

# Clinical value of histologic dating of the endometrium for personalized frozen-thawed embryo transfer (pFET) in patients with repeated implantation failure (RIF) in natural cycles

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## Research article

**Keywords:** histologic endometrial dating, endometrial receptivity, personal embryo transfer, window of implantation

**Posted Date:** March 30th, 2020

**DOI:** <https://doi.org/10.21203/rs.2.16202/v3>

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**Version of Record:** A version of this preprint was published on September 11th, 2020. See the published version at <https://doi.org/10.1186/s12884-020-03217-y>.

# Abstract

**Background:** Displacement of the window of implantation (WOI) has been proposed as an important factor contributing to RIF. However, histologic dating of the endometrium as a diagnostic tool of endometrial receptivity has been questioned.

**Methods:** This is a prospective intervention trial that entailed 205 infertile patients from July 2017 to December 2017. Endometrial biopsies from 50 good-prognosis patients were conducted on day 3 (n=6), 5 (n=6), 7 (n=26), 9 (n=6) or 11 (n=6) post-ovulation (PO+3/5/7/9/11) of the previous natural cycle before their conventional frozen-thawed embryo transfer (FET) cycle. We conducted endometrial biopsies of 155 RIF patients on day PO +7.

**Results:** The verification of the Noyes criteria for endometrial dating was conducted at different times (PO +3/+5/+7/+9/+11) on 41 good-prognosis patients who achieved an ongoing pregnancy in their first conventional FET cycle after endometrial biopsy. The agreement between two pathologists for endometrial biopsy dating in infertile patients was determined to be acceptable (weighted kappa = 0.672,  $P < 0.001$ ). The rate of out-of-phase dating on day PO+7 was significantly higher in RIF patients than good-prognosis patients (31.6% vs . 3.8%,  $P=0.003$ ). pFET was performed in 47 RIF patients diagnosed to be out of phase, and the cumulative live-birth rate was 61.7%.

**Conclusions:** Histologic endometrial dating of RIF patients in natural cycles may be a biomarker for a receptive endometrium in diagnosing the displacement of WOI.

## Background

Repeated implantation failure (RIF) is a particular challenge that is defined upon ultrasonographic examination as the absence of a gestational sac 5 or more weeks after an embryo transfer (ET) subsequent to three previous embryo transfers with high-quality embryos, or after the transfer of  $\geq 10$  embryos in multiple transfers [1]. RIF can be caused by both maternal and embryonic factors [2], and blastocyst culture and preimplantation genetic screening can partially improve the outcome of pregnancy through better embryo selection. The uterus, an important player in implantation that may be affected by polyps, intrauterine adhesions, uterine fibroids, adenomyosis, endometritis, and uterine malformations, has also been demonstrated to contribute to embryonic implantation failure [4]. Different strategies have been developed to improve the outcome of pregnancy for the aforementioned diseases, but unexplained RIF remains a challenge.

Endometrial receptivity has been frequently evaluated as one of the many uterine factors involved in RIF, but the relationship remains controversial. Several endometrial markers—such as the presence of pinopods, immunohistochemical biomarkers, endometrial blood flow and wave—have been used to determine uterine receptivity. These biomarkers may interact transiently with the embryo at implantation, but they appear to be unreliable in evaluating receptivity, particularly as precision indicators for use as clinical diagnostic tools [5-7].

The endometrium becomes receptive as a result of a series of timed hormonal events during the menstrual cycle. The exposure of the endometrium to progesterone after ovulation initiates morphologic and functional alterations that result in a change from a pre-receptive to a receptive endometrium. With the secretion of progesterone, subnuclear vacuoles are found in epithelial cells during the early secretory phase, the secretory products (in vacuoles) within the glandular epithelium cells discharge to the glandular lumen, and stromal edema becomes maximal in the middle secretory phase—all of which contributes to blastocyst adhesion and invasion. Edema is also less marked at this time, and a predecidual reaction begins around the blood vessels, which contributes to embryonic implantation. The morphologic changes observed histologically for each specific day after ovulation were described by Noyes and his colleagues in 1950 (designated as Noyes criteria) [8]. An endometrial biopsy that shows a difference of more than 2 days between the histologic dating and actual day after ovulation is considered to be “out of phase” [9]. However, the clinical application of the Noyes criteria is relatively limited, as an out-of-phase endometrium was found in 5%–50% of fertile patients [10-12]. The large variation in researchers’ results may be due to the inaccurate determination of the day of ovulation.

Previous investigators demonstrated that the use of classic histologic dating of endometrial biopsy samples to estimate the timing of the window of implantation and to adjust embryonic transfer time may increase the implantation rate in hormone replacement therapy (HRT) cycles of patients with out-of-phase classical dating of the endometrium [13]. The clinical value of histologic endometrial dating in RIF patients in natural cycles is, however, yet to be realized. In the present study, we investigated the clinical effects of pFET in unexplained RIF patients according to the histologic dating of endometrial biopsies under ultrasound-guided ovulation monitoring during natural cycles.

## Methods

### Study population

We evaluated a total of 205 infertile patients and created two phases for our study. In phase I, a total of 50 good-prognosis patients underwent endometrial biopsy at different time-points (PO+3/5/7/9/11) (Fig. 1). The histologic profiles of good-prognosis patients who were pregnant in their first conventional FET cycle were then collected as fertility parameters. For the good-prognosis patient group we enrolled women aged 20–35 who underwent FET in a natural cycle. In phase II, 155 patients with unexplained RIF were recruited to evaluate their endometrial dating on PO +7 (Fig. 1). According to an ESHRE PGD consortium, RIF was defined as the absence of a gestational sac upon ultrasonographic examination at 5 or more weeks subsequent to three embryo transfers with high-quality embryos, or after the transfer of  $\geq 10$  embryos in multiple transfers [1]. Patients with uterine abnormalities (double uterus, bicornuate uterus, unicornuate uterus, and uterine mediastinum), intrauterine adhesions, endometriosis, adenomyosis, hydrosalpinx, or uterine fibroids (submucosal fibroids, non-mucosal fibroids > 4 cm and/or endometrial pressure) were excluded from the unexplained-RIF group. In both groups, patients demonstrated a menstrual cycle length of 24–35 days and an indication for ovarian stimulation before in vitro fertilization/ intracytoplasmic sperm injection (IVF/ICSI). This study was approved by the Ethics

Committee of the Reproductive and Genetic Hospital of CITIC-XIANGYA (LL-SC-2017-007) (June 29, 2017). Although we began to recruit the patients on the initial release date of our clinical trial, we discovered an advantage of histologic dating of endometrial biopsy samples when we designed and organized the data of the first clinical trial (NCT03222830). We then increased the sample size of our study, which was of major importance in recruiting RIF patients and good-prognosis patients for histologic dating for the second clinical trial (NCT03312309).

### **Ovulation monitoring**

All patients were monitored throughout a natural cycle with a daily ultrasonographic scan from the 10<sup>th</sup>–12<sup>th</sup> days of the menstrual cycle when the largest follicular diameter was 16 mm, and until the dominant follicle disappeared. Urinary LH concentrations were assessed simultaneously when the follicular diameter was 16 mm. The day of dominant follicle disappearance was considered to be the day of ovulation (post-ovulation +0, PO+0).

### **Endometrial biopsy**

Endometrial biopsy was performed using a sterile pipelle (Laboratory CCD, China), and the tissue was stored in Hank's Balanced Salt Solution (Life Technologies, Grand Island, NY) on ice for further processing.

### **Histologic analysis and dating**

Endometrial tissue was rinsed in chilled PBS followed by 10% neutral-buffered formalin fixation and paraffin embedding (FFPE). FFPE tissues were sectioned at a 6-mm thickness for hematoxylin and eosin (H&E) staining. All H&E-stained endometrial biopsies were analyzed in a blinded manner for the evaluation of endometrial dating and glandular and stromal development. The verification of endometrial dating was established according to the Noyes dating criteria [8].

### **Personal frozen embryo transfer/conventional frozen embryo transfer protocol**

For the FET cycle, no more than two embryos were transferred to each patient. Embryos were warmed using a commercially available warming solution (Kitazato Biopharma), according to the instructions of the Kuwayama kit [14]. After warming, embryos were transferred to G1.5/G2.5 medium and cultured for 2–6 hours. Only cleavage-stage embryos that exhibited >50% intact blastomeres or blastocysts that re-expanded after warming were considered as surviving and suitable for transfer. The cleavage-stage embryos or blastocysts were transferred 3 or 5 days, respectively, after ovulation regardless of endometrial dating for good-prognosis patients, or 4-7 days after ovulation depending upon the endometrial dating of the RIF group. We applied luteal support when the dominant follicle disappeared and when we observed satisfactory endometrial development (thickness  $\geq 8$  mm as confirmed upon ultrasonographic examination): we administered 40 mg of dydrogesterone (Abbott Biologicals B.V.) until the 28<sup>th</sup> day of embryo transfer if a pregnancy test was positive.

## Clinical outcomes and statistical analysis

We defined the cumulative live-birth rate of repeated FET cycles during the study period as the probability of a live birth from all patients during the study period. Ongoing pregnancy was defined as at least one intrauterine gestational sac with cardiac activity by ultrasonography performed 6 weeks after ET. Biochemical pregnancy was defined as a positive hCG test in the absence of an intrauterine gestational sac. Analyses were performed using the statistical package SPSS, version 19.0 (SPSS) or (SAS®) version 9.3 (SAS Institute, Inc., Cary, NC, USA). Continuous variables are presented as means  $\pm$  SD, and comparisons were made using a one-way ANOVA or non-parametric statistical test. Categorical data were presented as a number (N) and percentage (%), and comparisons were made using a Chi-square or Fisher Exact-Probability test. A weighted kappa statistic was calculated to summarize the overall agreement between pathologist A and pathologist B. Bland–Altman plots were drawn to evaluate systematic biases of endometrial dating between pathologists A and B. We used GraphPad Prism7.0 to evaluate the intra-group difference (means  $\pm$  SD) in the control group.  $P < 0.05$  was considered to be statistically significant.

## Results

### Verification of Noyes criteria

Standard parameters of endometrial histologic dating were established from the good- prognosis patients who were pregnant in their first conventional FET cycle ( $n=41$ ). And all the good prognosis patients with ongoing pregnancy in this study have got live birth. With respect to PO/dating +3, gland nuclei were pushed to the center of the epithelial cells with cytoplasm above, with vacuoles below. For PO/dating +4, glandular nuclei returned to the basilar side of the cells. We noted that wisps of secretory material appeared in the lumina, and some vacuoles were pushed past the nucleus, apparently emptying their glycogen into the lumen. Mitosis and pseudostratification of nuclei were absent. For PO/dating +5, only a few vacuoles remained. For PO/dating +7, tissue edema—though variable in the proliferative phase— was characteristically notable in the mid-secretory stage, becoming evident rather suddenly. For PO/dating +9, the spiral arterioles (which were previously somewhat difficult to distinguish in the edematous stroma), became much more prominent. For PO/dating +11, pre-decidua began to differentiate under the surface epithelium (Fig. S1). These results were consistent with the Noyes dating criteria [8].

### The agreement of blinded endometrial dating and the criteria for endometrial dating

All the endometrial dating ( $n = 205$ ) were evaluated by two experienced pathologists. The inter-observer agreement was determined to be good (weighted kappa = 0.672; 95% CI 0.606–0.737;  $P < 0.001$ ). As shown in Fig. 2, Bland-Altman (B-A) plots of pathologist A and pathologist B highlighted trends regarding differences in endometrial dating between the two pathologists. The limits of agreement indicated that the difference value for dating an endometrial biopsy was  $\leq 1.76$ , but that the endometrial dating by both pathologists was clinically consistent when the value was  $\leq 2$ . Thus, the B-A plots suggested that

agreement was good between pathologists A and B when the value was  $\leq 2$  by using the Noyes criteria in the same patients.

### **Out-of-phase endometrial dating**

Endometrial dating standards for different days (PO+3/5/7/9/11) were established in good-prognosis patients who achieved an ongoing pregnancy in their first conventional FET cycle (n=41) (Fig.1). Two experienced pathologists confirmed the endometrial dating by Noyes criteria in the good prognosis patients group, showing that the endometrial dating at different times was significantly different. In contrast, the inner-group differences were so small that the endometrial dating of most good-prognosis patients showed a mean  $\pm$  SD between the lower and upper limits. The exception was only 1 endometrium of a good-prognosis patient that was biopsied on PO+7 but dating as PO+3, and who was pregnant in a conventional FET cycle (Fig. 3A).

Blinded histologic dating of endometrial biopsies from RIF (n = 155) or good-prognosis patients (n = 26) before frozen-thawed embryo transfer day PO+7. The rate of out-of- phase dating on day PO +7 was significantly higher (31.6% vs 3.8%, P = 0.003) in the RIF group relative to the good-prognosis group (Fig. 3B).

A total of 49 RIF patients were evaluated as being out of phase and 106 RIF patients were assessed as in phase on day PO +7. One third (n = 35) of in-phase patients were dated +7, while the remainder (n = 71) of in-phase patients were dated +5. In out-of-phase patients, 24% (n = 12) were dated +3 and 73% (n = 36, Fig. 3C) were dated +4 (Fig. 3C) or +5 (vacuoles remained) (Fig. 3C), and one patient was diagnosed as dated PO +10 (Fig. 3C).

### **Clinical outcomes in RIF patients with endometrial dating results per pFET**

The demographic characteristics and reproductive history of RIF patients—including age, body mass index, duration of infertility, and cause of infertility—are shown in Table 1. Previous failed cycles numbered  $3.6 \pm 0.7$ , with minimal and maximal values of 3 and 5 cycles, respectively. pFET was performed in 47 patients whose personal WOI was delayed by 3 (n = 35) or 4 (n = 11) days, or advanced by 3 days (n=1). Day-3 embryos or Day-5 blastocysts were then transferred using this strategy in natural cycles after 4 to 7 days of ovulation, resulting in a 57.4% (27/47) live-birth rate in the first transfer attempt.

RIF patients who failed to become pregnant after the first pFET had a second endometrial biopsy delayed by 1–2 days according to the results of the first endometrial dating of the RIF group. All of these five patients, then, showed their expected endometrial dating in their second endometrial biopsy. In the second pFET attempt, the live-birth rate was 40% (2/5), and thus the cumulative live-birth rate for personal FET was 61.7%.

## **Discussion**

Implantation is not a single event, but, is more accurately a cascade of interactions between the embryo and endometrium. The human endometrium is receptive to embryonic implantation during a narrow period of the menstrual cycle referred to as the WOI. The WOI had been assumed to be constant for all women, although investigators have recently demonstrated the existence of “displaced WOI” [15-18]. The classic method of dating the endometrium using defined histologic criteria was established in 1950 [8], but pFET studies in natural cycles using the Noyes criteria are presently lacking. In the present study we established the endometrial dating criteria only for pregnant patients in their first conventional FET cycle. All our good-prognosis patients were pregnant with histologic dates of days +5–7 on day PO+7, except for one patient whose histologic dating was PO+3, which is consistent with the definition of being in phase [9]. Endometrial dating was simultaneously evaluated by two experienced pathologists, and the inter-observer agreement was statistically determined to be acceptable. Thus, the endometrial dating criteria were easily mastered and utilized according to the Noyes criteria. Finally, our data indicated that intra-group variation in the good-prognosis patients was so low that we considered our results to be highly reliable. We determined the reproducibility and verifiability of our endometrial dating in the same patients with five RIF patients who failed to become pregnant after the first pFET; these patients underwent a second endometrial biopsy delayed by 1 or 2 days according to the first results of endometrial dating, and showed the expected endometrial dating of the second endometrial biopsy. The second biopsies of the five RIF patients were conducted within 4 months, which suggested that the results of endometrial dating might be repeatable in 4 months. Endometrial dating of a larger sample and longer period of time would still be needed to corroborate reproducibility. Displaced WOI may be detected by endometrial dating, and a subset of the unexplained-RIF patients may benefit from our study data.

This is the first pFET study to entail endometrial dating as verified using the Noyes criteria. Firstly, we verified the Noyes criteria by the endometrium samples of different time points from the good prognosis patients who were pregnant. Secondly, we compared the difference of the out of phase rate/displacement of WOI between RIF patients and good prognosis patients. Lastly but not least, we have individualized interventions(pFET) based on the verified criteria.

There were some limitations to our research. Although the result of previous donor oocyte IVF cycles suggested that recipients age  $\leq 39$  years had similar rates of implantation, clinical pregnancy, and live birth [19]. We couldn't exclude the impact of age on endometrium dating by recruiting the younger female (20-35 years) as good prognosis patients. The primary objective of our study was to use endometrial histology to detect the displacement of WOI in RIF patients, and to evaluate the clinical outcomes by pFET. However, there were no comparative data on clinical pregnancy rates or live-birth rates in women with RIF who had an adjusted FET, or on those who did not. We suggest that a randomized controlled study for RIF patients with out-of-phase dating be undertaken, and that further research be performed to determine the mechanism(s) underlying irregularities in the window of implantation.

## Conclusions

We observed an obviously increased percentage in WOI displacement in RIF patients compared with good-prognosis patients, leading to our proposition of pFET as a treatment strategy. Personal FET according the histologic dating of endometrium may improve the clinical outcomes of unexplained RIF patients.

## Abbreviations

pFET personalized frozen-thawed embryo transfer

RIF repeated implantation failure

WOI window of implantation

PO post-ovulation

ET embryo transfer

HRT hormone-replacement cycle

IVF/ICSI *in vitro* fertilization/intracytoplasmic sperm injection

ERA endometrial receptivity array

## Declarations

### Ethics approval

The study was approved by the Ethics Committee of the Reproductive and Genetic Hospital of CITIC-XIANGYA (LL-SC-2017-007) (June 29, 2017). Written informed consent was obtained from all participants.

### Consent for publication

Not applicable.

### Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

### Competing interests

The authors declare that they have no competing interests.

### Funding

The National Science Foundation of China (81501328). The funding organization had no role in the design of the study, data collection, analysis, interpretation of the data, or in the writing of this manuscript.

### **Authors' contributions**

All authors have read and approved the manuscript.

YL designed the experiment, recruited the samples, analyzed histologic samples, and wrote the manuscript; X FL performed the experiment; L JL, X XF, and R XG organized the data; Y BH analyzed histologic samples; G XL designed the experiment; and GL and FG designed and guided the experiment.

### **Acknowledgments**

We wish to thank the patients who agreed to the collection of their endometrial issues, and we also wish to thank the pathologists who agreed to participate in the procedures described herein.

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## Supplemental File Legends

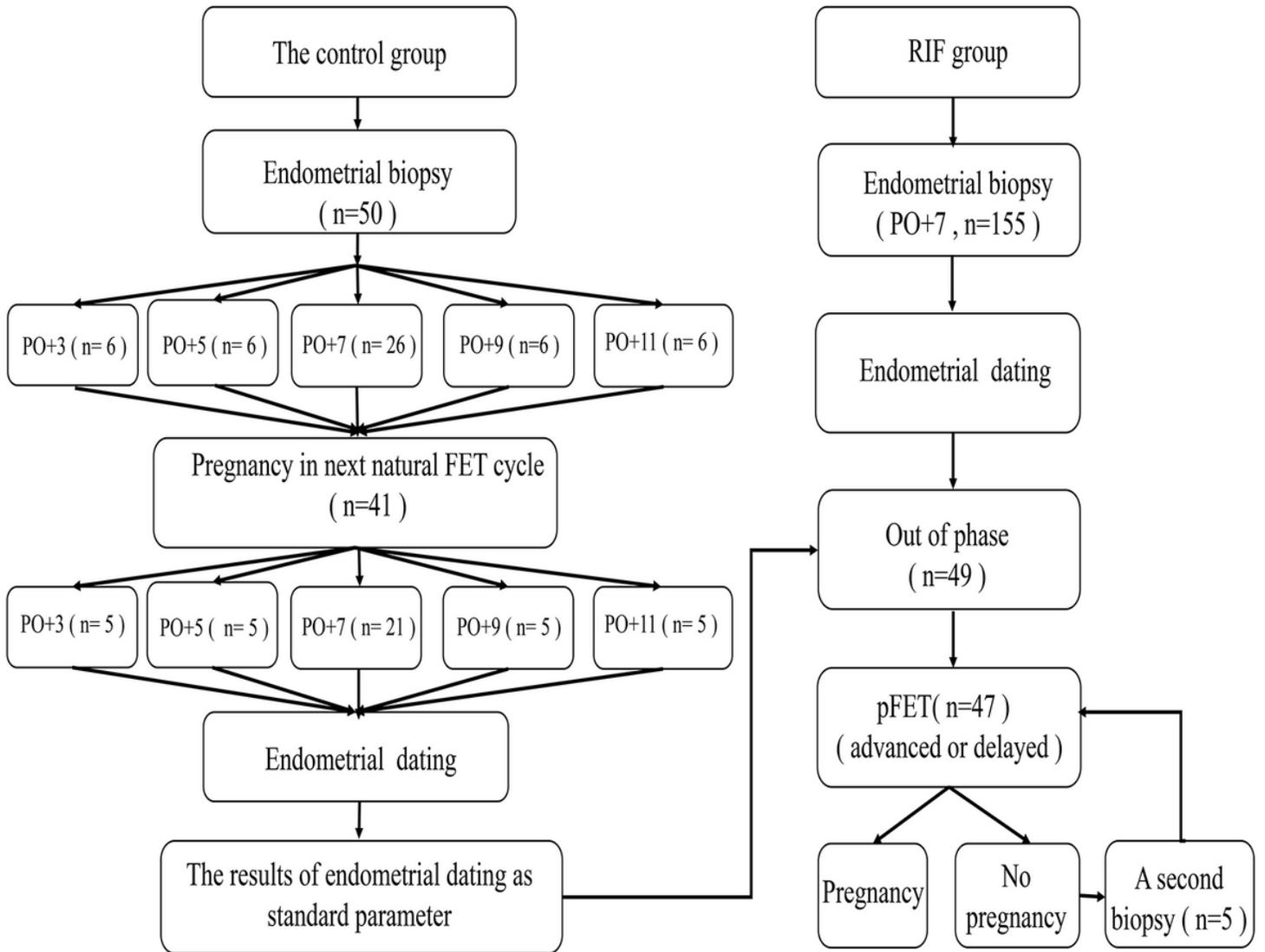
Figure S1. Endometrial specimen dating according to Noyes criteria (X 400). A (dating +3), gland nuclei were pushed to the center of the epithelial cells, with the cytoplasm above and vacuoles below (arrow). B (dating +4), gland nuclei returned to the basilar side of the cells, and some vacuoles (arrow) were pushed past the nucleus to apparently empty glycogen into the lumen. C (dating +5), few vacuoles remained, and the glandular cavity was filled with secretions (arrow). D (dating +7), tissue edema. E (dating +9), glands were highly distorted, jagged, or cauliflower-shaped (arrow). F (dating +11), pre-decidua (arrow) began to differentiate under the surface epithelium.

## Table

**Table 1** Summary of the clinical outcomes of RIF patients

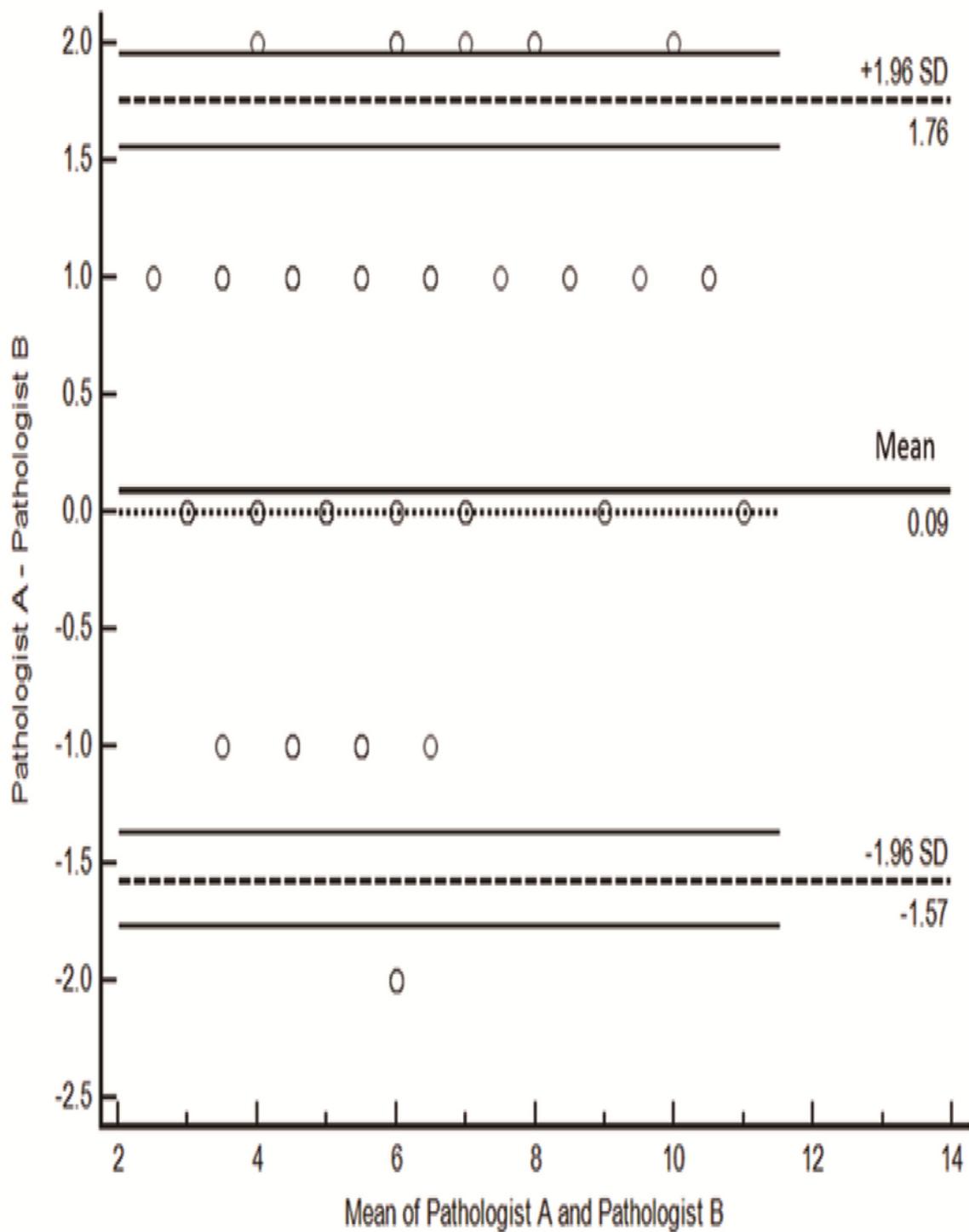
	RIF patients
No. of patients	155
Age (y)	33.0±3.7
Duration of infertility (year)	5.6±2.6
BMI (kg/m <sup>2</sup> )	20.9±1.6
Basal FSH level (mIU/ml)	6.4±2.7
Endometrial thickness on the day of embryo transfer (mm)	11.5±1.5
Cause of infertility	
Male factor	20/155 (12.9%)
Tubal factor	135/155 (87.1%)
No. of previous failed cycles	3.6±0.7
The out of phase rate	49/155 (31.6%)
Total patients with 1st pFET	47
High quality embryo rate	33/47 (70.2%)
Cleavage stage embryo	3/47 (6.4%)
Blastocyst	30/47 (63.8%)
Implantation rate after 1st pFET	32/67 (47.8%)
Ongoing pregnancies rate after 1st pFET	29/47 (61.7%)
Live birth rate after 1st pFET	27/47 (57.4%)
Biochemical pregnancies after 1st pFET	7/47 (14.9%)
Failed pregnancies after 1st pFET	11
No. of 2 <sup>nd</sup> biopsies at the specified day	5
2 <sup>nd</sup> expectant endometrial dating	5
Total patients with 2 <sup>nd</sup> pFET	5
Implantation rate after 2 <sup>nd</sup> pFET	3/7 (42.8%)
Ongoing pregnancies after 2 <sup>nd</sup> pFET	3/5 (60%)
Accumulative live birth rate after pFET	29/47 (61.7%)

# Figures



**Figure 1**

The patients recruited to the control and RIF groups.



**Figure 2**

Bland–Altman plots of variability according to pathologists A and B. The x-axis depicts the mean endometrial sample dating for pathologist A and pathologist B; the y-axis is the difference from the mean endometrial dating between pathologists A and B. The upper and lower lines on the B–A plots represent the limits of agreement and the mean difference  $\pm 1.96$  times its standard deviation. Thus, the distance from 0 and the width of the limits of agreement both indicate the magnitude of disagreement between

pathologists. Closer clustering to the mean indicates higher agreement. If the difference value for an endometrial dating is = 0, then the endometrial dating by pathologist A and pathologist B was identical.



### Figure 3

Endometrial dating in control and RIF groups. A: Inner-group differences in endometrial biopsy dating in the control group at different times (PO+3/5/7/9/11) were rare, as proven by two experienced pathologists. Using the mean  $\pm$  SD as the lower and upper limits to define a reference range for endometrial biopsy dating, 1 of 50 control women were below the range. B: The out-of-phase rate in the good-prognosis group was different from that for the RIF group. C: Endometrial dating according to the Noyes criteria in RIF patients (X 400). Dating +3, gland cytoplasm and nuclei above and vacuoles below (arrow); dating +4, glandular nuclei are in the center of the cells and glycogen vacuoles are seen on two sides of the gland nuclei (arrow); dating +5, vacuoles (arrow) remain in the basement membrane of the gland cells; dating +10, pre-decidua (arrow) begin to differentiate and spiral arteries increase.

## Supplementary Files

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