca cell angiogenesis has a primary role in follicular development.

As the corpus luteum formation begins, theca capillary sprouts begin to migrate toward and grow into folds of the stratum granulosum. The growth of new capillaries during luteal angiogenesis follows a cascade of events including changes

in basement membrane, migration and proliferation of endothelial cells and development of capillary lumina (79).

Increased vascularity in the EA follicles results in a greater vascular surface area for the exchange of transcapillary nutrients, gonadotropins, growth factors and other associated factors. Establishment of a vascular network around the developing follicles may be rate-timing step in the process of selecting the dominant follicle. An insufficient blood supply could act as the trigger that leads to follicular atresia (80).

Alternative production of angiogenic factors by follicular cells may stimulate vascular development in the dominant follicle (81).

A greater estrogen concentration in the follicular fluid of EA follicles compared with that in EI follicles indicates that EA follicles have a greater ability to produce estrogen during a period of relatively low serum gonadotropin concentrations. Estradiol-17B is recognized as the follicular growth, differentiation and survival factor which enhances aromatase activity, promotes expression of LH receptors and increases sensitivity of granulosa cells to FH and LH (82). The decrease in estrogen production observed during atresia of the dominant follicle was the direct result of decreased activity of the aromatase enzyme within granulosa cells (83).

VEGF-A has been demonstrated to regulate follicular growth directly, as the inhibition of VEGF-A during the follicular phase interrupt pre-ovulatory follicular development in several species.

VEGF -A concentration in follicular fluid (FF) increases significantly reaching high levels in bovine pre-ovulatory follicles suggesting that VEGF-A is a major angiogenic factor that is involved in regulation of the proliferation of capillaries accompanying the selection of the pre-ovulatory follicle supporting the growth of the dominant follicle (84).

Enhanced expression of VEGF-A with EA follicles was associated with enhanced estrogen concentration in the follicular fluid, a positive correlation between VEGF and estrogen in pigs was demonstrated (85). It was supported that follicular VEGF-A plays a role in vascularization during follicular deviation. It is postulated that VEGF-A interacts with several extra and intra follicular factors during the process of follicular selection to regulate vascular development and permeability and delivery of regulatory factors and nutrients to potentially dominant follicle.

VEG-A has a potentially important role affecting peri follicular angiogenesis and regulating intra follicular oxygen levels. Follicular VEGF-A levels are significantly correlated with the grade of peri follicular vascularity (86).

VEGF-A concentration in follicular fluid is positively correlated with gonadotropin concentrations, oocyte maturation and high fertilization rates in most studies (87). But in previous studies it was observed that the ability of follicular fluid to induce endothelial proliferation exceeded that of an equimolar concentration of purified VEGF and other angiogenic factors (88).

A prospective study reported that VEGF-A concentration in follicular fluid was significantly higher when the oocyte failed to fertilize (89), furthermore higher follicular fluid VEGF-A levels have also been associated with poor embryo morphology (90), and poor conception rate in IVF (91).

Follicular fluid is a product of both, the transfer of blood plasma constitutions that cross the blood follicular barrier and of the secretion activity of granulosa and thaca cells.

Follicular fluid provides a very important microenvironment for the development of oocytes. It is reasonable to think that some biochemical characteristics of the follicular fluid surrounding the oocyte may play a critical role in determining the oocyte quality and the subsequent potential to achieve fertilization and embryo development. The analysis of the follicular fluid components may also provide information on the metabolic changes in the blood serum, as the circulating biochemical milieu may be reflected in the composition of the follicular fluid (92).

1.3. Objective

The aim of this study is to compare the differential expression of VEGF-A in preovulatory follicular fluid of both normogonadotrophic and endometriotic patients undergoing ICSI and its relation to ICSI outcome.

2. Materials and methods

2.1. Patients

The study was conducted on ninety female patients recruited from the infertility clinic in Agial IVF/ICSI center.

- (1) Inclusion criteria: Age group between 20–and 40 years, normogonadotrophic females (tubal, male, unexplained infertility), and patients suffering from endometriosis.
- (2) Exclusion criteria: Hypogonadotrophic patients (class I ovulatory dysfunctions), hypergonadotrophic patients (class III ovulatory dysfunction) and uterine factor infertility.

The follicular fluid was collected on the day of oocyte retrieval and the serum was collected on the day of HCG administration from 90 patients who underwent ICSI. The patients were subdivided into two groups: Group A: 45 patients with normogonadotrophine as patients having tubal factor infertility, male factor infertility, and unexplained infertility, Group B: 45 patients with endometriosis.

After approval of the medical ethics committee and signing a written informed consent all patients were subjected to: full history taking, complete general examination, complete gynecologic examination and infertility workup including:

Husband's semen analysis, hystrosalpingography and trans-vaginal ultrasound:

1. Detection of antral follicle number and size in the early follicular phase (day 3).
2. Size, direction of the uterus as well as the thickness and pattern of the endometrium.
3. Serial folliculometry.
4. Hormonal profile in the early follicular phase (day 3) serum E2, FSH.

All patients underwent the same ovarian stimulation protocol (the long luteal phase protocol). The ovarian stimulation: the long down-regulation protocol (Suprefact[®], Hoechst) was used in all patients as a daily subcutaneous dose of 0.5 mg started on cycle day 21. Once a serum estradiol concentration was suppressed to ≤ 50 pg/ml, the dose was reduced to 0.2 mg

and continued until the day of HCG administration. Ovarian stimulation with recombinant FSH (Gonal F[®], Serono) 150 IU as well as urinary human menopausal gonadotrophin (u-HMG) (Merional[®], IBSA) 75 IU will begin following pituitary down-regulation. The standard initial dose will be 225 IU.

The ovarian response was monitored by serial serum estradiol concentrations and trans-vaginal ultrasound beginning on day 5 of stimulation until the day of HCG administration. Based on these results, the FSH/HMG dose and subsequent monitoring were individualized. Ovarian stimulation was continued until at least three follicles reached a mean diameter of ≥ 20 mm, at which time HCG (Choriomon[®], IBSA) 10,000 IU s.c. or i.m. was administered 36 h before oocyte recovery. Following oocyte retrieval, the patients received luteal phase support in the form of natural progesterone vaginally in a dose of 600 mg/day i.e.; two vaginal capsules taken three times daily (Prontogest Supp[®], Marcyl) to continue preparing the endometrium. After oocyte retrieval, the cumulus and corona radiata were removed mechanically under a stereomicroscope, after exposure to 80 IU/ml hyaluronidase solution for 30 s. ICSI was used to fertilize mature oocytes, this was followed by transfer of embryos in the appropriate time (Westregard et al., 2001).

Assessment of fertilization and cleavage

Oocytes were examined for fertilization 16–18 h after ICSI and cleavage of the oocytes was assessed on day 2 (48 h) and day 3 (72 h) before transfer into uterus.

2.2. The embryos will be graded on a scale of 1 to 4

2.2.1. Grade 1

The best embryos, containing even-sized, symmetrical blastomeres with no cytoplasmic fragmentation.

2.2.2. Grade 2

Even-sized, symmetrical blastomeres with < 10% cytoplasmic fragmentation.

2.2.3. Grade 3

Uneven-sized blastomeres with 10–15% cytoplasmic fragmentation.

2.2.4. Grade 4

Uneven-sized blastomeres with > 50% cytoplasmic fragmentation.

B-hCG was measured for the diagnosis of pregnancy 14 days after embryo transfer and then was measured serially to monitor the rise in its titer. Implantation was noted later by appearance of the gestational sac in the uterus using trans-vaginal ultrasonography (TVS).

2.2.5. Blood sampling

In all patients the blood was sampled on the day of HCG administration. The isolated sera were frozen and stored at -20°C for later hormone analysis (estradiol and progesterone).

2.2.6. Follicular fluid sampling

Oocyte retrieval was taken under vaginal ultrasound guidance. Only aspirates from follicles larger than 17 mm, uncontami-

nated with the blood were included in this study. Follicular fluid samples were centrifuged for 10 min, and the supernatants were stored at -20°C until hormone measurements were carried out (estradiol, progesterone and androstenedione) using commercially available kits. VEGF-A assay was performed using the ELISA technique for all the follicular fluid samples and the results were correlated to the ICSI outcome in both groups.

The primary outcome measures were: (a) The number of mature oocytes per cycle of induction, (b) the grading of embryos obtained, (c) implantation rate (number of sacs per number of embryo transfer), (d) serum of estradiol and progesterone level on the day of HCG administration and (e) follicular fluid level of estradiol, progesterone and androstenedione on the day of oocyte retrieval.

The secondary outcome measure was pregnancy rate which was diagnosed by:

1. Serum B-hCG assay 14 days after embryo transfer.
2. Clinical pregnancy was confirmed by observing fetal cardiac pulsations 2 weeks after positive pregnancy test by TVS.

In these cases, the administration of progesterone was continued up to week 12 of gestation.

2.3. Statistical analysis

The data were analyzed statistically using SPSS-17 (Statistical Package for Social Science “version 17” Chicago, USA). Means and standard deviation were used to describe data which were normally distributed, and Student’s *t*-test was used to detect the presence of significance between two groups. Pearson Correlation test was used to test linear relationship between two quantitative variables (group A and group B),

In all the statistical tests used, we used level of significance at 5%, below which the results are considered to be statistically significant.

3. Results

The different data of the patients were collected, analyzed and tabulated. Table 1 shows the differences between the two groups regarding the clinical and laboratory data. There was a statistically significant difference between the two groups only in embryos of class A4. There was also a statistically significant difference between the two groups when comparing the mean value of the follicular fluid VEGF-A concentrations. In group A, it ranged from 80 to 1449 (pg/ml) with a mean 529.4 ± 309.7 , while in group B, it ranged from 116 to 4400 (pg/ml) with a mean 1388.7 ± 1152 (*p* value = 0.0001).

There was no statistical significant difference between both groups as regards the outcome of pregnancy. Out of 45 cases in group A 18 cases (40%) were pregnant and 27 cases (60%) were not pregnant, however in group B out of 45 cases 19 cases (42.2%) were pregnant, and 26 cases (57.8%) were not pregnant. (Table 2)

When comparing the mean of different parameters between pregnant and non pregnant females in group A, there was no statistically significant difference between pregnant and non pregnant females as regards their age, the antral follicles,

Table 1 Differences between the two groups regarding the clinical and laboratory data.

	Group A	Group B	<i>t</i>	<i>p</i> -Value
Antral follicle number	3–25 12.5 ± 4.8	6–22 13.7 ± 3.4	1.2	0.22
Endometrial thickness	8–15 10.9 ± 1.56	8.3–15 11.4 ± 1.8	1.34	0.18
Oocyte number	2–27 11.75 ± 5.5	2–21 10.5 ± 4.4	1.08	0.28
M2	1–21 10.4 ± 4.9	1–18 7.6 ± 4.4	2.59	0.011*
M1	0–4 0.6 ± 0.9	0–3 1 ± 0.98	1.7	0.083
GV (germinal vesicles)	0–5 0.61 ± 1.03	0–5 0.7 ± 1.1	0.35	0.725
Class A	1–17 6.3 ± 4.35	1–20 6.1 ± 4.2	0.158	0.87
Class 4A	1–11 4.3 ± 3.6	1–11 1.6 ± 1.9	3.8	0.0001*
Number of ampoules				
Range	19–101	22–98		
Mean ± S.D.	53.7 ± 25.6	51 ± 21.5	0.45	0.65
Stimulation days	8–16 13 ± 1.5	8–15 12.9 ± 1.3	0.25	0.8
VEGF-A (pg/ml)	80–1449 529.4 ± 309.7	116–4400 1388.7 ± 1152.7	–4.38	0.0001*

Table 2 Pregnancy outcome.

	Group		Total	χ^2	<i>p</i>
	A	B			
<i>Pregnant</i>					
Count	18	19	37	0.046	0.83
% within group	40.0%	42.2%	41.1%		
<i>Non pregnant</i>					
Count	27	26	53		
% within group	60.0%	57.8%	58.9%		

endometrial thickness, parameters of oocyte maturity, embryo grading and VEGF as shown in Table 3.

When correlating between ICSI outcome and different studied parameters (Table 4), a positive correlation was detected between the ICSI outcome and endometrial thickness, it means that increasing the endometrial thickness was accompanied with an increase in the occurrence of pregnancy (Fig. 1). No correlation could be detected between the ICSI outcome and other studied parameters.

No correlations could be detected between VEGF-A expression and different studied laboratory parameters as shown in Table 5. However, A positive correlation was detected between VEGF-A and ICSI outcome in both groups (Fig. 2), it means that increasing the level of VEGF-A is accompanied with a decrease in the pregnancy rate ($p = 0.003^*$), a negative correlation was detected between VEGF-A and endometrial thickness (Fig. 3), a positive correlation was detected between VEGF-A and metaphase I oocytes, otherwise no other correlations could be detected.

4. Discussion

Despite much research into endometriosis some aspects of this disease remain controversial. The subfertility related to

endometriosis without any mechanical obstruction in mild to moderate disease has been found to be interesting. Subfertility can be caused by various reasons such as changed folliculogenesis (93,94), ovulatory dysfunction, hyperprolactinemia, luteal phase defect, inappropriate fertilization, early embryo development disorder (95), improper implantation and impaired micro follicular environment (96,97).

The key point in folliculogenesis and ovarian quality is ovarian angiogenesis. Vascular endothelial growth factor (VEGF-A) and interleukin (IL)-1 β are the mediators responsible for angiogenesis (98). VEGF-A is an endothelial cell-specific angiogenic protein that appears to play an important role in a variety of estrogen target tissues in regulating endometrial angiogenesis at a local level (99). VEGF-A expression was observed in serum, peritoneal fluid and endometrial cell cultures in women with endometriosis (100–102). A large number of studies have been investigated and observed that VEGF-A messenger ribonucleic acid and protein were significantly higher in women with endometriosis, which supported a key role for VEGF-A in the pathological angiogenesis in endometriosis (103–109). VEGF may have a pivotal role in the development and progression of endometriosis because: (a) it is expressed in human uterine epithelial and stromal cells and regulated by estrogen (110,111); (b) it is localized in the epithelium of endometriotic implants, particularly in red lesions (112,113); and (c) VEGF levels are elevated in the peritoneal fluid and serum of endometriosis patients (114–116). VEGF has even been proposed as a diagnostic and therapeutic target in endometriosis (117–119).

In the current study, it was found that the mean level of VEGF-A in the group of patients who were suffering from endometriosis (group B) (1388.7 ± 1152.7 pg/ml) was much higher than the group of patients without endometriosis (group A) (529.4 ± 309.7 pg/ml), and this go in agreement with Kilic et al., 2007 who in her prospective study investigated

Table 3 Comparison between the two groups' clinical and laboratory parameters regarding pregnancy occurrence.

	Occurrence of pregnancy	Group A			Group B		
		Mean \pm SD	I	<i>p</i>	Mean \pm SD	<i>t</i>	<i>p</i>
Age	Pregnant	30 \pm 5.1	1.87	0.06	30.1 \pm 4.5	1.01	0.3
	Non pregnant	33.1 \pm 5.6			31.65 \pm 5.3		
Antral follicle count	Pregnant	13.5 \pm 4.2	1.17	0.247	14 \pm 2.9	0.39	0.69
	Non pregnant	11.8 \pm 5			13.5 \pm 3.9		
Endometrial thickness	Pregnant	11.5 \pm 1.16	2.48	0.017*	11.5 \pm 1.9	0.33	0.75
	Non pregnant	10.5 \pm 1.65			11.3 \pm 1.7		
Oocyte number	Pregnant	13.4 \pm 4	1.7	0.09	11.3 \pm 3.4	1.06	0.3
	Non pregnant	10.6 \pm 6.1			9.8 \pm 5.1		
M2	Pregnant	11.9 \pm 3.9	1.7	0.08	8.6 \pm 5.3	1.4	0.16
	Non pregnant	9.3 \pm 5.4			6.5 \pm 2.8		
M1	Pregnant	0.5 \pm 6.18	-0.6	0.5	0.6 \pm 1	1.8	0.06
	Non pregnant	0.69 \pm 1			1.2 \pm 0.89		
GV	Pregnant	0.66 \pm 1.23	0.279	0.782	1.5 \pm 1.9	0.58	0.5
	Non pregnant	0.57 \pm .90			1 \pm 1.3		
Class A	Pregnant	7.3 \pm 3.23	1.4	0.16	6.4 \pm 3.2	0.4	0.6
	Non pregnant	5.5 \pm 4.9			5.8 \pm 5		
Class 4A	Pregnant	3.3 \pm 3.1	1.17	0.25	5.8 \pm 4.1	1.9	0.06
	Non pregnant	5.3 \pm 4			2.7 \pm 2.2		
Class A5	Pregnant	0.38 \pm 0.69	-1.06	0.293	1.2 \pm 2	0.7	0.4
	Non pregnant	0.76 \pm 1.39			0.6 \pm 0.5		
VEGF-A	Pregnant	556.1 \pm 326.6	0.467	0.64	1036 \pm 725	1.8	0.05*
	Non pregnant	511.6 \pm 302.9			1670 \pm 1353		
No. of ampoules	Pregnant	43.2 \pm 25.3	2.57	0.015*	46.2 \pm 22	1.3	0.19
	Non pregnant	64.1 \pm 21.9			55.8 \pm 20		
Stimulation days	Pregnant	12.5 \pm 1.6	2.14	0.04*	12.5 \pm 1.5	1.38	0.17
	Non pregnant	13.5 \pm 1.3			13.2 \pm 1.1	13.2 \pm 1.1	

Table 4 The correlation between occurrence of pregnancy and different studied parameters in both groups.

		Occurrence of pregnancy
Age	Pearson correlation (<i>r</i>)	0.06
	Sig. (2-tailed) (<i>p</i>)	0.55
Antral follicle	<i>r</i>	-0.013
	<i>p</i>	0.90
Endometrial thickness	<i>r</i>	0.29
	<i>p</i>	0.007*
Oocyte no.	<i>r</i>	-0.11
	<i>p</i>	0.3
M2	<i>r</i>	-0.27
	<i>p</i>	0.16
M1	<i>r</i>	0.03
	<i>p</i>	0.76
GV	<i>r</i>	-0.01
	<i>p</i>	0.89
Class A	<i>r</i>	-0.04
	<i>p</i>	0.66
Class 4A	<i>r</i>	0.15
	<i>p</i>	0.23
Class A5	<i>r</i>	0.15
	<i>p</i>	0.22
VEGF-A	<i>r</i>	-0.24
	<i>p</i>	0.02*
No of ampoules	<i>r</i>	0.004
	<i>p</i>	0.98
Stimulation days	<i>r</i>	-0.11
	<i>p</i>	0.511

follicular fluid vascular endothelial growth factor (VEGF-A) and tumor necrosis factor and (TNF α) concentrations in endometriosis, and their effect on the outcome of intracytoplasmic sperm injection (ICSI) on patients who had either a laparoscopic diagnosis of endometriosis ($n = 48$) or who had normal laparoscopic findings with unexplained infertility ($n = 43$). Kilic recorded a higher level of VEGF-A in females with infertility due to endometriosis (1008.6 ± 511.6 pg/ml) than those with unexplained infertility (687.1 ± 409.8 pg/ml) (120). Also, Reynolds et al. established that patients with endometriosis have higher intrafollicular VEGF-A levels than other infertile patients (121). Also previous research showed that patients with endometriosis had higher follicular concentrations of VEGF-A (122). In contrast, some studies showed that patients with endometriosis had lower concentrations of follicular fluid VEGF-A. Some showed no difference between the study and control groups (123).

Lee et al. investigated the relationship between ICSI outcome and VEGF-A concentration and it was proposed that VEGF-A could be a marker to predict poor ovarian response in ICSI cycles (124). In the present study, higher follicular fluid VEGF-A concentrations in the endometriosis group compared with the control group were found, making a negative correlation between VEGF-A concentration and ICSI outcome, with high concentrations associated with poor outcome thus suggesting that follicular fluid VEGF-A might be a marker of a decreased ovarian reserve and of a hostile follicular environment. The results of the present study are opposite to those of Attar et al., he concluded that values of intrafollicular

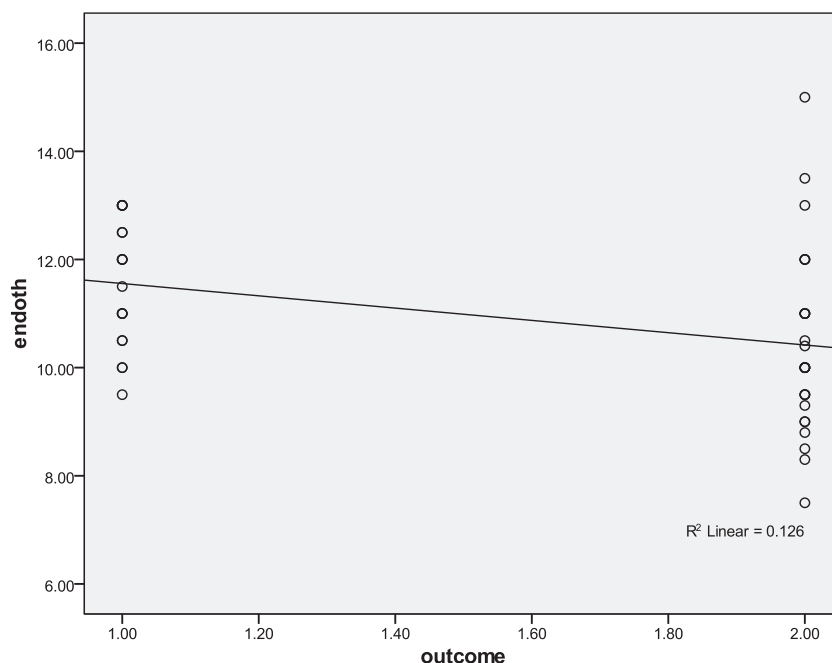


Figure 1 Correlation between ICSI outcome and endothelial thickness.

Table 5 The correlation between VEGF-A and different studied parameters in both groups.

		VEGF-A
Age	<i>r</i>	-0.02
	<i>p</i>	0.84
Antral follicle	<i>r</i>	0.06
	<i>p</i>	0.57
Endometrial thickness	<i>r</i>	-0.17
	<i>p</i>	0.12
Oocyte number	<i>r</i>	-0.09
	<i>p</i>	0.39
M2	<i>r</i>	-0.02
	<i>p</i>	0.86
M1	<i>r</i>	0.07
	<i>p</i>	0.52
GV	<i>r</i>	0.13
	<i>p</i>	0.27
Class A	<i>r</i>	-0.03
	<i>p</i>	0.78
Class 4A	<i>r</i>	0.21
	<i>p</i>	0.09
Class A5	<i>r</i>	-0.03
	<i>p</i>	0.76
No of ampoules	<i>r</i>	0.04
	<i>p</i>	0.80
Stimulation days	<i>r</i>	0.34
	<i>p</i>	0.04*
VEGF-A	<i>r</i>	-0.241
	<i>p</i>	0.022*

VEGF-A concentrations have no predictive value to evaluate ICSI outcome.

It has become increasingly clear that the follicular microenvironment of the maturing human oocyte is a determining factor for the implantation potential of an embryo deriving from

that oocyte. Indeed the quality and maturity of an oocyte are influenced by the level of intrafollicular oxygen content which, in turn, is proportional to the degree of follicular vascularity. The current work tried to establish whether there is a relationship between follicular fluid VEGF-A concentrations and reproductive outcome in patients undergoing ICSI. We found VEGF-A levels not to be significantly correlated with grade of oocyte maturity in both studied groups. In contrast to the results obtained by Monteleone et al. in a study that was done to establish whether there is a relationship between follicular fluid VEGF-A concentrations, perfollicular vascularity and reproductive outcome in normal responders under the age of 35 undergoing IVF and the results showed that oocytes obtained from follicles with the higher grade of vascularization also showed a higher rate of fertilization, embryos, a better quality and higher pregnancy rates were obtained in women with highly vascularized follicles referred his results to the fact that the quality and maturity of an oocyte are influenced by the intrafollicular level of oxygen content which, in turn, is proportional to the degree of follicular vascularity (125). The development of an adequate capillary network seems to depend at least in part on the action of vascular endothelial growth factor (VEGF-A). The contrary in results may be due to studying sufferers of endometriosis besides normal responders in the current study, while in the study of Monteleone et al., only normal responders to controlled ovarian hyperstimulation, i.e. presenting a number of follicles ≥ 3 were enrolled.

As regards the number of oocytes retrieved, there was observed that endometriotic cases (group B) retrieved a less number of oocytes compared to the non endometriotic group (group A) (10.5 ± 4.4 , 11.75 ± 5.5 , respectively), but this values did not make a statistically significant difference. This goes in agreement with Benifla et al. (126), who found a near number of oocytes between both endometriotic and non endometriotic patients (8.75 ± 6.2 , 8.46 ± 3.8 , respectively).

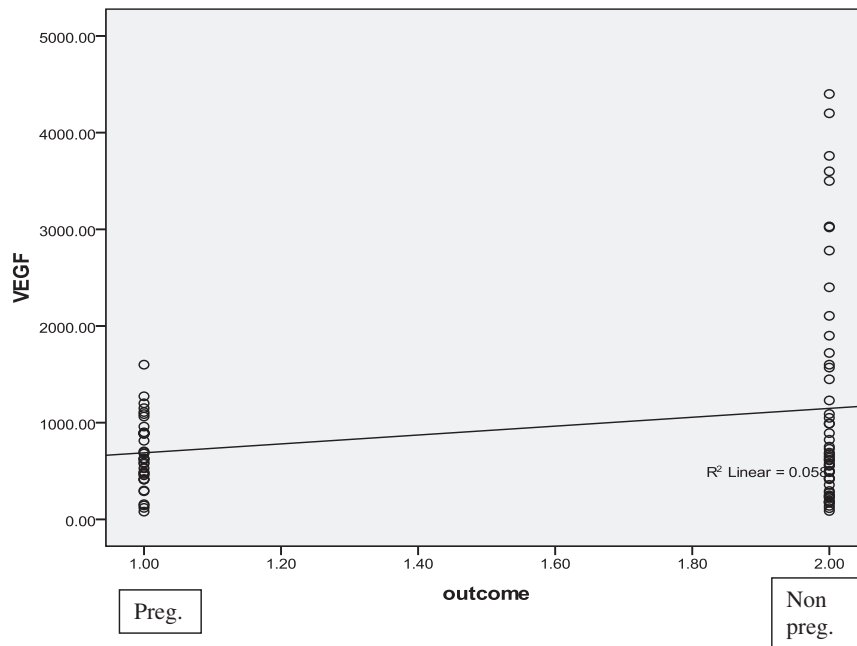


Figure 2 Correlation between VEGF-A and pregnancy outcome.

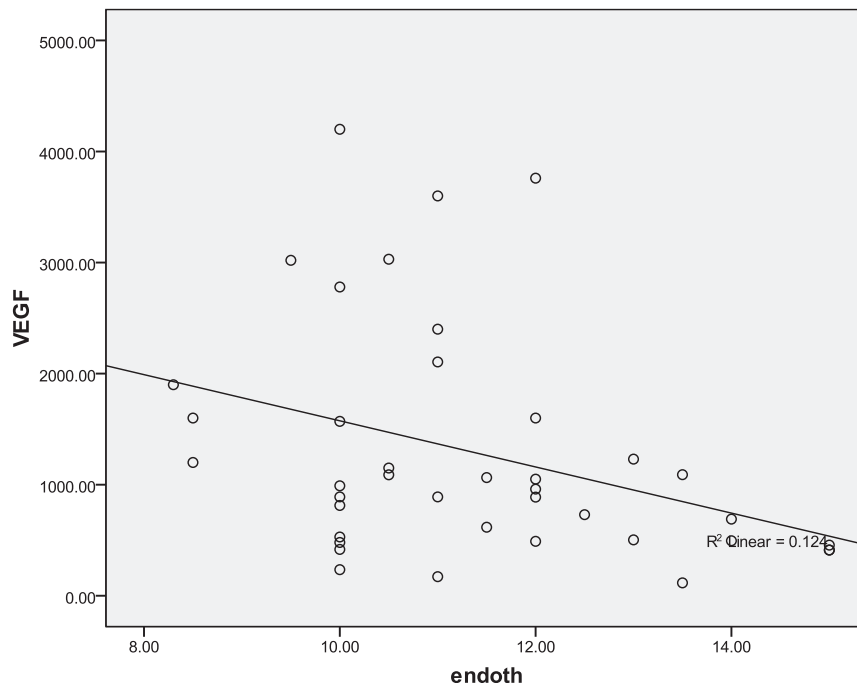


Figure 3 Correlation between VEGF-A and endometrial thickness.

A higher number of M2 oocytes (10.4 ± 4.9) in (group A) than endometriotic patients (group B) (7.6 ± 4.4), made a statistically significant difference between the two groups. ($p = 0.011^*$).

A higher embryo maturity class of A, 4A, A5 and B was recorded in the non endometriotic group (group A) than in the endometriotic group (group B) due to a statistically significant difference in between the two groups. No correlations could be recorded between oocyte maturity parameters or the embryo

grading with the level of VEGF-A. Neither the maturity of oocytes nor embryo grading affects the ICSI outcome in the current work in each of the studied groups. Overall, the viability and quality of oocytes (and derived embryos) could not represent strong predictors for implantation potential.

As regards the number of ampoules, there was no statistically significant difference between groups A and B ($p = 0.65$). Among group A, the mean number of ampoules in pregnant females was 43.2 ± 25.3 , in non pregnant females

was 64.1 ± 21.9 , this makes a statistically significant difference between pregnant and non pregnant females ($p = 0.015^*$). No correlation was found between the number of ampoules and VEGF-A either in group A or group B, unlike the results obtained by Benifla et al., who detected a positive correlation between the number of ampoules and VEGF-A (126).

There was a negative correlation between VEGF-A and ICSI outcomes in both of the studied groups. Our results suggest that VEGF-A in follicular fluid may predict ICSI outcome. This opposes the results obtained by Benifla et al. who found that VEGF-A did not affect the ICSI outcome, especially among patients < 40 years old.

5. Conclusion

The current study concluded that follicular fluid VEGF-A is correlated negatively with the ICSI outcome.

Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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