Clinical value of endometrial histological dating for personalized frozen-thawed embryo transfer (pFET) in patients with repeated implantation failure (RIF) in natural cycle

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Abstract
Background: The displacement of window of implantation (WOI) has been proposed as an important factor contributed to RIF. However, endometrial histological dating as the diagnostic tool of endometrial receptivity has been questioned. Methods: This is a prospective intervention trial including 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) of post-ovulation (PO+3/5/7/9/11) in the previous natural cycle before their conventional frozen-thawed embryo transfer (FET) cycle. The endometrial biopsies of 155 RIF patients were conducted on day of PO +7. Results: The verification of Noyes criterion for endometrial dating was conducted in different time (PO +3/+5/+7/+9/+11) from 41 good prognosis patients achieving ongoing pregnancy in the first conventional FET cycle after the endometrial biopsies. The agreement between two pathologists for endometrium dating in infertile patients was determined to be good (weighted kappa = 0.672; P < 0.001). The rate of out-of-phase dating on the day of 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 accumulative live birth rate was 55.7%. Conclusions: The endometrial histological dating in RIF patients in natural cycle may be a endometrial receptive biomarker for diagnosing the displacement of WOI.

Background
Repeated implantation failure (RIF) is a situation of particular challenge, which is defined as the absence of a gestational sac on ultrasound at 5 or more weeks after embryo transfer (ET) after 3 embryo transfers with high quality embryos or after the transfer of ≥10 embryos in multiple transfers [1]. RIF can be caused by both maternal and embryonic factors [2]. Blastocyst culture and preimplantation genetic screen can partially improve the outcome of pregnancy through better embryo selection. The uterus, an important player in implantation which may be affected by polyps, intrauterine adhesions, uterine fibroids, adenomyosis, endometritis, and uterine malformations, has also been demonstrated to contribute to embryo implantation failure [4]. Different strategies have been developed to improve the outcome of pregnancy for the above diseases, but unexplained RIF is
still challenging.

For a long time, the endometrial receptivity was frequently evaluated as another aspect of uterus factor in RIF patients but was also controversial. Many endometrial markers, such as pinopods, immunehistochemical biomarkers, endometrial blood flow and wave, have been used to determine the receptivity. They may interact transiently with the embryo at implantation but appear not to be enough reliable markers of receptivity evaluation as the precision indicators of 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 morphological and functional alterations that result in the change from a pre-receptive to a receptive endometrium. The morphological changes observed on histology for each specific day after ovulation were described by Noyes and his colleagues in 1950 (described as Noyes criterion) [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 criterion is relatively limited. That is because out of phase endometrium was found in 5%-50% of fertile patients in previous publications [10-12]. The large variation results may be resulted from the inaccurate determination of the day of ovulation.

Previous study demonstrated that the use of classic histologic endometrial dating to estimate the timing of the window of implantation and to adjust embryo transfer time accordingly may increase the implantation rate in hormone replacement cycle (HRT) cycles of patients with out of phase in classic endometrial dating [13]. The clinical value of the endometrial histological dating in RIF patients in natural cycle is still to be answered. In this study, we tried to investigate the clinical effects of pFET in unexplained RIF patients according to the endometrial histological dating of endometrial biopsies under ultrasound-guided ovulation monitoring in natural cycle.

Methods

**Patient population**

There were two phases in our study. In phase I, a total of 50 good prognosis patients underwent
endometrial biopsy in different time-points (PO+3/5/7/9/11) (Fig.1). The histological profiles of good prognosis patients who were pregnancy in their first conventional FET cycle would be collected as fertile parameters. The good prognosis patients group enrolled women aged 20-35 underwent FET in natural cycle. In phase II, 155 unexplained RIF patients were recruited to evaluate their endometrial dating on PO +7 (Fig.1). According to ESHRE PGD consortium, RIF is defined as the absence of a gestational sac on ultrasound at 5 or more weeks after 3 embryo transfers with high quality embryos or after the transfer of ≥10 embryos in multiple transfers[1]. Patients with uterine abnormalities (double uterus, bicornuate uterus, unicornuate uterus and uterine mediastinum), intrauterine adhesions, endometriosis, adenomyosis, hydrosalpinx, and uterine fibroids (submucosal fibroids, non-mucosal fibroids > 4 cm and/or endometrial pressure) were excluded from the unexplained RIF patient group. In both groups, patients had a menstrual cycle length of 24-35 days and an indication for ovarian stimulation before in vitro fertilization/ intracytoplasmic sperm injection[IVF/ICSI]. The study was approved by the Ethics Committee of the Reproductive and Genetic Hospital of CITIC-XIANGYA (LL-SC-2017-007) (June 29, 2017). We began to recruit the patients on the initial released date of our clinical trial. However, we found the advantage of endometrial histological dating when we designed and organized the data of first clinical trial (NCT03222830). Then we increased the sample of the study which major in recruiting RIF patients and good prognosis patients for endometrial histological dating by the second clinical trial (NCT03312309).

Ovulation monitoring

All patients were monitored throughout a natural cycle with daily ultrasound scan from the 10-12th day of the menstrual cycle onward when the follicular diameter was 16mm until the dominant follicle disappeared. At the same time, urinary LH was used since the follicular diameter was 16mm. The day of dominant follicle disappeared was considered as 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.
Histological analysis and dating

Endometrial tissue was rinsed in chilled PBS followed by 10% neutral buffered formalin-fixed and paraffin embedded (FFPE). FFPE tissues were sectioned at 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 were established according to the Noyes dating criterion [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 instruction of Kuwayama kit [14]. After warming, embryos were transferred to G1.5/G2.5 medium and cultured for 2-6 hours. Only cleavage stage embryos which exhibited >50% blastomeres intact 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 after ovulation regardless of endometrium dating in good prognosis patients. The cleavage stage embryos or blastocysts were transferred 4-7 days after ovulation depending on the endometrium dating in RIF group. Luteal support was applied when the dominant follicle disappeared with satisfactory endometrial development (thickness ≥ 8 mm, confirmed by ultrasound examination). Then 40mg dydrogesterone [Abbott Biologicals B.V.] was given by oral until the 28th day of embryo transfer if the pregnancy test was positive.

Clinical outcomes and statistical analysis

The accumulative live birth rate of repeated FET cycles during the study period was defined as the probability of a live birth from all cycles during the study period. Ongoing pregnancy was defined as at least one intrauterine gestational sac with cardiac action by ultrasound performed 6 weeks after ET. Biochemical pregnancy was defined as positive hCG without any 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 was presented as mean ± SD, and comparisons were made by one-way ANOVA or non-parametric statistical. Categorical data was
presented as number (N) and percentage (%), and comparisons were made by the Chi-square test or Fisher Exact 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 endometrium dating between pathologist A and pathologist B. GraphPad Prism 7.0 was used to evaluate the inner-group difference mean ± SD in control group. P<0.05 was considered statistically significant.

Results

The verification of Noyes criterion

Standard parameter of endometrial histological dating were established from the good prognosis patients (n=41). PO/dating +3: gland nuclei are pushed to the center of the epithelial cells with cytoplasm above, and vacuoles below. PO/dating +4: Gland nuclei are returning to the base of the cells. Note wisps of secretory material are appearing in the lumina. Some vacuoles are pushed past the nucleus on their way to empty glycogen into the lumen. Mitosis and pseudostratification of nuclei are absent. PO/dating +5: few vacuoles remain. PO/dating +7: Tissue edema, though variable in the proliferative phase, is characteristically marked in the mid-secretory stage, becoming evident rather abruptly. PO/dating+9: the spiral arterioles, previously somewhat difficult to distinguish in the edematous stroma, become much more prominent. PO/dating +11: Pre-decidua begins to differentiate under the surface epithelium (Fig. S1). The results was consistent with the Noyes dating criterion [8].

The agreement of blinded endometrium dating and the criteria for endometrium dating

All the endometrium dating (n=205) was evaluated by two experienced pathologists. The intra-observer agreement was determined to be good (weighted kappa = 0.672; 95% CI 0.606 -0.737; P < 0.001). As showed in Fig. 2, Bland-Altman (B-A) plots of pathologist A and pathologist B highlighted trends regarding differences of endometrium dating between the different pathologists (pathologist A and pathologist B). The limits of agreement indicated that the difference value for an endometrium dating was ≤1.76, but the endometrium dating of pathologist A and pathologist B was consistent when the value was ≤ 2 in clinical. Thus, the B-A Plots suggested that the agreement was good
between pathologist A and pathologist B which the value was ≤ 2 by using the Noyes criteria in the same patients.

**Out of phase of endometrial dating**

Endometrial dating standard for different days (PO+3/5/7/9/11) were established in good prognosis patients who achieved ongoing pregnancy in first conventional FET cycle (n=41) (Fig.1). Two experienced pathologists confirmed the endometrial dating by Noyes’ criterion in the good prognosis patients group, which showed that the endometrial dating in different times were significant distinct. In contrast, the inner-group differences were so few that the endometrium dating of most good prognosis patients were ranged in mean± SD (between the lower and upper limit). Only one endometrium of good prognosis patient biopsied in PO+7 was evaluated as dating+3 who was pregnancy in the conventional FET cycle (Fig. 3A).

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

Altogether 49 RIF patients were evaluated as out of phase and 106 RIF patients were assessed as in phase on the day of PO +7. One third(n=35) in phase patients were dating +7, the other two thirds (n=71) in phase patients were dating +5. In out of phase patients, 24%(n=12) were dating +3 (Fig. 3C),73%(n=36) were dating +4 (Fig. 3C) or dating +5 vacuoles remain (Fig. 3C), and one patient was diagnosed as dating +10 (Fig. 3C).

**Clinical outcome in RIF Patients with endometrial dating results by pFET**

The demographic characteristics and reproductive history RIF patients, including age, body mass index, duration of infertility, cause of infertility, were shown in Table 1. The previous failed cycles were 3.6±0.7 cycles, with the minimum and maximum value were respectively three cycles and five cycles. In 47 patients whose personal WOI was delayed three (n=35)/four (n=11) days or advanced three days(n=1), the pFET was performed. Day 3 embryos or Day 5 blastocysts were transferred with 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 were failure to pregnancy after the first pFET have a second endometrial biopsy by delayed one/two days according to the first result of endometrial dating in RIF group. And all the 5 patients were the expectant endometrial dating in the second endometrial biopsy. In the second pFET attempt, the live birth rate was 40% (2/5). And thus the accumulative live birth rate for personal FET were 55.7%.

Discussion

This is the first pFET study according to the endometrial dating by the verification of Noyes criterion. The implantation is not a single event, but, more accurately, a cascade of interactions between the embryo and endometrium. The human endometrium is receptive to embryo implantation during a narrow time of the menstrual cycle referred to as the WOI. It has been assumed that the WOI is constant in time in all women. Recent study have demonstrates the existence of a displaced WOI [15-18]. The classic method of dating the endometrium using defined histological criteria was established in 1950 [8]. But such pFET studies in natural cycle according to the Noyes criterion are lacking. The endometrial dating criterion were established only in pregnancy patients in the first conventional FET cycle in this study. All the good prognosis patients were pregnancy with histological dating +5-7 on the day of PO+7 except one patient whose histological dating was +3 which is consistent with the definition of in phase[9]. At the same time, all the endometrium dating was evaluated by two experienced pathologists which the intra-observer agreement was determined to be good. So the endometrium dating criteria was easily learned and used according to Noyes’ criterion. Finally, our data indicated that intra-group variation in good prognosis patients group was so little that our results are highly reliable. The reproducibility and verifiability of endometrial dating in the same patients were determined by 5 RIF patients who were failure to pregnancy after the first pFET, have a second endometrial biopsy by delayed one/two days according to the first result of endometrial dating suggested that the expectant endometrial dating in the second endometrial biopsy. And the second biopsies of five RIF patients were conducted within 4 months, which suggested that the results of endometrial dating maybe repeatable in 4 months in this study. The endometrial dating of larger
sample and longer time reproducibility study was needed.

Recent data from transcriptomic microarray (described as endometrial receptivity array, ERA) have shown that the receptive phase may be delayed in about 25 percent of RIF patient [19-25]. In that case, delaying FET according to the endometrial delay (pFET) may lead to improved clinical pregnancy rate (42%-51.7%), especially in women with multiple failure cycles and apparently good quality embryos. But the widespread use of ERA is hampered by high cost and complex transcriptomic analysis techniques. As shown in recent literature that endometrial transcriptomics could demonstrate the two RIF causes: molecular displacements and molecular disruptions [26]. Asynchrony (displacement) and pathology (disruption) are both possible in RIF [26]. Maybe the other 70% RIF patients who the endometrial dating was in phase in our study could be benefit from the molecular disruptions cause of endometrial transcriptomics.

There were some limits in our research. The main objective of this study was to use endometrial histology to detect the displacement of WOI in RIF patients and evaluate the clinical outcomes by pFET. However, there was no comparative data on clinical pregnancy rate and live birth rate in women with RIF who have had adjusted FET and those who have not to further support clinical significance. Randomized controlled study was needed in RIF patients with out of phase dating. Further research will be necessary to determine the mechanism of changeable window of implantation.

Conclusions
There is an obviously increased percentage of WOI displacement in RIF patients compared with good prognosis patients, leading to the concept of pFET as a treatment strategy.

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 the participants.

Consent for publication
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 on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
All authors have read and approved the manuscript.
YL designed the experiment, recruited the samples, analyzed histological samples and written the paper;
X FL performed the experiment;
L JL, X XF and R XG organized data;
YH analyzed histological samples;
G XL designed the experiment;
GL designed and guided the experiment;
FG designed and guided the experiment.

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Tables

Table 1 Summary of the clinical outcomes of RIF patients

| RIF patients                                      |
|---------------------------------------------------|
| No. of patients                                  | 155 |
| Agey                                             | 33.0±3.7 |
| Duration of infertility (year)                   | 5.6±2.6 |
| Body mass index (kg/m$^2$)                       | 20.9±1.6 |
| Cause of infertility                             |
| Male factor                                      | 20/155(12.9%) |
| Tubal factor                                     | 135/155(87.1%) |
| No. of previous failed cycles                    | 3.6±0.7 |
| No. of out of phase                              | 49/155(31.6%) |
| Total patients with 1st pFET                     | 47 |
| High quality embryo rate                         | 33/47(70.2%) |
| Cleavage stage                                   | 3/47(6.4%) |
| Event                                      | Rate          |
|--------------------------------------------|---------------|
| Embryo 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 2nd biopsies at the specified day   | 5             |
| 2nd expectant endometrial dating           | 5             |
| Total patients with 2nd pFET               | 5             |
| Implantation rate after 2nd pFET           | 3/7 (42.8%)   |
| Ongoing pregnancies after 2nd pFET         | 3/5 (60%)     |
| Accumulative live birth rate after pFET    | 29/52 (55.7%) |

**Figures**
The patients of control group and RIF group were recruited.
Figure 2

Bland–Altman plots of variability according to pathologist A and pathologist B. The x-axis is the mean endometrium dating for pathologist A and pathologist B; the y-axis is the difference from the mean endometrium dating between the pathologist A and pathologist B. The upper and lower lines on the B–A plots represent the limits of agreement, and the mean difference ±1.96 times its standard deviation. Thus, the distance from zero and the width of the limits of agreement both indicate the magnitude of disagreement between the pathologist A and pathologist B. Closer clustering to the mean indicates higher agreement. If the difference value for an endometrium dating is = 0, then the endometrium dating of pathologist A and pathologist B was same.
Figure 3

Endometrial dating in control group and RIF group. A: Inner-group differences of endometrium dating in control group in different times (PO+3/5/7/9/11) were few by two experienced pathologists. Using mean ± SD as lower limit and upper limit to define reference range of endometrium dating, 1 of 50 controls woman below the range. B: endometrium dating of control group was relatively concentrated, with only one case being out of phase; Most of the endometrium dating of the RIF group tended to be delayed. C: Endometrial dating according to Noyes criteria in RIF patients[X 400].

- **dating +3**: gland cytoplasm and nuclei above and vacuoles below (arrow).
- **dating +4**: gland nuclei are in the middle of the cells. glycogen vacuoles are seen in two sides of gland nuclei (arrow).
- **dating +5 vacuoles remain**: vacuoles (arrow) are remain in the basement of gland cells.
- **dating +10**: pre-decidua (arrow) begins to differentiate and spiral arteries increase.

Supplementary Files

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