Original Article

Detection of endometrial and subendometrial vasculature on the day of embryo transfer and prediction of pregnancy during fresh in vitro fertilization cycles

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Objectives: Successful implantation depends on interaction between a blastocyst and a receptive endometrium. Endometrial vasculature is important in the early endometrial response to blastocyst implantation, and vascular changes can affect uterine receptivity. This study aims to investigate whether vascular parameters measured using three-dimensional power Doppler ultrasound (3D PD-US) could predict pregnancy following fresh in vitro fertilization and embryo transfer (IVF–ET) using a gonadotropin releasing hormone (GnRH) agonist long protocol.

Materials and methods: This prospective observational study enrolled 236 nulliparous women who underwent a first IVF–ET using a GnRH long protocol with stimulation by recombinant FSH (rFSH) from May 2009 to April 2012. After excluding two cases of tubal pregnancy, 234 women were in either a pregnant group (n = 113) or a nonpregnant group (n = 121). Color Doppler ultrasound and 3D PD-US examinations were performed on the day of embryo transfer. Main outcomes were pulsatility index (PI), resistance index (RI), systolic/diastolic ratio (S/D) of the uterine artery, vascularization index (VI), flow index (FI), and vascularization flow index (VFI) of the endometrium and subendometrial region. Measurements were analyzed relative to IVF–ET outcome (pregnant vs. nonpregnant).

Results: No significant differences were observed in patient age, infertility duration, body mass index (BMI), basal FSH levels, number of retrieved oocytes or good quality embryos, or endometrial thickness or volume between the two groups. The pregnant group had higher endometrial VI, FI, and VFI scores than the nonpregnant group (p = 0.001, p = 0.000, p = 0.021, respectively). By contrast, neither sub-endometrial region VI, FI, and VFI scores (p = 0.770, p = 0.252, p = 0.451), nor uterine artery PI, RI, or S/D scores (p = 0.256, p = 0.527, p = 0.365) differed between groups. Cut-off values of endometrial VI, FI, and VFI scores were 0.95, 12.94, and 0.15 for pregnancy achievement.

Conclusion: Three dimensional PD-US was a useful and effective method for assessing endometrial blood flow in IVF cycles. Good endometrial blood flow on the day of embryo transfer might be associated with high pregnancy success with a GnRH long protocol, because this is indicative of endometrial receptivity in fresh IVF cycles.

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Introduction

Favorable maternal conditions and embryo quality are important for successful implantation. Problems that can originate from the host environment include abnormal uterine anatomy, maternal medical conditions, and a nonreceptive endometrium; all can have adverse effects on the cross-communication between the embryo
and the endometrium [1]. The endometrium is critical for successful implantation through interaction with the embryo [2]. Endometrial–embryo interactions can be altered if the embryo is defective, which can result from either paternal sperm factors or oocyte abnormalities.

Uterine receptivity is defined as the endometrial conditions for blastocyst attachment and implantation that occur during limited periods. The endometrium undergoes a complex series of changes during the menstrual cycle, with a short period of receptivity [3]. In this endometrial transition period, embryo implantation is supported by the ovarian hormones estrogen and progesterone, which modulate endometrial events in a spatiotemporal manner. The hormones affect numerous factors involved in implantation in humans [4]. In spite of our poor understanding of the molecular mechanism involving endometrial–embryo interactions, some interactions might influence endometrial neovascularization [5].

To increase advantageous endometrial–embryo interactions, the endometrium must become thicker, with richer vascularity. Endometrial blood flow reflects uterine receptivity because the endometrium is the site of embryonic implantation [5]. During in vitro fertilization (IVF) and embryo transfer (IVF–ET) cycles, implantation is a major determinant of success or failure. Up to two-thirds of implantation failures are estimated to be caused by defects in endometrial receptivity [6]. Efforts have been made to evaluate endometrial receptivity in endometrial and sub-endometrial blood supplies, especially during intrauterine insemination and IVF–ET cycles [7]. This study evaluated whether endometrial, subendometrial, and uterine blood flow parameters measured using three-dimensional power Doppler ultrasound (3D PD-US) and color Doppler ultrasound were useful in predicting pregnancy in fresh cycles using a GnRH long protocol. In comparison with previous studies, the uterine receptivity with vasculatures targeting restricted protocol and purified women experienced IVF–ET were evaluated for the first time.

### Materials and methods

#### Patient population

A total of 236 infertile women, aged 26–41 years old, who underwent a first IVF–ET with a GnRH long protocol with stimulation by recombinant follicle stimulating hormone (FSH) (rFSH) from May 2009 to April 2012 were enrolled in the fertility centers of three university hospitals. Couples were considered for inclusion after chart review, excluding the following conditions: (1) infertility attributed to endocrine abnormalities such as hyperprolactinemia, polycystic ovarian syndrome, and hyperthyroidism; (2) other diagnosed chronic diseases; (3) previous gynecologic operation for ovarian or uterine pathology; or (4) inadequate data for analysis.

The inclusion criterion was that the infertile couple had no known infertility factors. Women with endometriosis, ovarian cyst, tubal obstruction, severe adenomyosis, uterine fibroid, endometrial polyp, intrauterine adhesion, severe pelvic adhesion, or low resolution on ultrasound examination were excluded. All patients were included for only a single cycle to avoid selection bias. In this prospective observational study, no therapeutic interventions except routine procedures were performed on patients. A careful ultrasound evaluation just before embryo transfer (ET) was performed. Ethics approval was granted by the Institutional Review Board of Kosin University, Busan, Korea.

#### Controlled ovarian stimulation and IVF–ET procedures

All patients underwent a GnRH agonist (Lucrin; Abbott, Rungis Cedex, France) long protocol for controlled ovarian stimulation (COS) with daily injection of rFSH (Gonal-F; Serono, Geneva, Switzerland or Puregon; Organon, Oss, The Netherlands). After proper pituitary regulation and desensitization using the GnRH agonist during the previous midluteal phase, COS was initiated at the beginning of menstrual cycle Day 3. The daily dose of rFSH was customized according to serum E2 concentration and the follicular growth and number assessed by transvaginal ultrasound. Final oocyte maturation was induced by intramuscular administration of 10,000 IU of hCG (Pregnyl; Organon) when at least two follicles reached >18 mm in mean diameter on transvaginal ultrasound.

Oocyte retrieval was carried out under transvaginal ultrasound-guided aspiration at 36 hours after hCG injection. Retrieved oocytes were fertilized by either conventional insemination or intracytoplasmic sperm injection (ICSI). Fertilization was performed 3–6 hours after oocyte retrieval in IVF medium (Quinn’s Advantage media; SAGE BioPharma, Badminster, NJ, USA) with either ICSI or conventional insemination according to the presence or absence of male factor infertility. At 24 hours after oocyte retrieval, normal fertilization was confirmed by the presence of two pronuclei with two distinct or fragmented polar bodies.

Embryos were cultured in culture medium (Sage sequential media; SAGE BioPharma) to Day 3 after fertilization. After assessing the quality of cultured embryos, two or three good-quality embryos were transferred using a catheter (Cook catheter; Cook Medical, Bloomington, IN, USA) with abdominal ultrasound guidance on Day 3 after fertilization, adjusted for patient age, individual history, or preferences. Embryo quality was measured according to the following parameters: number of blastomeres, rate of fragmentation, and multinucleation of blastomeres. Other embryos were transferred from cleavage medium to blastocyst medium.

All patients received daily progesterone support (IM P 50 mg or vaginal 600 mg of micronized progesterone) from the day of oocyte retrieval.

#### Ultrasound investigation

Except for routine serial ultrasound examination, adjunctive 3D PD-US (ACCUVIX XQ, Medison, Seoul, Korea) and color Doppler evaluation by vaginal 3-D probe were performed to assess endometrium, subendometrium, and uterine arterial vascularity on the day of ET. All 3D PD-US and Doppler evaluation scans were carried out by uniform ultrasound modes and given to a single investigator for volume and Doppler analysis. The results of adjunctive ultrasound assessment did not affect subsequent clinical management procedures because the clinicians did not know the ultrasound evaluation results.

With color Doppler in the two-dimensional (2D) mode, flow velocity waveforms were obtained from the ascending main branch of the uterine artery on the left and right sides of the cervix in a longitudinal plane. The cursor of the Doppler was positioned where vessels with good color signals were identified on the screen. The PI, RI, and S/D ratio of the uterine artery were calculated electronically. Almost no differences in uterine PI, RI, and S/D ratios were seen between the left and right sides. Thus, analyses used the vascular indices of the right side uterine artery.

When a longitudinal view of the uterus was obtained, the power Doppler mode was turned on. The areas of interest were the endometrium and subendometrial regions within 5 mm of the echogenic endometrial borders. The setting conditions were: frame average, 5; balance, 16; sensitivity, 15; scale, 0.6 kHz; filter, 1; density, low; gray, 60. The 3D mode was activated and the area of interest was adjusted, and 3D volume data were obtained by automatic sweep with angle set to 90° to ensure inclusion of a
complete uterine volume encompassing the entire sub-
endometrium. The patient and 3D vaginal probe were kept as still 
as possible during volume acquisition. The resultant multiplanar 
display was examined to ensure that the area of interest had been 
captured in its entirety. Built-in VOCAL (Virtual Organ Computer-
Aided Analysis) software for 3D power Doppler histograms were 
used to measure blood flow indices. The manual mode of the 
VOCAL Contour Editor was used to cover the entire 3D volume of 
the endometrium with a 15° rotation step. A total of 12 endometrial 
slices were obtained outlining the endometrium at the myoendo-
mertial junction from fundus to internal os (Fig. 1). Three vascular 
indices calculated automatically by 3D PD-US were vascularization 
index (VI), flow index (FI), and vascularization flow index (VFI). VI, 
FI, and VFI of the subendometrial region, which was considered to 
be within 5 mm from the endometrial border, were obtained by 
editing to shell imaging (Fig. 1).

Pregnancy confirmation and outcome measures

To assess IVF treatment outcomes, serum β-hCG was measured 
12 days after ET. A rise in serum β-hCG (>20 IU) was considered 
positive for pregnancy and a consecutive serum β-hCG test was 
done. Clinical pregnancy was defined by transvaginal ultrasound 
observation as the presence of gestational sacs and fetal cardiac 
activities after 6 weeks of gestation. Ongoing pregnancy was

![Fig. 1. Three-dimensional power Doppler images by Virtual Organ Computer-Aided Analysis software. (A) Endometrial blood flow parameters; and (B) subendometrial blood flow parameters on the day of embryo-transfer.](image-url)
defined as a living fetus on ultrasound examination after 14 weeks of gestation.

Statistical analysis

All results are presented as mean and standard deviations. Comparisons between groups were carried out with Student t test for continuous variables and χ² test for categorical variables. Correlation was estimated using Pearson’s correlation coefficient. The receiver operating characteristic (ROC) curve was applied to determine the predictive value of endometrial vascular parameters. The cutoff levels for variables without significance in Pearson’s correlation coefficient analysis were not calculated. The significance level for all analysis was p < 0.05. Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

A total of 236 cycles that used a fresh GnRH agonist protocol met the study criteria, with two women having tubal pregnancies; these were terminated with a laparoscopic operation. IVF–ET led to 103 clinical pregnancies with multiple pregnancies in 11 cases, including a single case of triplets. A mean of 2.4 embryos were placed. The pregnancy rate was 43.6% per application of the GnRH long protocol, and the implantation rate was 20.6%.

The 103 intrauterine pregnancies were classified as the pregnant group; the other 131 cases were classified as the nonpregnant group. Table 1 summarizes clinical characteristics including age, duration of infertility, body mass index (BMI), basal FSH levels, basal LH levels, basal E2 levels, E2 levels on hCG administration day, total rFSH dosage, number of retrieved oocytes, use of ICSI, endometrial thickness, endometrial volume, and number of transferred embryos for the pregnant and the nonpregnant groups. None of these clinical characteristics had significant differences between the groups. Vascular parameters of the uterine artery are listed in Table 2. Both groups were similar in uterine PI, RI, and S/D ratio. Vascular parameters of the uterine artery are listed in Table 2. Both groups were similar in uterine PI, RI, and S/D ratio. Table 3 shows endometrial and subendometrial blood flow indices for both groups. Endometrial blood flow was lower in the nonpregnant patients compared to the pregnant patients. Endometrial VI, FI, and VFI were all significantly higher in the pregnant group, whereas subendometrial VI, FI, and VFI were similar for the two groups. The ROC curve was analyzed for endometrial and subendometrial blood flow parameters to assess their predictive values for pregnancy. The area under the curve was significantly different for endometrial VI, FI, and VFI, but not for subendometrial VI, FI, and VFI (Table 3). The best predictive rate was achieved for endometrial VI >0.95, with a sensitivity of 71.7% and a specificity of 68.9%; endometrial FI >12.94, with a sensitivity of 72.5% and specificity of 68.2%; and endometrial VFI >0.15, with a sensitivity of 65.4% and specificity of 72.8% (Fig. 2).

Discussion

For evaluating restricted tissue angiography, 3D PD-US is the most useful diagnostic tool, both for showing and calculating relevant parameters. This advanced ultrasonographic technology has been investigated in clinical practice for several years [3,5,8]. An advantage of power Doppler ultrasound is extreme sensitivity to low or slow vascular flow, which can identify overlapping vessels. This technique displays total flow in a confined area, providing images similar to angiography [9]. The three-dimensional display with power Doppler imaging allows clinicians to see dimensions interactively, rather than reconstructing sectional images. In restricted areas, the calculated vascularity based on power Doppler ultrasound can be used as a comparison for various clinical applications. For infertility, the restricted area of the endometrium is important for endometrial receptivity.

In artificial reproductive technology (ART), the predictive value of endometrial thickness for pregnancy is still controversial. Several studies demonstrated a significant relationship between endometrial thickness and pregnancy rate, whereas others showed none, possibly because of different protocols. Endometrial thickness can be influenced by mechanical stimulation such as

| Table 1 | Clinical characteristics for pregnant and non-pregnant groups using a fresh GnRH agonist protocol. |
|-------------------|-----------------------------------|-----------------------------------|
|                    | Pregnant group (n = 103)            | Non-pregnant group (n = 131)      |
| Age (y)            | 32.16 ± 3.81                       | 33.24 ± 5.28                      | 0.103*   |
| Duration of infertility (y) | 2.84 ± 1.87          | 3.21 ± 1.92                      | 0.265*   |
| Body mass index (kg/m²) | 23.29 ± 1.12       | 24.58 ± 1.42                     | 0.566*   |
| Basal FSH (U/mL)   | 6.25 ± 1.69                       | 6.67 ± 2.11                      | 0.078*   |
| Basal LH (U/mL)    | 4.99 ± 3.21                       | 3.69 ± 2.92                      | 0.007*   |
| Basal E₂ (pg/mL)   | 25.51 ± 15.32                     | 26.13 ± 16.05                    | 0.884*   |
| E₂ on hCG administration day (pg/mL) | 2029.51 ± 1231.75 | 1898.77 ± 985.75                 | 0.053*   |
| Total dosage of rFSH | 2515.23 ± 1025.25                      | 2827.12 ± 1535.64                | 0.078*   |
| No. of retrieved oocytes | 13.45 ± 6.52                  | 11.79 ± 7.14                     | 0.054*   |
| Use of ICSI        | 47 (45.6)                         | 61 (46.5)                        | 0.644**  |
| Endometrial thickness (mm) | 12.72 ± 3.19              | 12.39 ± 2.58                     | 0.365*   |
| Endometrial volume (mL) | 6.52 ± 2.85                 | 6.31 ± 2.49                      | 0.081*   |
| No. of transferred embryos | 2.35 ± 0.52              | 2.46 ± 0.60                      | 0.097*   |

Data are presented as n (%) or mean ± SD. *By Student t test. **By χ² test. ICSI = intracytoplasmic sperm injection.

| Table 2 | Uterine vascularity indices evaluated by Doppler velocimetry in pregnant and nonpregnant groups on the day of embryo transfer. |
|-------------------|-----------------------------------|-----------------------------------|
| Parameters        | Pregnant group (n = 103)            | Non-pregnant group (n = 131)      |
|                   | P*                                |
| Pulsatility index | 2.14 ± 0.52                       | 2.48 ± 0.78                      | 0.256    |
| Resistance index  | 0.91 ± 0.45                       | 0.87 ± 0.38                      | 0.527    |
| Systolic/diastolic ratio | 5.67 ± 1.49               | 5.29 ± 1.85                      | 0.365    |

Data are presented as mean ± SD. *By Student t test.

| Table 3 | Endometrial and subendometrial vascular parameters and cutoff values for pregnancy evaluated by three-dimensional power Doppler ultrasound on the day of embryo transfer. |
|-------------------|-----------------------------------|-----------------------------------|
| Parameters        | Pregnant group (n = 103)            | Non-pregnant group (n = 131)      |
|                   | Area Cut-off p**               | Area Cut-off p**               |
| Endometrial VI (%) | 0.87 ± 1.14                      | 0.31 ± 0.63                      | 0.001    |
| Endometrial FI (%) | 18.72 ± 14.74                     | 10.06 ± 9.07                     | 0.000    |
| Endometrial VFI (%) | 0.30 ± 0.80                      | 0.06 ± 0.16                      | 0.021    |
| Subendometrial VI (%) | 1.47 ± 1.66                      | 1.38 ± 1.63                      | 0.770    |
| Subendometrial FI (%) | 27.62 ± 13.69                     | 24.87 ± 11.85                    | 0.252    |
| Subendometrial VFI (%) | 0.49 ± 0.58                      | 0.41 ± 0.50                      | 0.451    |

Data are presented as mean ± SD. *By Student t test. **By Pearson correlation test. Area = area under the receiver operating characteristics curve; FI = flow index; VFI = vasculation index; VI = vascularization index.
the effect of endometrial thickness or volume on the pregnancy occurred with an endometrial volume used to predict the pregnancy success rate [11], based on the high volumes. Three-dimensional ultrasound can be both a tool for ART and between the myometrium and endometrium for distinguishing these common in ART. This technique provides accurate contrast be-

Several factors may affect the endometrial neomicrovascularization, which are age, medication such as aspirin, omega-3, or sildenafil, menstrual days, individuality, smoking, serum testosterone, and anti-Müllerian hormone [18]. Little information is known regarding the mechanisms and control of angiogenesis in the endometrium. Supraphysiological estrogen level in serum may have negative effects on endometrial angiogenesis. Endometrial and subendometrial blood flows were considered significantly lower in the stimulated cycles than the natural cycles [19]. Thus, minimal stimulation is expected for the better outcome. Smoking is, also, considered prohibiting endometrial vascularization [18]. In the life style modification, smoking must be discouraged.

The radial artery is the smallest detectable artery using color Doppler. Endometrial flow is from the radial artery, which turns after passing through the myometrial—endometrial junction [19]. Implantation occurs in the endometrium, and a brief investigation of the uterine artery is not representative of pregnancy success [3]. Many studies have investigated the correlation between vascular indices such as PI of the uterine artery or spiral artery [20–22]. However, Doppler studies have not been conclusive of pregnancy success. However, targeted evaluations of the endometrium have suggested novel areas for research on ART. The assessment of blood flow or microvascularization in the endometrium is possible with power Doppler ultrasound, and these parameters can be quantified as markers of the target area. Power Doppler imaging is generally superior to color Doppler imaging for detecting low-velocity flow and visualization of small vessels [23]. The combination of three-dimensional ultrasound and power Doppler imaging offers a clinically feasible, fast, simple, and reproducible tool for examining the blood supply to the entire endometrium or regions of interest [5]. This method can also quantize vascular parameters such as VI, FI, and VFI, which are calculated from the total number of color voxels and flow intensity [24].

Many studies examined endometrial and subendometrial vascularization for a correlation with IVF outcomes [9, 25, 26]. Vascularity differs by cycle and by COS protocol. In this study, the indicative category was restricted to unexplained infertile couples with no medical disease, a GnRH long protocol, and fresh IVF–ET cycles. Repetitive recruitment was not used to avoid selection bias. The complete enumeration survey was undertaken during a relatively short period at three infertility centers, and the analysis of ultrasound data transmitted from the centers was performed by a single blinded investigator using software and a desktop computer. After a follow-up period, another investigator analyzed the relationship with pregnancy.

This study is unique in examining endometrial and subendometrial blood flows using 3D PD-US to determine their relationship with a pure GnRH agonist long protocol in fresh IVF–ET cycles. The results on the vascularity of targeted areas could not be compared to other studies because the calculated parameters might differ with setting modes. However, the relationship between endometrial blood flow and pregnancy rate in this study suggested that higher vascularity is advantageous for IVF–ET success in a GnRH agonist long protocol. These results were similar to a study with a pure thawing ET protocol [5]. Although blood flow

hysterosalpingography or previous curettage, or by the ovarian stimulation regimen [8]. Endometrial volume has been the most important parameter for endometrial receptivity for monitoring COS, with some researchers suggesting an endometrial thickness cutoff of 7–8 mm [10].

Three-dimensional transvaginal ultrasound is increasingly common in ART. This technique provides accurate contrast between the myometrium and endometrium for distinguishing these features and calculating the endometrial volume or targeted volumes. Three-dimensional ultrasound can be both a tool for ART and used to predict the pregnancy success rate [11], based on the high reproducibility of the 3D measurement parameters. The accepted volume for endometrial receptivity has a threshold of 3.2 ml for establishing a pregnancy, with a negative predictive value of 96% in frozen–thaw cycles. However, the positive predictive value is reported to be only 28.6% [12]. In another study, no pregnancy occurred with an endometrial volume <1 ml in IVF–ET cycles [13].

Endometrial vascularization is not conclusive, but favorable for the effect of endometrial thickness or volume on the pregnancy rate in ART. Thus the impact on pregnancy of endometrial vascularization should be investigated [14,15]. Endometrial vascularization is considered to be important in the early endometrial response to blastocyst implantation, and vascular changes can influence uterine receptivity [16]. Endometrial neomicrovascularization significantly increases during the follicular and early luteal phases, suggesting that endometrial vascular changes affect endometrial growth and implantation [17].

Fig. 2. Receiver operating characteristic curves of vascular parameters for pregnancy in three-dimensional power Doppler ultrasound. (A) Endometrial VI, FI, and VFI and (B) subendometrial VI, FI, and VFI. FI = flow index; ROC = receiver operating characteristic; VFI = vascularization flow index; VI = vascularization index.
parameters using 3D PD-US are relative values, the cutoff values for pregnancy success were VI ≥0.95, FI ≥12.94, and VFI ≥0.15 in the endometrium, but not in the subendometrium. These data suggested that increased blood flow in the endometrium reflected favorable endometrial receptivity.

In this study, comparisons showed no significant differences between pregnant and nonpregnant groups in basic clinical characteristics including age. No correlation was observed between pregnancy success and endometrial thickness, endometrial volume, subendometrial, or uterine artery vascularity between the two groups. Relatively young women visited the infertility centers, decreasing the age difference between the groups and clarifying the results. However, a limitation of this study was it included only women treated with a fresh GnRH agonist long protocol and an area length of 5 mm in the subendometrial region. In spite of the advantage of the prospective study design, the simplified protocols cannot be applied to IVF success in other protocols such as a GnRH antagonist protocol, thawing ET cycle, or mixed protocol. In addition, this study did not compare rFSH types. Further, large-scale studies are expected on IVF success measuring multiple vascular parameters in several endometrial and subendometrial sections, using various COS protocols.

In conclusion, 3D PD-US was an effective and useful tool for mapping endometrial and subendometrial blood flow, even on the morning of ET. Excellent endometrial blood supply, which is indicative of endometrial receptivity, was associated with successful IVF outcome. No correlation was observed between subendometrial or uterine artery blood flow and pregnancy. These findings suggest that endometrial evaluation with 3D PD-US can be a useful prognostic protocol for pregnancy in infertile women undergoing a fresh GnRH agonist long protocol.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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