Role of Vascular Endothelial Growth Factor (VEGF) and Doppler Sub-endometrial Parameters as Predictors of Successful Implantation in Intracytoplasmic Sperm Injection (ICSI) Patients

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ABSTRACT

Objective: to investigate the expression of vascular endothelial growth factor (VEGF) in patients having infertility due to low endometrial acceptance, and to correlate it to non-invasive ultrasound variables, endometrial thickness, and sub-endometrial Doppler parameters (PI, RI, Vs/Vd).

Methods: 80 women all under the age of 40 underwent ICSI-ET; all patients were exposed to ovarian stimulation protocols. The oocytes were retrieved using an ultrasound guide, and were fertilised via injection of sperm inside the follicle (ICSI). VEGF serum level was analysed at day of embryo transfer by ELIZA test, and sub-endometrial evaluation was conducted via two-dimension power Doppler ultrasound (2D PD-US), by measuring resistance index (RI) and pulsatility index (PI) on the day of embryo transfer.

Results: There was a significantly higher VEGF level and endometrial thickness in pregnant (433± 207 and 9.72± 1.35) women, compared to non-pregnant (276± 165 and 8.95 ± 1.21) respectively as *p*-values were (0.001 and 0.01)). Additionally, there were significantly lower RI and PI in pregnant (0.584 ± 0.124 and 0.829 ± 0.301) women compared to non-pregnant (0.651±0.132 and 1.006±0.335) women, as *p*-values were (0.02 and 0.02, respectively). The level of E2 was on the day of embryo transfer and Vs/Vd in pregnant women (1402± 524 and 3.14 ± 3.75) and in the non-pregnant group (1296± 611 and 3.82 ± 3.07), as *p*-values were 0.41 and 0.38, respectively.

Conclusion: The combined analysis of endometrial receptivity was completed, and the serum level of VEGF and sub-endometrial evaluation with 2D PD-US was defined by measuring resistance index (RI) and pulsatility index (PI) on the day of embryo transfer. These can serve as useful prognostic methods for the detection of endometrial receptivity and pregnancy outcomes in infertile women undergoing ICSI protocols, and will be helpful for candidate counselling about postponing embryo transfer and cryopreservation, which may serve as a better option, to be recommended for the next cycle, when achieving better endometrial Doppler parameters.

Keywords: VEGF; Sub-endometrial doppler; infertility (Siriraj Med J 2020; 72: 33-40)

INTRODUCTION

Infertility means the inability to have a baby despite regular unprotected sex.[1] According to NICE guidelines, two years of having regular sex without the presence of a known abnormality in both partners is necessary for establishing the diagnosis of infertility.[2] Management of

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http://dx.doi.org/10.33192/Smj.2020.05

these patients have changed and improved dramatically, ranging from assistedreproduction technology to in vitro fertilisation (IVF). Intracytoplasmic sperm injection (ICSI), has helped tremendously in management of infertility due to male factors.[3] The problem of IVF arise in the failure of transfer of embryo. That's way research has focused on finding the best conditions in the embryo and the endometrium that will allow proper transfer. Discovering specific and accurate biomarkers is a primary concern to many researchers. Additionally, knowledge of the human implantation window length on days (6-10) post-ovulation, the effect of steroid hormones (oestrogen, progesterone, gonadotropins) on genetic factors, the age of a woman, and functional and morphological markers pertaining to endometrial receptivity are of crucial significance to all future studies aimed at identifying endometrial receptivity[4], studying endometrial receptivity by non-invasive methods like vaginal ultrasonography. Conventionally, pulsed Doppler sonography is used to evaluate uterine and endometrial blood flow, but non standard results have been reported for the measurement of sub-endometrial resistance index (RI) and pulsatility index (PI) at the second endometrial zone (zone 2), with serum level of estradiol at day of HCG and angiogenic factor VEGF at day of embryo transfer. This variation is correlated with endometrial receptivity and pregnancy outcome, and can contribute to making a judgment as to whether an embryo should be transferred, or whether to cryopreserve it until it reaches an optimum level of endometrial receptivity.

Due to the limited mobility of the intravaginal transducer in the small vaginal cavity, Normal ultrasound is of limited value. Real-time U/S allows study of two factors related to implantation: the thickness of endometrium and the pattern of endometrial morphology.[5] Ultrasound is crucial for predicting endometrial preparation before transfer of embryo (ET), in both fresh IVF cycles and frozen-thawed embryo transfer cycles. Frequently used parameters for endometrial sonographic assessment involve endometrial thickness, endometrial patterns, and Doppler indices.[6] At the myometrial-endometrial junction, there is a sub-endometrial region that is detected on ultrasound examination as a thin hypoechoic layer between the echogenic endometrium and myometrium; this is referred to as the junctional zone, inner myometrium, sub- endometrial halo, or sub- endometrial layer, and can be identified by either ultrasound or MRI. Research has confirmed that the sub-endometrial haloaround around the endometrium is representative of the innermost layer of the myometrium.[7] The vascularity zones are identified as follows. Zone 1: vessels are detected in the myometrium around the endometrium; zone 2: vessels penetrate the hyperechogenic endometrial edge; zone 3: vessels reach the internal hypo echogenic zone; zone 4: vessels reach the endometrial cavity, when colour mapping of the endometrial and sub-endometrial regions is absent; this means a definite implantation failure or a considerable decline in the implantation rate.[8] Doppler studies have revealed that the resistance in arteries of the endometrium is significantly reduced during the mesoluteal phase, which is the period when embryo implantation is possible. The changes in blood vessels may have a vital role in the implantation process, as they are present from the start of the embryo implantation. Vascular endothelial growth factor (VEGF), belongs to proteins binding heparin that attach to endothelial cells, and which lead to proliferation and new blood vessel formation. The VEGF also causes release of cytokines by the endothelial cells leading to dilatation of blood vessels. It acts as an angiogenic factor to promote angiogenesis in various tissues.[9] Consequently, this explains the crucial role of angiogenesis in different female reproductive processes, e.g., development of a dominant follicle, formation of a corpus luteum, endometrial growth, as well as implantation. As such, local angiogenesis is considered a major prerequisite for implantation and subsequent conception.[10] Angiogenesis was reported as it is least occurring during the menstrual phase, then there will be an increase during the early proliferative phase, reaching maximum in mid-cycle then decrease near the end of the cycle.[10,11]

MATERIALS AND METHODS

This is a prospective study was conducted in the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University. The study was approved by the Local Medical Ethical Committee of the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University. Eighty women undergoing an IVF/ICSI cycle were included in this study. At the day of embryo transfer, two dimensional Power Doppler (2D-PD) ultrasound was conducted for sub-endometrial blood flow zone 2, with serum VEGF level analysis.

IVF/ICSI procedures

Step-by-step descriptions of IVF procedures:

1. Controlled ovarian hyper stimulation (COH)
2. Oocyte retrieval (OCR)
3. Fertilisation and embryo culture
4. Embryo quality
5. Embryo transfer (ET)

Three different types of controlled ovarian hyperstimulation (COH) were used according to the demographic parameters of patients, starting a long agonist protocol, from day 21 of the preceding cycle and using a GnRH agonist (Decapeptyl® 0.1 mg, Ferring Co., Germany); then, on the second day of the menstrual cycle, stimulation was started with a daily dose of 150-450 IU rFSH (follitropin alfa, Gonal F®, Merck Serono). Secondly, a short agonist protocol was initiated, beginning with a GnRH agonist from cycle day one-to-two, then starting a daily dose of 150-450 IU rFSH (follitropin alfa, Gonal F®, Merck Serono). The third option used an antagonist protocol by starting with a daily dose of 150-450 IU rFSH (follitropin alfa, Gonal F®, Merck Serono), and when follicles reached 13-14 mm in size, a GnGH antagonist was started (Cetrotide®, Merck Serono). Patients were monitored by transvaginal sonography (TVS). HCG (Ovitrelle® 250 microgram, Merck Serono) was given when three or more follicles reached a diameter of 18 mm. Oocytes retrieval was performed using a transvaginal probe 34-36 hours following the HCG injection, immediately prior to the rupture of follicles. Oocytes were aspirated by transvaginal ultrasound guided oocyte retrieval (TUGOR); oocytes at retrieval can be either the germinal vesicle (GV), which is the most immature, or from metaphase I (MI), at which the oocyte is an immature egg. The absence of a polar body or germinal vesicle indicates it to be at the MI stage, which is an intermediate stage between the GV and MII (mature) stages, or metaphase II oocyte (MII), which is mature. Generally, prior to OCR, a semen sample is prepared for sperm extraction, following a minimum of two days and a maximum of seven days' sexual abstinence. Testicular sperm extraction (TESE) is a surgical sperm retrieval procedure used in infertility treatment for men with azoospermia.

At the IVF laboratory, aspirated follicles were examined. Flushing was performed, and the follicles are kept one-to-two hours in a 37°C/CO_2 incubator. Later, all oocytes underwent denudation and grading in a laminar flow cabinet. Thereafter, a needle was carefully inserted through the shell of the egg into its cytoplasm, then kept in the CO_2 incubator while waiting for the results of cell division, which would be detected with the aid of a Nikon ICSI microscope. Following insemination, zygotes were observed for 18-20 hours to check for the presence of two pronuclei, and for 25-29 hours to confirm the existence of early cleavage, which was correlated with higher implantation rates. At day one, the presence of two pronuclei was considered a good prognostic sign.

At day two-or-three post-OCR, and according to the number and grading of the embryos, patients were prepared for the transfer. The patient's serum was obtained for VEGF serum level, two dimensional transvaginal ultrasound scans were done to measure endometrial thickness, regularity, and echogenicity, and sub-endometrial blood flow colour Doppler indices (PI, RI and Vs/Vd) were measured. The measurement involved both endometrial layers, excluding the surrounding low amplitude echo layer; three measurements were taken and the average value was recorded. A pulsed Doppler system was used for blood flow analysis. Sub-endometrial vessels were visualised at the endometrial periphery, sometimes penetrating the hyperechogenic endometrial edge, or even reaching the endometrial cavity.

We measured the endometrial thickness on the day of ET as we want it to be more accurately correlated with the level of VEGF which was measured on the same day.

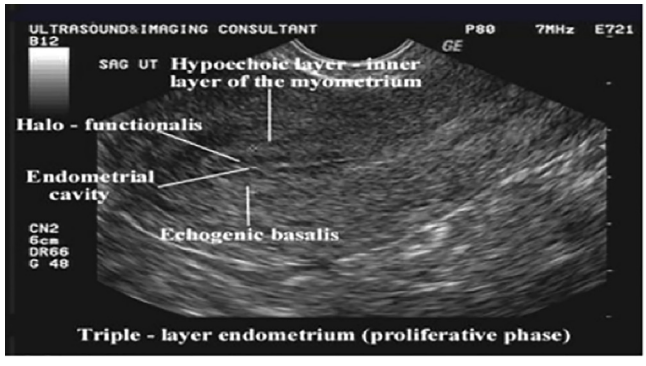

Fig 1. Ultrasound review reveals myometrial, endometrial, and sub-endometrial zones.

Blood flow velocity waveforms from the sub-endometrial vessels were obtained by placing the Doppler gate over the coloured area at zone 2 and activating the pulsed Doppler function.

$$RI = \frac{PSV-EDV}{PSV} \quad PI = \frac{PSV-EDV}{mv} \quad mv = \frac{PSV+EDV}{2}$$

Abbreviations: RI = resistance index; PSV = peak systolic velocity; EDV = end-diastolic velocity; PI = pulsatility index; mv = mean velocity

RESULTS

Demographic parameters and hormonal profile

Regarding the demographic parameters (age, BMI of patients, and duration of infertility) and the basal hormonal profile level (FSH, LH, PRL, E2, and testosterone), the statistical analysis showed no significant differences between pregnant and non-pregnant ICSI patient groups, and *p*-values of 0.516, 0.06, and 0.622, and

0.289, 0.495, 0.334, 0.782, 0.568, and 0.342, respectively (Table 1).

Vascular endothelial growth factor (VEGF) and Doppler parameters

Table 2 demonstrates the differences between some measurements in pregnant and non-pregnant women in this study. Endometrial thickness, RI, PI, and VEGF showed statistically significant differences, with *p*-values equaling 0.01, 0.021, 0.015, and 0.001, while Vs/Vd showed no statistically significant differences, with a *p*-value of 0.385.

Correlation of VEGF with other endometrial receptivity parameters

Table 3 shows that the Pearson correlation for VEGF, and E2 at day of HCG administration and endometrial thickness at day of embryo transfer, showed a positive association above zero (r= 0.325, 0.227), with a statistically significant value of association (*p*-value equal to 0.003 and 0.043). Pearson correlation for VEGF and RI, PI, and Vs /Vd showed a weak negative association below zero (r= -0.168, -0.11, -0.088), with no statistically significant value of association (*p*-value equal to 0.137, 0.331, and 0.436).

Correlation of endometrial thickness with other endometrial receptivity parameters

The Pearson correlation of endometrial thickness with Vs/Vd showed a positive association above zero (r=0.23), with a statistically significant association value (*p*-value 0.04), while Pearson correlation for endometrial thickness with RI, PI, and E2 at D. of HCG administration showed weak association above zero (r=0.062, 0.079, and 0.129), with no statistically significant association value (*p*-value=0.586, 0.489, and 0.253), as shown in Table 4.

Correlation of E2 at day of embryo transfer with endometrial receptivity parameters

Regarding E2 level at day of HCG administration, its Pearson correlation with RI, PI, and Vs/Vd showed weak negative association below zero (r=-0.01, -0.031, and -0.107, respectively), with no statistically significant association value (*p*-value=0.933, 0.786, and 0.346, respectively), as shown in Table 5.

DISCUSSION

The data for the present study show that the pregnant group had higher levels of VEGF compared to the non-pregnant group, as shown in Table 2. The rate of endometrial blood flow during the normal female reproductive cycle has been correlated with increased expression of angiogenic factors (e.g., VEGF). These are members of a family of heparin binding proteins that act directly on the endothelial cells, and induce proliferation and angiogenesis.[12] Zenneni et al. found that patients with primary infertility had a much less immunohistochemical expression of VEGF in the secretory endometrium.[13] In another study, Hannan et al confirmed the presence of thirty different types of mediators present in the myometrial cavity during the menstrual cycle, of which VEGF was much less in uterine fluid obtained during implantation from patients with infertility.[11] Schild et al. state that it is important to have good emdometrial vascularization inorder to implant.[14] In the current study, there was positive correlation between VEGF and E2 at day of HCG administration (Table 3), which is supported by authors who have noted a maximum expression of VEGF in the stroma during proliferative phase, with a peak glandular VEGF expression during the secretary phase.[15]

Additionally, in the present study, we noticed a clear positive association between VEGF and endometrial thickness at day of embryo transfer (Table 3), which is also concluded in a study conducted by Miwa et al., which indicates a 'thin' endometrium characterised by high blood flow impedance of the uterine radial artery, poor epithelial growth, decreased VEGF expression, and poor vascular development.[16] Thus, there is a positive association between endometrial thickness and VEGF; the angiogenic factor plays a role in endometrial receptivity, and this elucidates higher endometrial thickness for pregnant women compared to non-pregnant women, as well as a significant value association with Vs/Vd in our study (Table 4).

In the current study, pregnant women had a thicker endometrium than non-pregnant women. Researchers found that there is a reduced success rate of IVF in patients with a thin endometrium, even if there was no previous intrauterine surgery or infection. Some studies found that a thin EMT negatively affects pregnancy rates following fertility treatment[17], while other studies were unable to confirm this, indicating that the use of endometrial thickness as a tool for deciding on cycle cancellation or the freezing of all embryos, or cessation from further IVF treatment, appears unjustified.[18,19]

Some groups did not find a correlation between age, the number of follicles, and gonadotropin ampoules with endometrial thickness; however, in all age ranges, the chance of pregnancy was higher with an endometrial thickness of 6<ET≤10 mm.[20] Higher conception ratesoccured in patients with endometrial thickness of 10 mm and above.

TABLE 1. Comparison of demographic parameters and hormonal profile between pregnant and non-pregnant ICSI patient groups by unpaired t-test.

Parameters	Pregnant N = (36) Mean ± SD	Non-pregnant N = (44) Mean ± SD	*P*-value
Age (yrs)	28.89 ± 6.07	29.77 ± 5.97	0.52
BMI (kg/m^2)	26.05 ± 2.85	27.31 ± 2.94	0.06
Duration of infertility (yrs)	5.64 ± 3.79	6.07 ± 3.95	0.62
FSH (mIU/ml)	7.59 ± 1.94	7.02 ± 2.80	0.29
LH (mIU/ml)	4.89 ± 2.06	5.31 ± 3.36 0.49	
E_2 (pg/ml)	37.3 ± 13.6	34.3 ± 13.4	0.33
PRL (ng/ml)	15.87 ± 6.18	15.46 ± 7.03	0.78
TSH (mIU/ml)	2.275 ± 0.625	2.176 ± 0.920	0.57
Testosterone (ng/dl)	1.039 ± 0.682	1.50 ± 3.06	0.34

Abbreviations: n=number, SD=standard deviation, yrs=years, BMI=body mass index, FSH=follicle stimulating hormone, LH=luteinized hormone, E_2=estradiol hormone, PRL=prolactin hormone, TSH=thyroid stimulating hormone

TABLE 2. Comparison between pregnant and non-pregnant ICSI patient groups with ultrasound Doppler parameters and VEGF level by unpaired t-test.

Parameters	Pregnant N = (36) Mean ± SD	Non-pregnant N = (44) Mean ± SD	*P*-value
Endometrial thickness (mm)	9.72 ± 1.35	8.95 ± 1.21	0.0
RI	0.584 ± 0.124	0.651± 0.132	0.0
PI	0.829 ± 0.301	1.006± 0.335	0.02
V_s/V_d	3.14 ± 3.75	3.82 ± 3.07	0.38
VEGF (pg/ml)	433 ± 207	276 ± 165	0.01

Abbreviations: n=number, SD=standard deviation, RI=resistance index, PI=pulsatility index, V_s=peak systolic velocity, V_d=diastolic velocimetry, VEGF=vascular endothelial growth factor

TABLE 3. Correlation between VEGF with E2 at day of HCG administration, and ultrasound Doppler parameters at day of embryo transfer by Pearson correlation test.

		E_2 at Day of HCG administration (pg/ml)	RI	PI	V_s / V_d	Endometrial thickness (mm)
VEGF (pg/ml)	r	0.325	-0.168	-0.11	-0.088	0.23
	P	0.003	0.137	0.331	0.436	0.04

Abbreviations: r=Pearson correlation, *P*=p-value, E_2==estradiol hormone, HCG=human chorionic gonadotropin hormone, RI=resistance index, PI=pulsatility index, V_s=peak systolic velocity, V_d=diastolic velocimetry, VEGF=vascular endothelial growth factor.

TABLE 4. Correlation between endometrial thickness with E2 at day of HCG administration, and ultrasound Doppler parameters at day of embryo transfer by Pearson correlation test.

		E_2 at day of HCG administration (pg/ml)	RI	PI	V_s/V_d
Endometrial thickness (mm)	r	0.129	0.062	0.079	0.23
	P	0.253	0.586	0.489	0.04

Abbreviations: r=Pearson correlation, *P*=p-value, E_2=estradiol hormone, HCG=human chorionic gonadotropin hormone, RI=resistance index, PI=pulsatility index, V_s=peak systolic velocity, V_d=diastolic velocimetry

TABLE 5. Correlation between E_2 at day of HCG administration with ultrasound Doppler parameters at day of embryo transfer by Pearson correlation test.

		PI	RI	V_s/V_d
E_2 at day of HCG administration (pg/ml)	r	-0.01	-0.031	-0.107
	P	0.933	0.786	0.346

Abbreviations: r=Pearson correlation, *P*=p-value, E_2=estradiol hormone, HCG=human chorionic gonadotropin hormone, RI=resistance index, PI=pulsatility index, V_s=peak systolic velocity, V_d=diastolic velocimetry

Another study found that when the is an endometrium of less than 7 mm it is better to do cryopreservation, however if the endometrium is thin but with a good texture (triple-line pattern), other factors should be considered such as the quality of the embryo. This is in agreement with the results of the current study.[21]

The sub-endometrial RI and PI in the present study were significantly lower in pregnant patients than in non-pregnant patients. This is supported by other researchers, who found patients that became pregnant were characterised by a significantly lower resistance index; these results were obtained from sub-endometrial vessels by transvaginal colour Doppler ultrasonography.[22] The reason for this is because sub-endometrial vascularity increased significantly at day of embryo transfer, due to the effect of stimulated hormones on endometrial angiogenesis.[23] Jain et al. (2015) found that serum VEGF levels rose alongside with an increase in Doppler vascular penetration zones (zone 2, intermediate vascularity), which implies that serum VEGF concentrations can be used as a marker of endometrial receptivity. No conception was noted in patients with poor or intermediate vascularity, as identified in Doppler vascular zone 2.

This means that vascular endothelial growth factor (VEGF) is a major regulator of endothelial cell proliferation, angiogenesis, vasculogenesis, and capillary permeability. A concurrent rise in serum VEGF level was observed alongside an increase in Doppler vascular penetration zones. A receptive endometrium is a reflection of good endometrial vascularity, which signifies serum VEGF as a marker of endometrial receptivity.[24,25] This is supported by our results as shown in Table 2, where a high level of VEGF is associated with low RI, PI, and VS/Vd, and an elevated level of E2 at day of embryo transfer, as well as a thick endometrium.

CONCLUSION

Combined analysis of endometrial receptivity was conducted for the present research. The serum level of VEGF, and sub-endometrial evaluation with 2D PD-US, by measuring resistance index (RI) and pulsatility index (PI) at day of embryo transfer, can serve as useful prognostic methods for the detection of endometrial receptivity and pregnancy outcome in infertile women undergoing ICSI protocols, and will be helpful for candidate counselling with regard to postponing embryo transfer and cryopreservation, which may be a better option for a future cycle, when better endometrial Doppler parameters can be achieved.

ACKNOWLEDGMENTS

The authors would like to thank International Islamic University Malaysia for funding publication of this project under grant number PRIGS 18-03-0030.

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