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ORIGINAL RESEARCH

# Impact of Transfer Timing on Day-6 Blastocyst Pregnancy Outcomes in HRT Cycles of 2021–2024

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**Background:** The achievement of a healthy live birth from a singleton pregnancy has become the primary objective of modern Assisted Reproductive Technology (ART). Consequently, the effective utilization of a single blastocyst is of paramount importance. However, the optimal approach to improving clinical pregnancy rates for Day-6 (D6) blastocysts in the hormonal replacement therapy frozen embryo transfer (HRT-FET) cycle remains unclear.

**Methods:** This study retrospectively analyzed 990 D6 single blastocyst HRT-FET cycles conducted at the Reproductive Medicine Center of Nanjing Drum tower Hospital from January 2021 to April 2024. Patients were categorized based on the timing of embryo transfer (on the 6th or 7th day of progesterone administration), and both univariate and multivariate regression analyses were employed to assess the impact of transfer timing on clinical pregnancy outcomes.

**Results:** The results revealed no significant differences in baseline characteristics or treatment cycle parameters between the two groups based on transfer timing. Univariate analysis identified several factors, including the age of both partners, infertility etiology, anti-Müllerian hormone (AMH) levels, antral follicle count (AFC), and the number of prior ART cycles, which may influence clinical pregnancy outcomes. After adjusting for these variables, multivariate regression analysis indicated that the timing of endometrial preparation for D6 single blastocyst transfer did not significantly affect clinical pregnancy rates.

**Conclusion:** In conclusion, our retrospective data suggest that transferring single D6 blastocysts on either the sixth or seventh day of progesterone administration in HRT-FET cycles yields comparable clinical pregnancy outcomes. Until further prospective evidence emerges, our findings do not support routine delay of D6 blastocyst transfers in HRT cycles.

**Keywords:** day-6 blastocyst, HRT-FET, transfer timing, clinical pregnancy rate

## Introduction

Complications in multiple gestations, such as elevated rates of cesarean sections, preterm deliveries, and low birth weight infants, significantly heighten risks to both maternal and fetal health. As a result, reducing the occurrence of multifetal pregnancies has increasingly become a priority in modern assisted reproductive technologies (ART), alongside efforts to enhance clinical pregnancy rates.[1,2] Single embryo transfer (SET) has emerged as a key strategy to optimize outcomes, aiming to achieve the goal of a singleton live birth.[3]

In fresh cycles, the pregnancy rate associated with Day 5 blastocysts (D5) is generally higher than that observed with Day 6 blastocysts (D6), suggesting that the developmental speed of blastocysts may serve as a predictor of their implantation potential. Slower-developing blastocysts tend to have lower pregnancy rates, likely due to a mismatch between embryonic development and endometrial receptivity.[4–6] In contrast, frozen-thawed cycles offer an advantage by better synchronizing endometrial development. Some studies report no significant differences in clinical pregnancy rates, implantation rates, or live birth rates between D5 and D6 frozen-thawed blastocyst transfers,[7–9] while others indicate that clinical pregnancy, implantation, and live birth rates are significantly higher following D5 transfer compared to D6

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transfer.[8,10–12] Many researchers[13] suggest that the extended in vitro culture time for D6 blastocysts makes them more vulnerable to external adverse environmental factors, leading to increased DNA damage, impaired developmental potential, delayed development, and marginally reduced embryo quality. Moreover, slower-developing embryos are more likely to exhibit aneuploidy and other genetic abnormalities.[14,15] Despite evidence showing that D5 blastocysts have superior implantation and pregnancy rates compared to D6, it does not imply that D6 blastocysts should be discarded, as D6 transfers still yield relatively high clinical pregnancy rates.[16] This is particularly relevant for patients with diminished ovarian reserve, poor embryo quality, or those unable to tolerate repeated IVF cycles. Therefore, the freezing and transfer of D6 blastocysts warrant further attention. Is there a clinical strategy to enhance the pregnancy rate of D6 blastocyst transfers?

The success of frozen embryo transfer (FET) largely depends on the synchronization of embryonic development with endometrial receptivity.[17,18] The human endometrium has a limited window for accepting an embryo for implantation, typically between days 20–24 of the menstrual cycle, corresponding to 7–11 days after the Luteinizing hormone (LH) peak. Optimizing the timing of embryo transfer is a crucial factor in improving pregnancy rates in FET cycles. Hormone replacement therapy (HRT) cycles are commonly employed to prepare the endometrium for FET by simulating normal menstrual cycle hormonal fluctuations, thereby adjusting the implantation window. During the FET cycle, prolonging the duration of progesterone-induced endometrial transformation can reduce uterine contractions, enhance endometrial receptivity, and shorten the interval between embryo implantation and development, thus contributing to favorable pregnancy outcomes. Most studies agree that transferring D5 blastocysts on the 5th day of progesterone supplementation (P5) achieves a stable clinical pregnancy rate.[19,20] Some researchers[21,22] suggest that approximately 25% of infertile patients experience a delayed implantation window, advocating for later embryo transfers in these cases. At our center, we typically perform D5 blastocyst transfers on the 6th day (P6) of endometrial transformation, which has been associated with a higher clinical pregnancy rate. Given that D6 blastocysts develop for one additional day compared to D5 blastocysts, we hypothesize that delaying D6 transfers until P7 might improve clinical pregnancy outcomes. Therefore, we conducted a retrospective study on D6 single embryo transfers at the Reproductive Medicine Center of Nanjing Drum Tower Hospital between 2021 and 2024 to investigate the impact of transfer timing on clinical pregnancy outcomes. This study aims to determine whether a slight delay in the timing of embryo transfer following progesterone-induced endometrial transformation can potentially enhance pregnancy outcomes.

# Material and Methods

## Patients

This retrospective study analyzed FET cycles performed between January 2021 and April 2024 at the Reproductive Medicine Center of Nanjing Drum Tower Hospital (Figure 1). All patients underwent endometrial preparation using a HRT protocol, which included oral administration of estradiol (Femoston: 2 mg estradiol alone or 2 mg estradiol combined with 10 mg dydrogesterone; Abbott, USA). In each cycle, a single D6 blastocyst was transferred. Prior to the initiation of the HRT-FET cycle, all patients received a comprehensive clinical evaluation to exclude contraindications to medication or pregnancy. The exclusion criteria were as follows: Concurrent use of additional hormone replacement regimens; Presence of hydrosalpinx, endometrial pathology, or uterine cavity abnormalities (including submucosal fibroids, intrauterine adhesions, or fibroids distorting the uterine cavity); Diagnosis of moderate to severe endometriosis or adenomyosis; Undergoing preimplantation genetic testing (PGT).

## Efficacy

This study was approved by the Ethics Committee of Nanjing Drum Tower Hospital, affiliated with Nanjing University Medical School (Approval No. 2021-384-01). This study utilized anonymized medical records obtained from previous clinical practice, which were reviewed and approved by an ethics committee confirming compliance with the exemption from informed consent requirements. As a retrospective study, it carries minimal risk of adverse effects on participants' rights and interests, with comprehensive privacy protection measures already implemented. Our study fully protects the privacy of patient data and strictly complies with the Declaration of Helsinki.

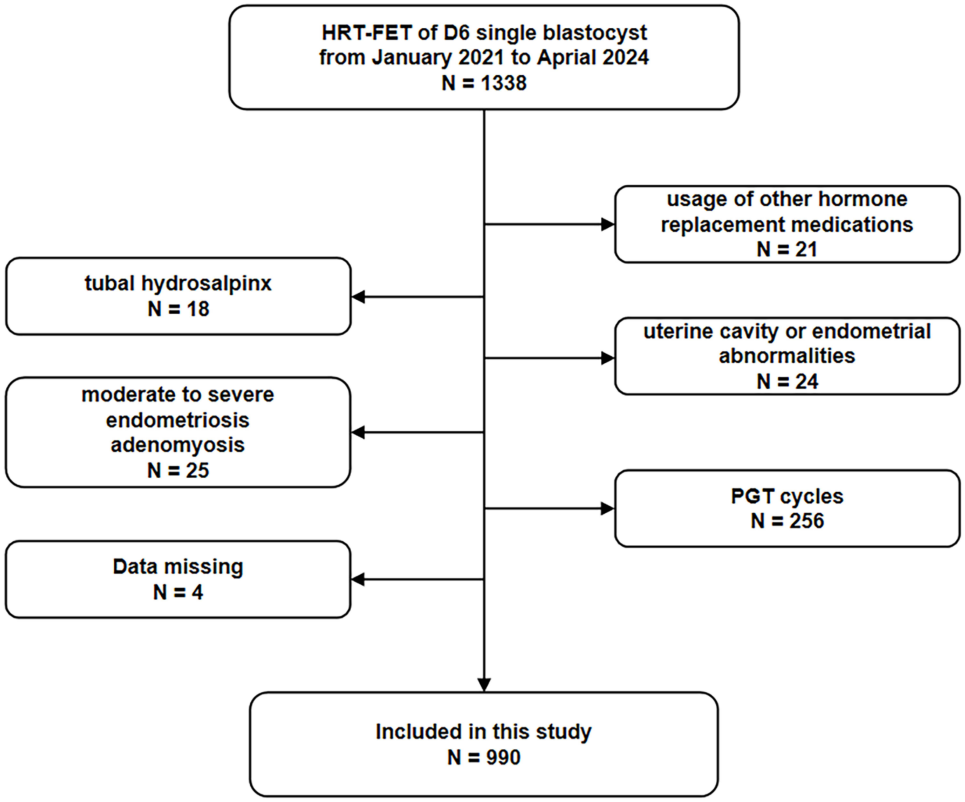


**Figure 1** A flow chart of this study.

## Endometrial Preparation And thawed Embryo Transfer

Oral estradiol (Femoston, Abbott Laboratories, USA; 2 mg, t.i.d. for 14 days) was initiated on days 2–3 of the menstrual cycle. During this phase, serum estradiol ($E_2$) and progesterone (P) levels, along with endometrial thickness, were closely monitored. In cases where the endometrium was found to be thin, the dose of estradiol was increased to 4 mg, administered b.i.d. for 4–6 days. Once the endometrial thickness reached the target level, endometrial transformation was induced by the combination of oral estradiol/dydrogesterone tablets (Femoston, estradiol 2 mg + dydrogesterone 10 mg, t.i.d. for 6–7 days) and intramuscular progesterone injections (60 mg, q.d. for 6–7 days). On day 6 or 7 of endometrial transformation, a D6 blastocyst was thawed and transferred. Following embryo transfer, hormonal support was continued with Femoston (estradiol 2 mg + dydrogesterone 10 mg, t.i.d.) and vaginal progesterone sustained-release gel (90 mg, q.d). Serum β-human chorionic gonadotropin (β-hCG) was measured two weeks post-transfer to assess biochemical pregnancy. In patients with a positive β-hCG result, transvaginal ultrasound was performed four weeks post-transfer to confirm clinical pregnancy and determine the number of implanted embryos. Once pregnancy was confirmed, luteal phase support was maintained until two months post-transfer, with ongoing monitoring for any pregnancy-related complications.

## Statistical Analysis

The primary outcome of our study was the clinical pregnancy rate, and the secondary outcome was the early miscarriage rate. In this study, HRT-FET cycles were categorized into two groups according to the timing of embryo transfer: Group A (transfer on day 6 of progesterone administration, P6) and Group B (transfer on day 7, P7). Univariate analyses were performed to identify factors associated with clinical pregnancy outcomes following single D6 blastocyst transfer in HRT-FET cycles. Variables identified as significant in univariate analysis were subsequently evaluated using multivariate logistic regression to assess the independent impact of embryo transfer timing. Continuous variables with a normal distribution were analyzed using the independent-samples *t*-test, whereas non-normally distributed variables were assessed using the Mann–Whitney *U*-test. Categorical variables were compared using the chi-square test. Data are


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presented as mean ± standard deviation (SD), and statistical significance was defined as a two-tailed P-value < 0.05. All statistical analyses were conducted using EmpowerStats (www.empowerstats.com; X&Y Solutions, Boston, MA, USA) and R software (version 3.6.0; http://www.r-project.org).

## Results

A total of 990 embryo transfer cycles were included in this study, comprising 679 cycles in Group A (P6) and 311 cycles in Group B (P7). Baseline characteristics were largely comparable between the two groups (Table 1). The mean female age was 32.71 ± 4.67 years in Group A and 32.88 ± 4.38 years in Group B (P = 0.595), while the mean male age was 33.72 ± 5.25 years and 34.08 ± 4.88 years, respectively (P = 0.304). Body mass index (BMI) did not differ significantly between the groups (23.54 ± 8.56 kg/m² vs 23.42 ± 3.20 kg/m², P = 0.807). The distribution of infertility types was similar (P = 0.526), with primary infertility observed in 54.05% of Group A and 56.27% of Group B. The duration of infertility was also comparable (3.49 ± 2.69 years vs 3.59 ± 2.56 years, P = 0.569). With respect to reproductive endocrine parameters, anti-Müllerian hormone (AMH) levels were 3.74 ± 3.19 ng/mL in Group A and 3.62 ± 3.20 ng/mL in Group B (P = 0.629). Baseline follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels showed no significant differences (P = 0.778 and P = 0.305, respectively), and the antral follicle count (AFC) was also similar between the groups (P = 0.821). Notably, the number of embryo transfer cycles was significantly higher in Group B compared to Group A (2.27 ± 1.26 vs 2.06 ± 1.06, P = 0.005). Endometrial thickness was comparable (9.99 ± 1.63 mm

**Table 1** Comparison of Characteristics Between Group A and Group B

| Transfer Time | Group A: P6 | Group B: P7 | P-Value |
|---|---|---|---|
| **N** | 679 | 311 | |
| **Female age (years)** | 32.71 ± 4.67 | 32.88 ± 4.38 | 0.595 |
| **Male age (years)** | 33.72 ± 5.25 | 34.08 ± 4.88 | 0.304 |
| **BMI (kg/m$^2$)** | 23.54 ± 8.56 | 23.42 ± 3.20 | 0.807 |
| **Infertility type** | | | 0.526 |
| **Primary infertility** | 367 (54.05%) | 175 (56.27%) | |
| **Secondary infertility** | 312 (45.95%) | 136 (43.73%) | |
| **Infertility duration (years)** | 3.49 ± 2.69 | 3.59 ± 2.56 | 0.569 |
| **AMH (ng/mL)** | 3.74 ± 3.19 | 3.62 ± 3.20 | 0.629 |
| **Basal FSH (IU/L)** | 7.33 ± 2.93 | 7.27 ± 2.85 | 0.778 |
| **Basal LH (IU/L)** | 6.16 ± 4.16 | 5.87 ± 3.90 | 0.305 |
| **AFC (N)** | 18.77 ± 10.48 | 18.94 ± 11.08 | 0.821 |
| **Number of cycles (N)** | 2.06 ± 1.06 | 2.27 ± 1.26 | 0.005 |
| **Endometrial thickness (mm)** | 9.99 ± 1.63 | 9.93 ± 1.69 | 0.592 |
| **P level before transformation (ng/mL)** | 0.14 ± 0.12 | 0.18 ± 0.18 | 0.002 |
| **$E_2$ level before transformation (pg/mL)** | 278.71 ± 331.99 | 255.81 ± 252.55 | 0.281 |
| **LH level before transformation (IU/L)** | 11.39 ± 7.76 | 12.32 ± 10.00 | 0.214 |
| **Clinical pregnancy rate** | 311 (45.80%) | 132 (42.44%) | 0.324 |
| **Early miscarriage rate** | 55 (17.68%) | 31 (23.48%) | 0.158 |

**Abbreviations**: BMI, Body Mass Index; AMH, Anti-Mullerian Hormone; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; AFC, Antral follicle count; $E_2$, oestrogen; P, progesterone.

vs 9.93 ± 1.69 mm, P = 0.592). However, serum progesterone (P) levels prior to endometrial transformation were significantly elevated in Group B (0.18 ± 0.18 ng/mL vs 0.14 ± 0.12 ng/mL, P = 0.002), whereas estradiol ($E_2$) and LH levels did not differ significantly between the groups (P = 0.281 and P = 0.214, respectively). The clinical pregnancy rate was 45.80% in Group A and 42.44% in Group B (P = 0.324), and the early miscarriage rates were 17.68% and 23.48%, respectively (P = 0.158), with neither outcome showing statistical significance.

Univariate logistic regression analysis was performed to evaluate potential predictors of clinical pregnancy (Table 2). Female age was significantly and negatively associated with clinical pregnancy (OR = 0.94, 95% CI: 0.91–0.96, P < 0.001), as was male age (OR = 0.96, 95% CI: 0.93–0.98, P < 0.001). BMI was not significantly associated with clinical pregnancy outcomes (OR = 1.01, 95% CI: 0.99–1.03, P = 0.406). Compared to women with primary infertility, those with secondary infertility had a significantly lower likelihood of clinical pregnancy (OR = 0.74, 95% CI: 0.57–0.95, P = 0.017). Infertility duration was not associated with clinical pregnancy (OR = 1.01, 95% CI: 0.96–1.06, P = 0.712). Among endocrine and ovarian reserve indicators, AMH demonstrated a positive association with clinical pregnancy (OR = 1.07, 95% CI: 1.02–1.12, P = 0.004), as did AFC (OR = 1.01, 95% CI: 1.00–1.03, P = 0.029). Baseline FSH, baseline LH, and endometrial thickness were not significantly associated with clinical pregnancy (all P > 0.05). The number of embryo transfer cycles was negatively associated with clinical pregnancy (OR = 0.89, 95% CI: 0.79–1.00, P = 0.042). Serum levels of P, $E_2$, and LH on the day before transfer were not significantly associated with clinical pregnancy outcomes (P > 0.1).

Multivariate logistic regression analysis was subsequently conducted to assess the association between embryo transfer timing and clinical pregnancy, adjusting for relevant baseline covariates identified in the univariate analysis (female age, male age, infertility type, AMH, AFC, and number of treatment cycles) (Table 3). Using day 6 embryo transfer as the reference, transfer on day 7 was not independently associated with clinical pregnancy (adjusted OR = 1.12, 95% CI: 0.82–1.53, P = 0.490).

**Table 2** Univariate Analysis of Clinical Pregnancy Outcomes

| | Statistics | Clinical Pregnancy OR (95% CI) P-Value |
|---|---|---|
| **Female age (years)** | 32.77 ± 4.58 | 0.94 (0.91, 0.96) <0.001 |
| **Male age (years)** | 33.83 ± 5.14 | 0.96 (0.93, 0.98) <0.001 |
| **BMI ($kg/m^2$)** | 23.50 ± 7.30 | 1.01 (0.99, 1.03) 0.406 |
| **Infertility type** | | |
| **Primary infertility** | 542 (54.75%) | 1 |
| **Secondary infertility** | 448 (45.05%) | 0.74 (0.57, 0.95) 0.017 |
| **Infertility duration (years)** | 3.52 ± 2.65 | 1.01 (0.96, 1.06) 0.712 |
| **AMH (ng/mL)** | 3.70 ± 3.19 | 1.07 (1.02, 1.12) 0.004 |
| **Basic FSH (IU/L)** | 7.31 ± 2.91 | 0.97 (0.93, 1.01) 0.175 |
| **Basic LH (IU/L)** | 6.07 ± 4.08 | 1.02 (0.99, 1.05) 0.237 |
| **AFC (N)** | 18.83 ± 10.67 | 1.01 (1.00, 1.03) 0.029 |
| **Number of cycles (N)** | 2.12 ± 1.13 | 0.89 (0.79, 1.00) 0.042 |
| **Endometrial thickness (mm)** | 9.97 ± 1.65 | 1.00 (0.93, 1.08) 0.971 |
| **P level before transformation (ng/mL)** | 0.15 ± 0.14 | 1.17 (0.49, 2.82) 0.726 |
| **$E_2$ level before transformation (pg/mL)** | 271.51 ± 309.28 | 1.00 (1.00, 1.00) 0.166 |
| **LH level before transformation (IU/L)** | 11.72 ± 8.63 | 1.01 (0.99, 1.03) 0.381 |

**Abbreviations**: BMI, Body Mass Index; AMH, Anti-Mullerian Hormone; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; AFC, Antral follicle count; $E_2$, oestrogen; P, progesterone.

**Table 3** Multivariate Regression Analysis of Clinical Pregnancy Outcomes

| Transfer Time | Clinical Pregnancy |
|---|---|
| | aOR (95% CI) P-Value |
| 6 days after endometrial transformation (P6) | 1 |
| 7 days after endometrial transformation (P7) | 1.12 (0.82, 1.53) 0.490 |

**Notes**: Adjust for: Female age, Male age, Infertility type, AMH, AFC, Number of cycles.

## Discussion

This study aimed to evaluate whether modifying the timing of embryo transfer could enhance clinical pregnancy outcomes following the transfer of D6 blastocysts. Specifically, we examined the impact of transferring D6 blastocysts on either the sixth or seventh day of endometrial transformation. Our findings revealed no significant difference in clinical pregnancy rate between the two groups, suggesting that postponing D6 blastocyst transfer to the seventh day may offer no added benefit.

Compared to cleavage-stage embryos, blastocysts undergo a selection process that mitigates developmental arrest at the 8-cell stage, thus eliminating embryos with limited developmental potential. Consequently, blastocyst transfer is more physiologically synchronized with endometrial receptivity, resulting in superior outcomes in fresh embryo transfer cycles.[23] With advances in laboratory protocols and clinical practices, cryopreservation has emerged as a cornerstone in ART. Enhanced vitrification techniques and improved post-thaw survival rates[24,25] have made single blastocyst transfer during FET cycles the preferred strategy. This approach yields high pregnancy and live birth rates while minimizing the risk of ovarian hyperstimulation syndrome (OHSS), multiple gestations, and related maternal and neonatal complications.[26–28] These benefits have contributed to a global surge in the adoption of FET cycles. The elective freeze-all strategy is increasingly employed in clinical scenarios such as OHSS prevention, PGT, elevated progesterone levels in the late follicular phase, and embryo-endometrial asynchrony.[29–32] Clinically, blastocyst formation typically occurs on day 5 or day 6 post-fertilization, with D5 and D6 blastocysts being most commonly utilized.[33] While some studies have reported no significant differences in clinical pregnancy, implantation, or live birth rates between D5 and D6 frozen-thawed blastocysts,[7,8] the preponderance of evidence suggests superior outcomes with D5 blastocysts.[10,11] It has been hypothesized that D6 cohorts are more likely to include embryos of lower developmental potential,[10,34] although morphologically high-quality D5 and D6 blastocysts may exhibit comparable clinical performance.[34] Nonetheless, considering the economic and physiological burden associated with repeated oocyte retrieval, optimizing the clinical utility of all viable embryos, including D6 blastocysts, is imperative. Enhancing the clinical performance of D6 blastocysts holds substantial clinical value, particularly given the robust outcomes associated with D5 transfers. Earlier expansion and implantation potential observed in D5 blastocysts may reflect more optimal synchronization with endometrial receptivity.[35] Moreover, HRT cycles have been associated with improved implantation outcomes for D6 blastocysts.[36] A critical determinant of FET success is the alignment between embryonic development and the window of implantation (WOI).[37] Improved synchronization in HRT-FET cycles may therefore enhance outcomes for D6 blastocyst transfers. Progesterone is a key modulator of the WOI,[37] yet there remains considerable variability regarding the optimal route, dosage, and timing of administration.[38,39] A Cochrane review by Glujovsky et al[40] demonstrated that initiating progesterone administration prior to the putative ovulation day markedly reduces pregnancy rates in frozen cleavage-stage transfers. Most comparative analyses of D5 and D6 blastocyst outcomes have employed identical progesterone administration timelines. Thus, it remains unclear whether adjusting the timing of D6 blastocyst transfer relative to progesterone exposure could yield improved outcomes. One study found similar live birth rates when blastocysts were transferred on either the sixth or seventh day of progesterone administration in HRT cycles;[41] however, a subgroup analysis revealed a significantly higher miscarriage rate for D6 blastocysts transferred on day 6.[42] Conversely, a randomized controlled trial by the same group reported no significant difference in clinical pregnancy rates between 5- and 7-day endometrial preparation regimens. However, the 7-day group exhibited a 4.9% lower pregnancy rate, a difference that did not reach statistical significance. Notably, this study did not perform a stratified analysis based on the

developmental timing of the transferred blastocysts.[39] In our retrospective analysis of single D6 blastocyst HRT-FET cycles, postponing transfer by one day did not enhance clinical pregnancy rate. Clinical pregnancy rates were comparable between groups, and no reduction in miscarriage rates was observed.

This study is subject to several limitations. First, the sample size for D6 blastocyst transfers on the seventh day of progesterone exposure was relatively limited, which may affect the statistical power of our findings. Second, due to constraints in our data collection infrastructure, we were unable to access comprehensive information regarding the originating oocyte retrieval cycles, including embryo quality metrics. This precluded a more granular analysis of potential confounders. Third, our study was restricted to HRT cycles and did not include natural or mild stimulation protocols - an area warranting future investigation. Most importantly, the retrospective nature of the analysis introduces potential bias, underscoring the need for well-designed prospective randomized controlled trials to confirm our findings and refine transfer timing strategies for D6 blastocyst FETs.

## Conclusion

In conclusion, our retrospective data suggest that transferring single D6 blastocysts on either the sixth or seventh day of progesterone administration in HRT-FET cycles yields comparable clinical pregnancy rates and early miscarriage rate. Until further prospective evidence emerges, our findings do not support routine delay of D6 blastocyst transfers in HRT cycles.

## Abbreviations

ART, Assisted Reproductive Technology; SET, Single embryo transfer; FET, frozen-thawed embryo transfer; HRT, hormone replacement therapy; D5, Day 5; PGT, preimplantation genetic testing; $E_2$, estradiol; P, progesterone; β-hCG, β-human chorionic gonadotropin; SD, Standard Deviation; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; AMH, anti-müllerian hormone; AFC, antral follicle count; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; OHSS, ovarian hyperstimulation syndrome; WOI, window of implantation.

## Data Sharing Statement

The datasets generated and analyzed during the current study are not publicly available due to the special requirements of our hospital and our reproductive medicine center for the disclosure of patients' clinical data but are available from the corresponding author (Yue Jiang) on reasonable request via E-mail.

## Ethics Approval and Consent to Participate

This retrospective study received ethical approval from the ethics committee of Nanjing Drum Tower Hospital. All methods were carried out in accordance with relevant guidelines and regulations.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no competing interests.

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