

Assesment of Endometrial Vascularization with Immunohistochemical CD34 Assay in Recurrent Pregnancy Loss: A Case Control Study

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ABSTRACT

OBJECTIVES: We compared the endometrial vascularization in hysteroscopic endometrial samplings between recurrent pregnancy loss (RPL) and control group.

STUDY DESIGN: We prospectively evaluated hysteroscopic endometrial samplings from recurrent pregnancy loss and control groups. CD34 transmembrane protein was used for evaluating endometrial vascularization. The vascularization was assessed based on thickness of vessels, diameter of the largest vessel, and number of vessels per mm² in CD34-stained slides.

RESULTS: There was no significant difference in demographic findings and vascularization, such as largest vessel diameter (p: 0.572), and number of vessels per mm² (p: 0.982) between the two groups.

CONCLUSION: The cycling endometrium is a highly angiogenic tissue and may play a role in the etiology of RPL. However, we find a weak relationship between endometrial vascularization and recurrent pregnancy loss.

Keywords: Recurrent pregnancy loss, Vascularization, Endometrium, CD34

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Introduction

Recurrent pregnancy loss (RPL), a condition of three or more consecutive pregnancy losses before the pregnancy reaches 20 weeks, is observed in 1% of women in their reproductive age (1). Recently, RPL is defined as two consecutive pregnancy losses, a health problem that affects 5% of women. Many studies have been conducted to find an association between RPL and genetic, immune, infectious, and endocrine disorders (2-6). However, its etiology is not known in more than 50% of the RPL cases (2). In this study, we investigate the con-

tribution of vascularization to RPL (6). Endometrial tissue is regenerated dynamically and continuously during menstrual cycle, making vascularization essential to the endometrium. Previous studies have investigated vascularization in decidua and placenta in RPL cases and its indirect association with various factors (7,8). The main purpose of the present study is to compare endometrial angiogenesis/vascularization in RPL and healthy fertile women. To analyze the endometrial vascularization in non-pregnant RPL cases, we used CD34, which is a transmembrane protein (9). It was previously used for evaluation of vascularization in decidua and comparison of parietal and basal decidua vascularization (7,10). In this study, we aim to demonstrate the effect of vascularization in RPL.

Material and Method

Selection of patients and inclusion and exclusion criteria

Patients with a history of RPL, wishing to have a child, and referred to the outpatient clinic of Obstetrics and Gynecology Department of a tertiary research hospital were included in the study. Recurrent abortion was considered as at least 2 or more consecutive spontaneous pregnancy losses before 20 weeks of gestation (11). The controls were healthy women referred to the outpatient clinic for annual routine gynecologic control during the same period. Women with a history of abortion, irregular menstruation, and who received hormonal therapy 3 months prior to biopsy were excluded. (Figure 1). Patients incidentally detected with an endometrial pathology, such as polyp, in office hysteroscopy examination were excluded from the study. Women who had an abortion at


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least 4 months before were included in the patient group. For all patients selected in the study, sexual intercourse after the last menstrual period was forbidden.

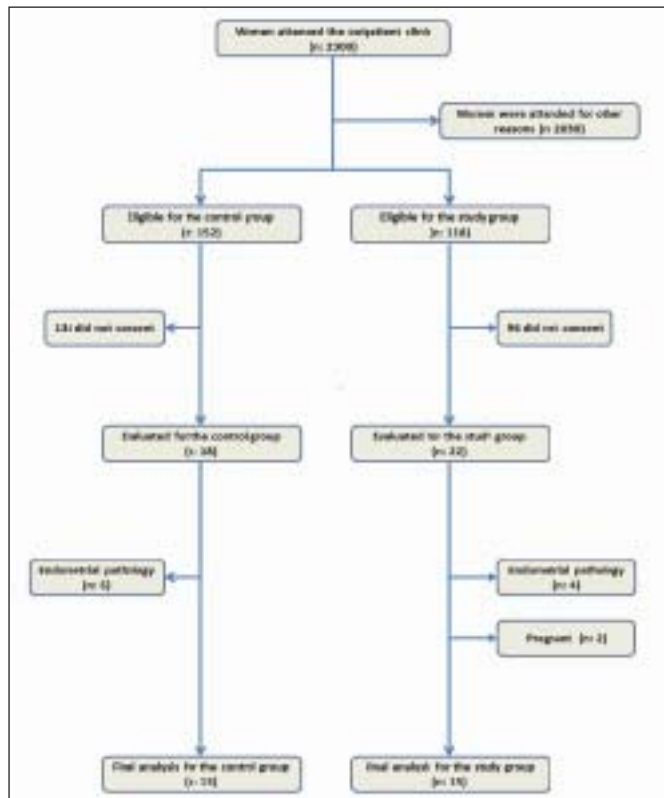


Figure 1: Patient selection

Preparation of tissue specimens

Tissue specimens were taken during office hysteroscopy using hysteroscopic forceps and a rigid standard hysteroscopy (Karl Storz) was used for the procedure. The endometrial biopsy specimens were taken during the luteal phase of menstrual cycle.

This study was approved by the institutional ethics review board (reference number: 2012/33) and registered with ClinicalTrials.gov (identifier: NCT01735331). Oral and written information was given to the participating patients; subsequently, their written informed consent was taken.

Histopathological assessment

The tissue specimens were fixed in 10% formalin and embedded in paraffin. Sections of 4-5- μ m thickness were processed for microscopic slides. The slides were stained with hematoxylin and eosin. For immunohistochemical assays, the sections were passed through alcohol and xylol protocol at room temperature for deparaffinization. The sections were kept in 0.3% w/v H₂O₂ in distilled water and washed with PBS for 3-5 min (PBS: Phosphate buffered saline, pH 7.2 - 7.4). The sections then underwent antigen retrieval procedure with citrate buffer (pH 6) in a microwave oven at medium setting for 15 min. Subsequently, the chalet with closed lid was cooled at room temperature for 45 min. Then, the sections

were passed through distilled water and PBS. The sections were incubated with normal blocking serum for 8-10 min; subsequently, the solution on the tissue was discarded and area around the tissue was wiped dry. Primary antibody binding was determined for CD34; the reaction was revealed with DAB, and the slides were counterstained with Mayer's hematoxylin and mounted.

Evaluation of vascularization

A single experienced pathologist who was unaware if patients were from RPL or control group performed all pathological assessments. A microscope (BX51, Olympus America, Inc., Melville, New York) was used to examine the tissue sections; 4-6 tissue sections from each patient were examined for evaluation. In each section, eight fields with 200x magnification were studied. The vascularization was evaluated according to the thickness of vessels, diameter of the largest vessel, and the number of vessels per mm² in CD34-stained slides (Figure 2 and 3).

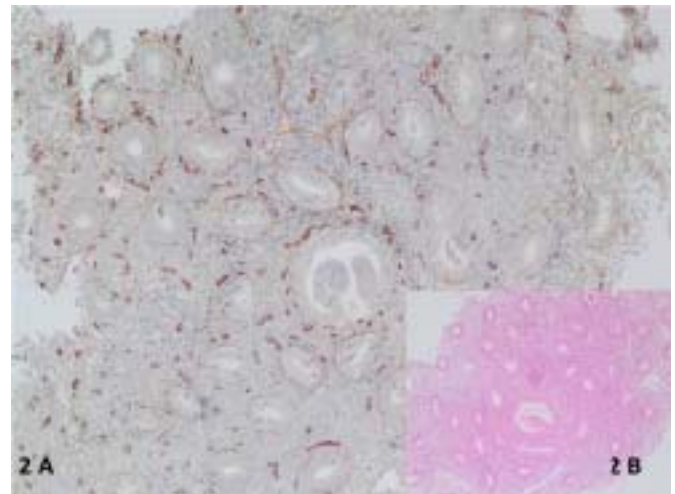


Figure 2A: CD34 staining revealed vascular structure of endometrium (x10), B: H&E stain section of same slide (x10).

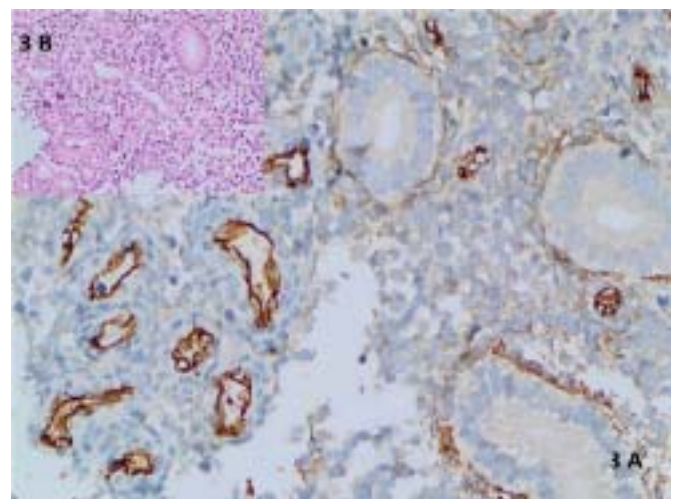


Figure 3A: Thick wall vessels (CD34 stained x40), the vascularization was evaluated according to the thickness of vessels in CD34-stained slides, B: H&E stained section of same slide (x40).

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences v.18.0 for Windows (SPSS, Inc., Chicago, IL). Variables were investigated by using visual and analytical methods (Kolmogorov-Smirnov test/Shapiro Wilk's test) to determine their normal distribution. The Student's t-test was used to compare the normally distributed variables. Because diameter of the large vessels was not normally distributed, Mann-Whitney U test was used to compare the groups. Chi-square test or Fisher's exact test was appropriately used to compare the proportions in case and control groups. A p-value of 0.05 was considered as statistically significant.

Results

Age, number of gravidity, and abortions in the patient and control groups are summarized in table 1. There was no abortion in the control group. In the RPL group, median number of abortions was three, and its interquartile range was 2-4. There was no difference in age and number of gravidity between the case and control groups. Pregnancies of all women in the control group ended in healthy births. In the RPL group, the thickness of vessels per magnification field was 53.3%, the diameter of the largest vessel was 0.16 ± 0.6 mm, the vessel count per magnification field was 30 (range: 47-22); there was no significant difference in vascularization between the two groups (Table 2).

Discussion

The aim of this study was to compare endometrial vascularization of women in RPL and control groups with successful pregnancies. Our results show that there was no difference in endometrial vascularization between the two groups.

A previous study compared endometrial vascularization in

healthy fertile women in RPL cases using 3-dimensional Doppler angiography, and reported a significant decrease in vascularization in the RPL cases (12). Similarly, another indirect comparative study by Doppler angiography reported an impairment of endometrial vascularization in antiphospholipid antibody-positive RPL cases (13). In the present study, we directly assessed vascularization in the tissue. Our comparison was optimal because number of pregnancies in both the groups was equal, patients had regular menstrual periods, and we applied strict criteria to avoid other factors that could affect vascularization.

Direct assessment of endometrial vascularization was previously conducted by two studies. One study involved histopathological evaluation of decidual vascularization in women with missed abortion that were diagnosed by sonographic examination and clinical findings (8). The control group consisted of women with viable intrauterine pregnancy referred for medical abortion, and the decidual vascularization in both the groups was compared. The prognosis of pregnancies in the control group could not be foreseen. The prevalence of abortion in normal populations is 8%-20% (14) and some patients in the control group could have suffered abortion in the absence of medical abortion. This makes the control group unreliable; thereby, rendering the study imperfect.

In the other study, vascularization in endometrial pipelle biopsy material of women with a history of RPL was compared with vascularization in hysterectomy specimens of controls (8). For optimal evaluation, it is essential that the patients have regular menstrual periods. The possible presence of irregular menstruation or menopause in the control group, and higher mean age of the hysterectomy group exposes limitations in the study. In contrast, the subjects in the patient and control groups in our study were selected almost perfectly.

Table 1: Demographic features of the patients

	RPL group (n=15)	Control group (n=13)	p value
Age	31.4±6.8	35.6±7.6	0.137 ^a
Gravidity	4 (3.0-5.0)	3 (4.5-2.0)	0.156 ^b
Live birth	1 (1.0-0.0)	3 (4.5-2.0)	< .000 ^b

RPL: Recurrent pregnancy loss, Row data are mean ± standard deviation, a: Student's t test; Row data are median (interquartile range), b: Mann-Whitney Rank Sum test

Table 2: Comparison of vasculogenesis in the recurrent pregnancy loss and control groups

	RPL group (n=15)	Control group (n=13)	p value
Largest vessel diameter	0.16±0.6	0.14±0.9	0.572 ^a
Thick vessel count per magnification field	53.3%	23.1%	0.102 ^b
Vessel count per magnification field	30 (47-22)	28 (22.5-40.5)	0.982 ^c

RPL: Recurrent pregnancy loss, Row data are mean ± standard deviation a: Student's t test, Row data are proportion, b: Chi-square test, and Row data are median (interquartile range), c: Mann-Whitney Rank Sum test

A major limitation of our study is the limited number of patients included. This was because of the strict criteria of patient selection. In addition, some subjects in the control group refused the invasive methods of hysteroscopy and biopsy because they showed no pathological finding and had no history of abortion. Nevertheless, we could make statistically significant evaluation. Future studies on etiologic factors other than endometrial vascularization or different methods of assessing vascularization are required. Different studies continue to be carried out in the literature that address this issue.

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