The influence of morphologic grading and COS protocol on the outcome of Day 5 versus Day 6 single fresh blastocyst transfers: a retrospective analysis of clinical outcomes

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Research Article

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Abstract

Introduction The purpose of this study was to evaluate whether the blastocyst morphologic grading and the protocol of controlled ovarian stimulation (COS) would influence effect estimates, aiming to provide guidance when choosing blastocyst transfer.

Methods The clinical data of 612 patients who received single fresh blastocyst transplantation for first cycle, as well as the data of 253 patients who had already delivered were analyzed retrospectively. The patients were divided into two groups according to blastocyst formation time (D5 or D6). The following subgroup analyses were performed: (i) the morphologic grading of blastocyst. and (ii) the protocol of COS.

Results We observed that D5 single embryo transfer (SET) were associated with higher clinical pregnancy rate (CPR, 59.04% VS 31.73%) and live birth rate (LBR, 43.90% VS 24.04%) than D6 SET following fresh cycle. Patients in D5 group experienced more good blastocysts transfer (45.47% VS 13.46%) and less poor blastocysts transfer (9.64% VS 45.19%) than patients in D6 group. As to early stage and good quality blastocysts, the CPR and LBR were similar between D5 and D6 group. GnRHa antagonist protocol had a demonstrable inferiority comparing with the GnRHa follicular or luteal phase protocol with regard to the CPR and LBR in D6-SET group.

Conclusions The analysis found that ovarian reserve of patients in D6-SET group was comparatively worse than that of patients in D5-SET group and D6-SET patients represented a subgroup of infertility patients usually having relatively poor embryo quality.

Introduction

With gradual improvement in *in vitro* embryo culture systems, blastocyst has many advantages as it contains a re-screening process, therefore implantation rate may be higher after blastocyst transplantation, and the rate of live births could thus be increased. As the main goal of IVF is the birth of a single healthy child, single embryo transfer (SET), either elective or mandatory, has become the major strategy in many IVF centers since it could reduce the rate of multiple pregnancies which are known to be associated with adverse maternal and neonatal outcomes [1, 2, 3, 4] .

Blastocyst development rate is not consistent for in vitro development. Embryos that are cultured in vitro usually develop to the blastocyst stage 5 days after fertilization, but slower embryos can achieve blastulation on Day 6 or even later [5, 6, 7]. The meta-analysis conducted by Bourdon M et al concluded that CPR and LBR after D5 blastocyst transfers were significantly higher when compared with D6 embryos in either fresh or frozen-vitrified ET cycles [8]. Up to now, few studies have focused on whether the blastocyst morphologic grading would influence clinical outcomes following fresh blastocyst transfers. The few studies to date which have attempted to analyze the downstream fetal effects of each morphologic characteristic individually have been focused on frozen blastocysts [9, 10]. High-quality SBT is increasingly recommended to patients because of its acceptable pregnancy outcomes and significantly reduced multiple pregnancy rate compared to double blastocyst transfer (DBT) [11]. Nevertheless, there is no consensus on whether this transfer strategy is suitable for different COS protocols. To our knowledge, fewer studies comparing outcomes of D5 versus D6 SET in fresh cycles according to embryo quality (with the Gardner morphological analysis) or COS protocol are available. Therefore, this study aimed to explore the effects of blastocyst development speed, morphology and COS protocols on pregnancy and neonatal outcomes during the fresh SBT cycle and ultimately provide references for clinical transfer strategies.

Materials And Methods

Subjects

Data of 612 patients who received first cycle fresh SBT at the Centre for Reproductive Medicine, Wuhan Kangjian Maternal and Infant Hospital for the period of January 2015 to December 2020 were analyzed retrospectively. Patients with the following infertility criteria in first IVF (in vitro fertilization) cycle were included: (1) uterine tube factor; (2) endometriosis and metrial adenomyosis; (3) male factor; (4) ovulation dysfunction (including polycystic ovary syndromes, etc.); or (5) unexplained infertility. Exclusion criteria were: (1) performed D5/D6 blastocyst transfer in repeated fresh cycle; (2) frozen-thawed blastocyst transplantation; (3) performed D5/D6 fresh DBT; (4) performed D7 blastocyst transplantation; (5) drug or alcohol abuse, smoking, and a history of chronic medical disease (heart diseases, hepatonephric dysfunction). Among the 612 patients enrolled according to the above criteria, 253 patients had given birth at the time of analysis. The patients in the SBT groups were further sub-grouped by blastocyst morphology and COS protocols.

Stimulation protocols

GnRH (Gonadotropin-releasing hormone) antagonist protocol: COS was performed with administration of 150–225 IU/day recombinant FSH (r-FSH) from Day 2 or 3 of the cycle. Daily injections of 0.25 mg GnRH antagonist Ganirelix Acetate (Orgalutran, Merck Sharp & Dohme Limited, US) were administered at the presence of at least one follicle measuring > 14 mm until ovulation induction.

Long-acting GnRH agonist(GnRHa)follicular phase protocol: Patients received a single dose of 3.75 mg long-acting triptorelin acetate (Decapeptyl; Ferring, Saint-Prex, Switzerland) on Day 2 of the cycle. 28 days after the initiation of GnRHa, when complete pituitary desensitization was achieved, COS was started with administration of r-FSH.

Long-acting GnRHa luteal phase protocol: Pituitary suppression was achieved by a injection of 1 mg triptorelin acetate (Decapeptyl; Ferring, Saint-Prex, Switzerland) in the midluteal phase of the preceding cycle. Patients came back 14 days later. Once the downregulation was achieved, COS was started with administration of r-FSH as above.

For protocols above, final oocyte maturation was induced by injection of 5000 to 8000 IU hCG (HCG, Livzon, China) or injection of 0.2 mg triptorelin (Decapeptyl; Ferring, Saint-Prex, Switzerland), as soon as two to three leading follicles reached 17–18 mm in size. Oocyte retrieval following COS was carried

out 36h after ovulation trigger. Embryo transplantation was performed under ultrasound guidance, one embryo was transplanted per cycle. Serum HCG was tested on the 14th day. Ultrasound was performed on the 28th to 30th day of transplantation.

Luteal phase support

Vaginal micronized progesterone tablets (Utrogestan) 200 mg three times daily were administered for luteal phase support from Day 1 after oocyte retrieval onwards, until 7 weeks of pregnancy, after which the dose was gradually reduced and discontinued 1 week later.

Ethical approval

The study was approved by the institutional review board on July 21, 2021. This study was conducted in accordance with the Declaration of Helsinki and conducted with the approval of the Ethics Committee of Wuhan Kangjian Maternal and Infant Hospital. Due to the retrospective nature of the study, written informed consent was not obtained from the participants.

Setting

Baseline characteristics, cycle characteristics, and obstetric and neonatal data were extracted from the electronic medical record. Records were ascertained from patients who received fresh D5/D6 SET during the study period. All study data were collected by authorized staff and stored in a restricted directory on the hospital's network system. A flowchart is presented in Fig.1.

Outcome measures

The primary outcome of this study was CPR and LBR. Secondary endpoints included rates of multiple pregnancy, spontaneous early miscarriage, and neonatal outcomes. Neonatal outcomes included preterm birth, birth weight, height, and low birth weight. Live birth was defined as the delivery of any viable infant who was 28 weeks of gestation or older, and twins delivered by one mother were calculated as one live birth. Biochemical pregnancy loss was defined as loss of pregnancy after conception, β-hCG level > 5 mIU/mL, and before visualization of a gestational sac on transvaginal ultrasound. Clinical pregnancy was defined as the presence of gestational sac transvaginal ultrasound at 6-8 weeks of transplantation. Gestational age was calculated from the day of oocyte retrieval which was defined as Day 14 of the cycle. Stillbirth was defined as intrauterine or intrapartum death of a child born at a gestational age ≥ 28 weeks (elective terminations not included). Preterm birth was a birth between 28 and 37 completed weeks of gestation. The cut-off for low birthweight was 2500g at birth. Hypertensive disorders of pregnancy (HDP) included pregnancy induced hypertension, preeclampsia and haemolysis elevated liver enzymes and low platelets (HELLP) syndrome. Abnormal placentation included placenta praevia and placental abruption.

Blastocyst culture and scoring

Blastocysts were scored in the light of the Gardner criteria [12], including degree of expansion and quality of the inner cell mass (ICM) and trophectoderm cells （TE）by two embryologists with over 5 years of experience. This system consists of a number indicating the degree of blastocoel or blastocyst expansion: stage 1 = early blastocyst, blastocoel < 50% total embryo volume; stage 2 = early blastocyst, blastocoel > 50% total embryo volume; stage 3 = full blastocyst, blastocoele fully occupies the embryo; stage 4 = expanded blastocyst; stage 5 = hatching blastocyst; stage 6 = hatched blastocyst, For blastocysts at Grades 3 to 6, the ICM and TE were also graded. The ICM was graded as follows: A = many cells tightly compacted; B = several cells loosely adhered; C = very few cells; D = no cells or degenerate or necrotic cells), and a second letter grade designating the grade the TE: A = continuous layer of small identical cells; B = noncontinuous layer with fewer cells; C = noncontinuous layer with few small cells and large cells; D = sparse distribution of large or flat or degenerate cells. Blastocyst was recorded as high quality embryo if they reached at least an expansion stage 3 with A or B for ICM and TE. In our fertility center, lower-grade embryos such as stage 1 or grade CC were identified as unavailable for transfer, then, blastocysts were divided into four groups based on their morphologic grading on day 5: group 1 = early stage (stage 2) blastocysts, group 2 = good (3-6, AA/AB/BA); group 3 = fair (3-6, BB), and group 4 = poor (3-6, AC/CA/BC/CB) [12].

Follow-up

All patients were given a telephone follow-up 1-3 months after childbirth. The follow-up contents included obstetric complications, neonatal birth date, delivery mode, gestational age, sex, birth weight, height, and birth defects, etc.

Statistical analysis

A descriptive statistical analysis was performed on assisted reproductive technology (ART) characteristics. Continuous data were presented as the mean value ± standard deviation (SD), and differences in variables were compared using Student's t-test or one-way analysis of variance (ANOVA). Categorical variables were presented by the number of cases and corresponding percentage and compared using the chi-square test and Fisher's exact test when the number of events was less than 5. Multivariate logistic regression analysis was used to study the association between clinical characteristics and CPR. A p-value < 0.05 was considered statistically significant. The statistical analysis was performed with the use of the Statistical Package for Social Science (SPSS) version 19.00.

Results

General information

The final D5 and D6 transplantation cycles had 508 and 104 cases respectively. The flow diagram is presented in Fig. 1. The average maternal age was 30.94 ± 4.61 years, average serum AMH was 4.88 ± 3.60 ng/ml, and average number of AFC was 15.81 ± 6.53, before stimulation start. There was no significant

difference in maternal age, BMI, AMH, and infertility years between the two groups (p > 0.05), meanwhile, the D5 group had significantly less primary infertility and higher number of AFC (16.09 ± 6.54 vs 14.45 ± 6.34, p = 0.02) than those of the D6 group (Table 1) .

Ovarian stimulation characteristics

When the SBT group was stratified by development speed, there was no difference in endometrial thickness, duration of stimulation, and total dose of gonadotropins (Gn) between the D5-SBT and D6-SBT groups (p > 0.05). However, oocytes retrieved, 2 pronucleus (PN), embryos available for transfer, and blastocyst formed in the D5-SBT group were more than those of the D6-SBT group (p < 0.05) (Table 1) .

Pregnancy outcomes

Comparing to D6 group, D5 group had significantly higher CPR (59.84% vs 31.73%, p < 0.001) and LBR (44.69% vs 25.00%, p < 0.001). In addition, no significant differences were observed in the rates of biochemical pregnancy loss rate (9.06% vs 8.65%, p = 0.90) , early miscarriage rate (22.04% vs 18.18%, 1.64% vs 0, p = 0.61), or late miscarriage rate (1.64% vs 0, p = 0.46) (Table 1).

Obstetric outcomes

There were no statistical difference in rate of caesarean section, Male/Female ratio, preterm labour, hypertensive disorder, and gestational age between D5 and D6 group. All 5 twins were premature delivery. Considering the small number of twins, only singleton data were analysed in the study. There was no significant difference between the D5-SBT and D6-SBT groups stratified by singleton in terms of gestational age, newborn height and weight, and proportion of low birth weight infants (p > 0.05). (Table 2).

Embryo transfer characteristics

There are statistically significant differences in the proportion of good quality blastocyst transplanted between D5 and D6 group. In the group of D5, patients more often experienced good blastocysts transfer (45.67% vs 13.46%, p < 0.001), and the fair blastocysts transfer ratio was similar (38.58% vs 37.5%, p = 0.85) compared with D6 group. In turn, patients in D6 group more often underwent poor blastocysts transfer (45.19% vs 9.65%, p < 0.001) (Table 3).

The influence of morphologic grading on the pregnancy outcomes of D5 vesus D6

According to the quality of blastocysts, the selected cases were subdivided into 4 groups:

for Group 1, the CPR and LBR were similar between D5 and D6 group. As to Group 2, We didn't find difference in the CPR（63.36% vs 50.00%, p = 0.33）and LBR (47.62% vs 42.86%, p = 0.74）between D5 and D6 group. According to Group 3 and Group 4, the CPR（63.26% vs 30.77%, p < 0.001; 48.98 vs 27.66, p = 0.03） and LBR（47.72 vs 25.64, p = 0.01; 34.69 vs 17.02, p = 0.049）were significantly greater in D5 group than those in D6 group (Table 3). Multivariate analysis demonstrated that progesterone on hCG day (OR: 0.323, 95% CI: 0.135-0.770, p = 0.011) was negatively related to CPR in Group3 and AFC (OR: 1.137, 95% CI: 1.017 - 1.270, p = 0.024) was positively related to CPR in Group4 (Supplementary Table 1).

The influence of different COS protocols on the pregnancy outcomes of D5 vesus D6

GnRH antagonist protocol: There were no remarkable differences between the groups in maternal age, BMI, AMH and endometrial thickness, while D5 group had significantly lower ratio of primary infertility, infertility years, and higher number of oocytes retrieved, 2PN, embryos available for transfer, and blastocyst formed (p < 0.05). The CPR (45.76% vs 10.00%, p = 0.01) was significantly higher in the D5 group than in the D6 groups (Table 4). Multivariate analysis of didn't find any factors related to CPR in GnRH antagonist protocol (Supp Table 2).

Long-acting GnRHa follicular phase protocol: The numbers of AFC, oocytes retrieved, 2PN, embryos available for transfer, and blastocysts formed were significantly higher in the D5 group than those in the D6 groups (p < 0.05). The CPR (67.59% vs 40.70%, p < 0.001) and LBR (51.38% vs 32.20%, p = 0.01) were significantly higher in the D6 group than those in the D5 groups, meanwhile, there were no distinct difference in the rate of biochemical pregnancy loss, early miscarriage, and preterm labour between D5 and D6 (Table 4). Multivariate analysis didn't find any factors related to CPR in GnRHa follicular phase protocol (Supp Table 2).

Long-acting GnRHa Luteal phase protocol: Patients in D5 group were younger (31.28 ± 4.38 vs 33.36 ± 4.34, p = 0.03) than the patients in D6 group. The numbers of embryos available for transfer, blastocyst formed, CPR (50.94% vs 28.00%, p = 0.03) was significantly higher in the D5 group than those in the D6 group, nevertheless, LBR (35.22% vs 24.00%, p = 0.27) showed no statistical difference between D5 and D6. The rate of biochemical pregnancy loss, early miscarriage, and preterm labour were similar between D5 and D6 (Table 4). Multivariate analysis revealed that endometrial thickness (OR 1.174, 95% CI: 0.023 - 1.346, p = 0.022) and 2PN (OR 1.192, 95% CI: 1.005 - 1.413, p = 0.044) were positively associated with CPR in GnRHa luteal phase protocol (Supple Table 2).

Discussion

The results of this study demonstrated that the transfer of D5 blastocysts was associated with prominently higher CPR and LBR when compared to transfer of D6 blastocysts after fresh cycles. This was in line with the findings of the previous studies [8, 13]. The meta-analysis performed by Bourdon M. et al identified a higher risk of miscarriage after D6 compared to D5 ET-in overall fresh and/or frozen cycles and in fresh only and frozen-vitrified ET cycles [8], which was not valid in this study.

There were no obvious difference in maternal age, AMH, BMI, endometrial thickness, etc, between the D5-SET and D6-SET groups. However, the number of AFC, oocytes retrieved, 2PN, embryos available for transfer, and blastocyst formed in the D5 group were significantly higher than those of the D6 group (p <

0.05), which gave an indication that ovarian reserve of patients in D6-SET group was relatively worse than that of patients in D6-SET group. Meanwhile, in fresh embryo transfer cycle, patients experienced D6 blastocyst transfer in our infertility center meant that they didn't have D5 blastocyst formed. From the data about blastocysts transfer characteristics, we found that patients in D6 group more often experienced poor quality blastocysts transfer (45.19% vs 9.65%, $p < 0.001$), which was't noted in previous articles and suggested that D6-SET patients represent a subgroup of infertility patients usually having relatively poor embryo quality. These results could partially explain the relatively lower CPR and LBR in D6 group.

Analysis of our data showed that D5 and D6 blastocyst had similar CPR and LBR for AA/AB/BA blastocysts; however, for blastocysts in the same morphology group including BB, BC/AC/CA/CB, we found superior implantation potential in favor of D5 embryos. Yang et al. performed preimplantation genetic screening (PGS) on 237 blastocysts and found that blastocyst development speed had no effect on the euploid rate for high-quality blastocysts (55.2 % vs. 55.3 %, $p > 0.05$). However, the rates of euploid and clinical pregnancy in the D5 group were higher than those in the D6 group for poor-quality blastocysts ($p > 0.05$) [14]. Although there was no statistical difference, it may be the reason for the lower pregnancy rate of the D6 poor-quality blastocysts [14,15,16]. Similarly, another study found that blastocyst frozen days (D5 or D6) had no impact on LBR for AA/AB/ BA blastocysts; however those frozen on day 5 had significantly better LBR than those frozen on day 6 for BB/BC/CB blastocysts [17]. These findings indicated that blastocyst development speed may have little predictive value for the developmental potential of high-quality blastocysts but may have a certain predictive value for poor-quality blastocysts.

Our results showed that the LBR of poor-quality blastocysts in the D5-SBT and the D6-SBT group could reach 34.69%, and 17.0% respectively. For poor-quality D5 blastocysts, SBT could be recommended because of the acceptable LBR and significantly reduced multiple pregnancy rates compared to DBT. For poor-quality D6 blastocysts, this study cannot specify accurately a better strategy to transfer D6-blastocysts. To draw a firm conclusion, an RCT should be performed to evaluate pregnancy chances after fresh D6 transfer [18,19] . It is expected that the more the genetic and molecular features of embryo development are characterized, the more the role of traditional morphology-based selection will be replaced in IVF [20-23]. Future research to identify non-invasive biomarkers of reproductive potential may further enhance blastocysts selection [24]. In clinical practice, some women obtain both D5 and D6 blastocysts after embryo culture. For these, it appears reasonable to transfer first D5 blastocysts in order to limit time to pregnancy. For those with only D6 blastocyts, chances of pregnancy may be lower but still remained and D6 blastocysts should be transferred.

Meanwhile, once a continuous pregnancy is reached, the blastocyst development speed and morphology do not affect neonatal outcomes. Bouillon et al. [25] suggested that the main outcomes of singletons after transfer of blastocysts with poor morphological grading were not associated with increased adverse obstetric and perinatal events. This information could be important to reassure couples who conceive following the transfer of poor-quality embryos. In this study, the gestational age, birth weight and length of D5 and D6 blastocysts were not significantly different after transplantation in the single-birth group. But, because of the limited case numbers, we can't draw a definitive conclusion [26-28]

Urged by the ongoing controversies on reproductive outcome using the different treatment regimens, we compared the efficiency of GnRH antagonist, GnRHa follicular phase, GnRHa luteal phase regimens in women after the first oocyte retrieval [29-33]. In all the three COS protocols, the main characteristics of the populations, such as maternal age, BMI, and AMH of D5 vesus D6 showed no statistical difference. In terms of biological parameters, the results of D6 were always lower for the number of embryos available for transfer and blastocyst formed. The results indicated that the GnRHa antagonist protocol has a demonstrable inferiority comparing with the GnRHa follicular or luteal phase protocol with regard to the CPR and LBR in D6-SET group ($p < 0.05$) . This was in line with the findings of large-scale retrospective population-based cohort survey [29, 30]. In view of these results, we changed the blastocyst transfer stragety in fresh cycle. We perform fresh D5 blastocyst transfer regularly in all the three COS protocols, nevertheless, fresh D6 blastocyst transfer was conducted just in GnRHa follicular phase and luteal phase protocol aiming to achieve comparatively ideal pregnancy outcomes.

There are some limitations to this study, including its retrospective design. First, the sample size included in each group is uneven; therefore, the results of the study may be biased, and further research is needed to confirm the conclusion of this study. Several methodological aspects support the results of our study: all the blastocysts were evaluated by the same trained embryologists in our IVF centre. As a consequence, the risk of variation in morphologic assessment was greatly reduced; all pregnancies were initiated after transfer of a single blastocyst. Such a strategy has the advantage of excluding the potential vanishing twin phenomenon after double embryo transfer [34, 35]. Due to the small number of our study, more larger, prospective and well conducted studies are warranted to confirm the reported findings.

Conclusion

Our study demonstrates that the CPR and LBR in the D5 group were significantly higher than those in the D6 group. The difference in neonatal outcomes between the two groups was not statistically significant. The subgroup analysis found that the superiority of day 5 SET compared with day 6 SET is influenced by the quality of blastocyst transferred and the COS protocols. The CPR and LBR of good quality blastocysts was similar between D5 and D6 group. Despite this, D5 blastocysts were significantly outperforming day 6 blastocysts with the same embryo grade including fair and poor quality for CPR and LBR, We pointed out that ovarian reserve of patients in D6-SET group was comparatively worse than that of patients in D5-SET group and D6-SET patients represented a subgroup of infertility patients usually having relatively poor embryo quality. The GnRHa antagonist protocol has a inferiority comparing with the GnRHa follicular or luteal phase protocol with regard to the CPR and LBR in D6-SET group.

Abbreviations

ART: Assisted reproductive technology; AMH: Anti-Müllerian hormone; AFC: Antral follicle counting; BMI: Body mass index; CPR: clinical pregnancy rate; COS: Controlled ovarian stimulation; DBT: double blastocyst transplantation; ET: Embryo transfer; FET: Frozen-embryo transfer; GnRH-a: Gonadotropin releasing hormone agonist; GnRH-ant: Gonadotropin-releasing hormone antagonist; IVF: In vitro fertilization; ICM: inner cell mass: ICSI: Intracytoplasmic sperm injection;TE: trophectoderm cells; LBR: Live birth rate; OHSS: Ovarian hyperstimulation syndrome; PGT-A:Preimplantation genetic testing for aneuploidy; RCTs:

Randomized controlled trials; rFSH: Recombinant follicle-stimulating hormone; SET: single embryo transfers (SET); SBT: single blastocyst transplantation; 2PN:2 pronucleus.

Declarations

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Authors' contributions

Zhilan Chen and Wei Li are responsible for the concept and the study design. Cong xiao and Yanmin Li performed the data collection, and Wei Li did the statistical analysis. Zhilan Chen drafted the manuscript. Aidong Gong contributed to the critical discussion, interpretation and editing of the manuscript.

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Availability of supporting data

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

Ethical Approval and Consent to participate

Not applicable

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Competing interests

The authors declare that they have no competing interests

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Tables

Table 1 Baseline patient characteristics and descriptive data of ovarian stimulation, oocytes and embryo transfer

Parameter	D5	D6	P-value
ET cycles (n)	508	104	
Maternal age (years)	30.84 ± 4.63	31.47 ± 4.48	0.20
BMI (kg/m2)	23.76 ± 7.94	22.78 ± 3.52	0.22
Infertility years (years)	3.03 ± 2.65	3.45 ± 3.02	0.16
Infertility type, n (%)			
Primary	198/508 (38.98)	51/104 (49.04)	0.06
Secondary	310/508 (61.02)	53/104 (50.96)	
Serum AMH (ng/ml)	4.99 ± 3.61	4.33 ± 3.52	0.09
AFC (n)	16.09 ± 6.54	14.45 ± 6.34	0.02
Total dose of Gn (IU)	2286.61 ± 892.24	2424.34 ± 976.76	0.16
Duration of stimulation (days)	11.32 ± 2.10	11.14 ± 2.27	0.45
E2 on hCG day (pg/ml)	2831.87 ± 1255.32	2756.74 ± 1286.99	0.58
Progesterone on hCG day (pg/ml)	0.74 ± 0.37	0.77 ± 0.40	0.58
Endometrial thickness (mm)	11.84 ± 2.49	11.73 ± 2.44	0.69
Oocytes retrieved (n)	15.57 ± 4.88	13.74 ± 5.71	0.001
Methods of insemination			
IVF	441/508 (86.81)	73/104 (70.19)	<0.001
ICSI	67/508 (13.19)	31/104 (29.80)	
2PN (n)	10.86 ± 4.07	8.78 ± 4.32	<0.001
Embryos available for transfer (n)	6.63 ± 2.97	3.54 ± 2.21	<0.001
Blastocyst formed (n)	5.84 ± 2.89	2.71 ± 2.14	<0.001
CPR, n (%)	304/508 (59.84)	33/104 (31.73)	<0.001
Biochemical pregnancy loss, n (%)	46/508 (9.06)	9/104 (8.65)	0.90
Early miscarriage rate, n (%)	67/304 (22.04)	6/33 (18.18)	0.61
Multiple pregnancy rate, n (%)	4/304 (1.32)	1/33 (3.03)	0.41
Ectopic pregnancy	5/304 (1.64)	1/33 (3.03)	0.57
Late miscarriage rate, n (%)	5/304 (1.64)	0	0.46
LBR, n (%)	227/508 (44.69)	26/104 (25.00)	<0.001
Single birth, n	223/508 (43.90)	25/104 (24.04)	
Twin birth, n	4/4	1/1	NA

AFC Antral follicle count, AMH Anti-Müllerian hormone, BMI Body mass index, bFSH basal follicle stimulating hormone, CPR Clinical pregnancy rate, E2 Estradiol, ET Embryo transfer, HCG human chorionic gonadotropin, Gn Gonadotropin, ICSI Intracytoplasmic Sperm Injection, IVF In vitro fertilization, LBR Live birth rate, PN Pronucleus

$p < 0.05$ was considered statistically significant

Table 2 Neonatal outcome in liveborns after fresh D5 and D6 transfer

Parameter	D5	D6	P-value
Mode of delivery, n (%)	227	26	
Caesarean section	162/227（71.37）	23/26（88.46）	0.06
Vaginal,	65/227（28.63）	3/26 （11.54）	
Preterm labour, n (%)	24/227（10.57）	4/26 （15.38）	0.51
Abnormal placentation (n)	1	1	NA
Gestational diabetes (n)	2	0	NA
Hypertensive disorder (n)	4/304 (1.32)	1/33 (3.03)	0.34
Perinatal death (n)	2	0	NA
twin-twin transfusion syndrome (n)	1	0	NA
Malformations (n)	2	0	NA
Male/Female ratio	129/102(1.26)	18/9 (2.00)	0.28
Singleton birth, n	223	25	
Gestational age (weeks)	38.62 ± 1.41	38.29 ± 1.44	0.26
Birth weight (g)	3276.84 ± 474.93	3264.20 ± 438.12	0.9
Height (cm)	49.96 ± 2.23	50.04 ± 0.89	0.86
Birth weight ≤2500 g , n (%)	11/223 (4.93)	1/25 (4.00)	0.84
Preterm labour, n (%)	20/223 (8.97)	3/25 (12.00)	0.62
Malformations (n)	2	0	NA
Twin birth, n	4	1	
Gestational age (weeks)	33.79 ± 3.20	36.43	NA
Birth weight (g)	1937.5 ± 635.69	2500 ±141.42	NA
Height (cm)	42.13 ± 7.97	48.0 ±0.00	NA
Birth weight ≤ 2500 g, n (%)	44720	44594	NA
Preterm labour	44655	44562	NA
Malformations (n)	0	0	NA

Table 3 Comparisons of ET cycles undergoing SBT between D5 and D6 groups stratified by blastocyst morphologic grading

Parameter	Group 1			Group 2			Group 3			Group 4	
	D5	D6	P	D5	D6	P	D5	D6	P	D5	D6
ET cycles (n)	31	4		231	14		197	39		49	47
Propotion of blastocysts transferred/Total blastocysts transferred , n (%)	31/508 (6.10)	4/104 (3.84)	0.37	231/508 (45.47)	14/104 (13.46)	< 0.001	197/508 (38.78)	39/104 (37.50)	0.81	49/508 (9.64)	47/1 (45.1
Maternal age (years)	30.32 ± 6.21	32.75 ± 4.27	0.46	30.83 ± 4.41	30.21 ±3.83	0.61	30.85 ± 4.55	33.08 ± 4.18	0.01	31.14 ± 4.98	30.4 4.61
BMI (kg/m2)	24.11 ± 4.92	19.25 ± 0.88	0.28	23.287 ± 4.57	23.44 ± 3.96	0.90	24.17 ± 11.44	23.03 ± 3.40	0.54	24.11 ± 3.82	22.67 3.54
Infertility years (years)	2.49 ± 1.91	3.66 ± 2.73	0.28	3.12 ± 2.86	3.42 ± 2.42	0.71	3.12 ± 2.46	4.10 ± 3.53	0.04	2.61 ± 2.74	2.89 2.71
Infertility type, n (%)											
Primary	10/31 (32.25)	1/4 (25.00)	0.77	96/231 (41.56)	8 (57.14)	0.25	71/197 (36.04)	17/39 (43.59)	0.37	21/49 □42.86□	25/4 □53.1
Secondary	21/31 (67.74)	3/4 (75.00)		135/231 (58.44)	6 (42.86)		126/197 (63.96)	22/39 (56.41)		28/49 □57.14□	22/4 □46.8
Serum AMH (ng/ml)	4.45 ± 3.62	6.00 ± 5.63	0.45	5.2715 ± 3.69	5.7092± 5.68	0.69	4.89 ± 3.72	3.92 ± 2.79	0.12	4.40 ± 2.66	4.15 3.09
AFC (n)	15.35 ± 6.00	18.75 ± 8.22	0.31	16.63 ± 6.37	16.57 ± 7.08	0.98	16.45 ± 6.10	13.95 ± 6.16	0.02	15.63 ± 6.29	13.87 6.05
Total dose of Gn (IU)	2479.84 ± 920.36	2212.50 ± 879.51	0.59	2177.08± 913.87	1963.93± 998.65	0.40	2332.05± 858.28	2466.36± 1000.80	0.39	2498.04 ± 856.44	2544 ± 944.3
Duration of stimulation (days)	11.48 ± 2.29	10.75 ± 0.96	0.54	11.26 ± 2.26	10.50 ± 1.51	0.21	11.31 ± 1.98	11.00 ± 2.36	0.38	11.47 ± 1.72	11.49 2.43
E2 on hCG day (pg/ml)	2649.23 ± 1140.25	3727.75 ± 1220.25	0.09	2977.99 ± 1265.9	2899.64 ± 956.81	0.82	2702.21 ± 1261.89	2923.84 ± 1357.66	0.32	2782.83 ± 1206.67	2492 ± 1283
Progesterone on hCG day (pg/ml)	0.66 ± 0.36	0.95 ± 0.46	0.16	0.76 ± 0.38	0.85 ± 0.34	0.40	0.73 ± 0.38	0.79 ± 0.43	0.40	0.78 ± 0.33	0.71 0.40
Endometrial thickness (mm)	12.09 ± 2.31	11.150 ± 3.01	0.46	11.87 ± 2.51	10.85 ± 2.39	0.15	11.87 ± 2.43	11.66 ± 2.13	0.62	11.45 ± 2.80	12.09 2.08
Oocytes retrieved (n)	15.03 ± 5.87	13.00 ± 2.16	0.21	16.23 ± 4.69	16.07 ± 5.74	0.91	15.23 ± 4.72	14.00 ± 5.31	0.15	14.18 ± 5.40	12.89 6.12
Methods of Insemination											
IVF	27/31 (87.09)	2/4 (50)	0.06	196/231 (84.85)	8/14 (57.14)	0.01	175/197 (88.83)	27/39 (69.23)	0.001	43/49 (87.76)	36/4 □76.6
ICSI	4/31 (12.90)	2/4 (50)		35/231 (15.15)	6 /14 (42.85)		22/197 (11.17)	12/39 (30.77)		6/49 (12.24)	11/4 (23.4
2PN (n)	10.39 ± 4.79	9.00 ± 2.16	0.58	11.60 ± 3.79	11.57 ± 4.60	0.98	10.49 ± 3.48	8.64 ± 4.34	0.01	9.14 ± 5.03	8.04 4.13
Embryos available for transfer (n)	4.55 ± 2.26	5.25 ± 0.5	0.16	7.69 ± 2.90	5.79 ± 2.12	0.02	6.11 ± 2.72	3.51 ± 1.90	<0.001	5.00 ± 2.72	2.74 2.06
Blastocyst formed (n)	4.52 ± 2.52	1.50 ± 1.0	0.03	6.72 ± 3.03	4.29 ± 4.12	0.01	5.43 ± 2.55	2.62 ± 1.35	<0.001	4.18 ± 2.40	2.43 1.70
Biochemical pregnancy loss, n (%)	6/31 (19.35)	1/4 (25.00)	1.00	19/231 (8.23)	3/14 (21.42)	0.12	16/196 (8.16)	3/39 (7.69)	1.0	5/49 (10.20)	2/47 (4.25
CPR, n (%)	9/31 (29.03)	1/4 (25.00)	1.00	147/231 (63.64)	7/14 (50.00)	0.33	124/196 (62.94)	12/39 (30.77)	< 0.001	24/49 (48.98)	13/4 (27.6
Early miscarriage rate, n (%)	2/9 (22.22)	0	NA	32/147 (21.80)	0	NA	27/124 (21.77)	2/12 (16.66)	0.74	6/24 (25.00)	4/13 (30.7
LBR, n (%)	7/31 (22.58)	1/4 (25.00)	1.00	110/231(47.62)	6/14(42.86)	0.74	94/197 (47.72)	10/39 (25.64)	0.01	17/49 (34.69)	8/47 (17.0

AFC Antral follicle count, AMH Anti-Müllerian hormone, BMI Body mass index, bFSH basal follicle stimulating hormone, CPR Clinical pregnancy rate, E2 Estradiol, ET Embryo transfer, HCG human chorionic gonadotropin, Gn Gonadotropin, ICSI Intracytoplasmic Sperm Injection, IVF In vitro fertilization, LBR Live

birth rate, PN Pronucleus

p < 0.05 was considered statistically significant

group 1 = early stage (stage 2) blastocysts; group 2 = good (3-6, AA/AB/BA); group 3 = fair (3-6, BB); group 4 = poor (3-6, AC/CA/BC/CB).

Table 4 Basic and treatment cycle characteristics in the three COS protocol

Parameter	GnRH antagonist protocol			GnRHa follicular phase protocol			GnRHa luteal phase protocol		
	D5	D6		D5	D6		D5	D6	
ET cycles (n)	59	20	P	290	59	P	159	25	P
Maternal age (years)	32.47 ± 5.16	32.20 ± 5.05	0.86	30.26 ± 4.57	30.42 ± 4.09	0.79	31.28 ± 4.38	33.36 ± 4.34	0.03
BMI (kg/m2)	23.85 ± 3.94	22.89 ± 4.10	0.36	23.60 ± 4.56	22.66 ± 3.52	0.14	24.01 ± 12.59	22.96 ± 3.15	0.67
Infertility years (years)	2.99 ± 2.01	5.01 ± 3.39	0.02	3.15 ± 2.82	2.72 ± 2.40	0.27	2.83 ± 2.53	3.91 ± 3.55	0.06
Infertility type, n (%)									
Primary	17/59 (28.81)	12/20 (60.00)	0.01	125/290 (43.10)	28/59 (47.45)	0.54	56/159 (35.22)	11/25 (44.00)	0.40
Secondary	42/59 (71.19)	8/20 (40.00)		165/290 (57.90)	31/59 (52.54)		103/159 (64.78)	14/25 (56.00)	
Serum AMH (ng/ml)	3.96 ± 3.39	2.95 ± 2.27	0.19	5.40 ± 3.89	4.48 ± 3.13	0.09	4.59 ± 3.03	5.10 ± 4.79	0.48
bFSH (mIU/ml)	6.74 ± 2.31	8.38 ± 2.36	0.01	6.27 ± 1.84	6.52 ± 2.06	0.35	6.22 ± 1.61	6.96 ± 1.90	0.04
AFC (n)	14.02 ± 7.47	12.65 ± 6.50	0.47	17.18 ± 6.33	15.00 ± 6.32	0.02	14.89 ± 6.20	14.60 ± 6.27	0.83
Total dose of Gn (IU)	2276.39 ± 979.51	2793.75 ± 991.07	0.05	2300.00 ± 846.07	2364.42 ± 965.18	0.88	2265.99 ± 944.56	2270.24 ± 958.30	0.98
Duration of stimulation (days)	10.03 ± 1.68	10.25 ± 2.69	0.67	11.44 ± 2.27	11.39 ± 2.24	0.60	11.57 ± 1.74	11.28 ± 1.82	0.44
E2 on hCG day (pg/ml)	2854.20 ± 1286.06	2607.82 ± 1478.74	0.47	2795.05 ± 1250.47	2770.11 ± 1238.27	0.89	2891.72 ± 1258.30	2854.16 ± 1272.37	0.89
Progesterone on hCG day (ng/ml)	0.80 ± 0.37	0.87 ± 0.47	0.52	0.70 ± 0.36	0.74 ± 0.41	0.60	0.79 ± 0.38	0.76 ± 0.33	0.72
Endometrial thickness (mm)	11.05 ± 2.54	11.77 ± 2.32	0.26	12.13 ± 2.40	12.02 ± 2.49	0.73	11.60 ± 2.56	10.97 ± 2.36	0.27
Oocytes retrieved (n)	13.90 ± 5.91	9.85 ± 5.64	0.01	16.03 ± 4.82	15.17 ± 5.48	0.22	15.33 ± 4.46	13.48 ± 4.92	0.06
Methods of Insemination, n (%)									
IVF	53/59 (89.83)	16/20 (80.00)	0.25	248/290 (85.51)	42/59 (71.18)	0.01	140/159 (88.05)	15/25 (60.00)	0.00
ICSI	6 /59 (10.16)	4/20 (20.00)		42/290 (14.48)	17/59 (28.81)		19/159 (11.94)	10/25 (40.00)	
2PN (n)	9.88 ± 4.70	5.85 ± 3.95	0.001	11.04 ± 4.03	9.44 ± 4.38	0.01	10.89 ± 3.86	9.56 ± 3.56	0.11
Embryos available for transfer (n)	5.90 ± 3.31	2.15 ± 1.46	0.001	6.70 ± 2.99	3.73 ± 2.12	< 0.001	6.76 ± 2.77	4.20 ± 2.52	0.001
Blastocyst formed (n)	5.34 ± 2.64	1.75 ± 0.97	0.001	6.02 ± 3.05	3.10 ± 2.50	< 0.001	5.70 ± 2.66	2.56 ± 1.61	0.001
CPR, n (%)	27/59 (45.76)	2/20 (10.00)	0.01	196/290 (67.59)	24/59 (40.70)	< 0.001	81/159 (50.94)	7/25 (28.00)	0.03
Biochemical pregnancy loss, n (%)	5/59 (8.47)	3/20 (15.00)	0.42	22/290 (7.59)	5/59 (8.47)	0.82	19/159 (11.59)	1/25 (4.00)	0.32
Early miscarriage rate, n (%)	4/27 (14.81)	1/2 (50.00)	0.32	44/196 (22.45)	5/24 (20.83)	1.0	19/81 (23.46)	0	NA
Ectopic pregnancy	1/27 (3.70)	0	NA	1/196 (0.50)	0	NA	3/81 (3.70)	1/7（14.29）	0.22
LBR, n (%)	22/59 (37.29)	1/20 (5.00)	0.01	149/290 (51. 38)	19/59 (32.20)	0.01	56/159 (35.22)	6/25 (24.00)	0.27
Preterm <37 weeks, n (%)	2/59 (9.09)	0	0.85	15/149 (10.07)	2/19 (10.53)	0.95	5/56 (8.93)	1/6 (16.67)	0.54

AFC Antral follicle count, AMH Anti-Müllerian hormone, BMI Body mass index, bFSH basal follicle stimulating hormone, CPR Clinical pregnancy rate, E2 Estradiol, ET Embryo transfer, HCG human chorionic gonadotropin, Gn Gonadotropin, ICSI Intracytoplasmic Sperm Injection, IVF In vitro fertilization, LBR Live birth rate, PN Pronucleus

$p < 0.05$ was considered statistically significant

Figures

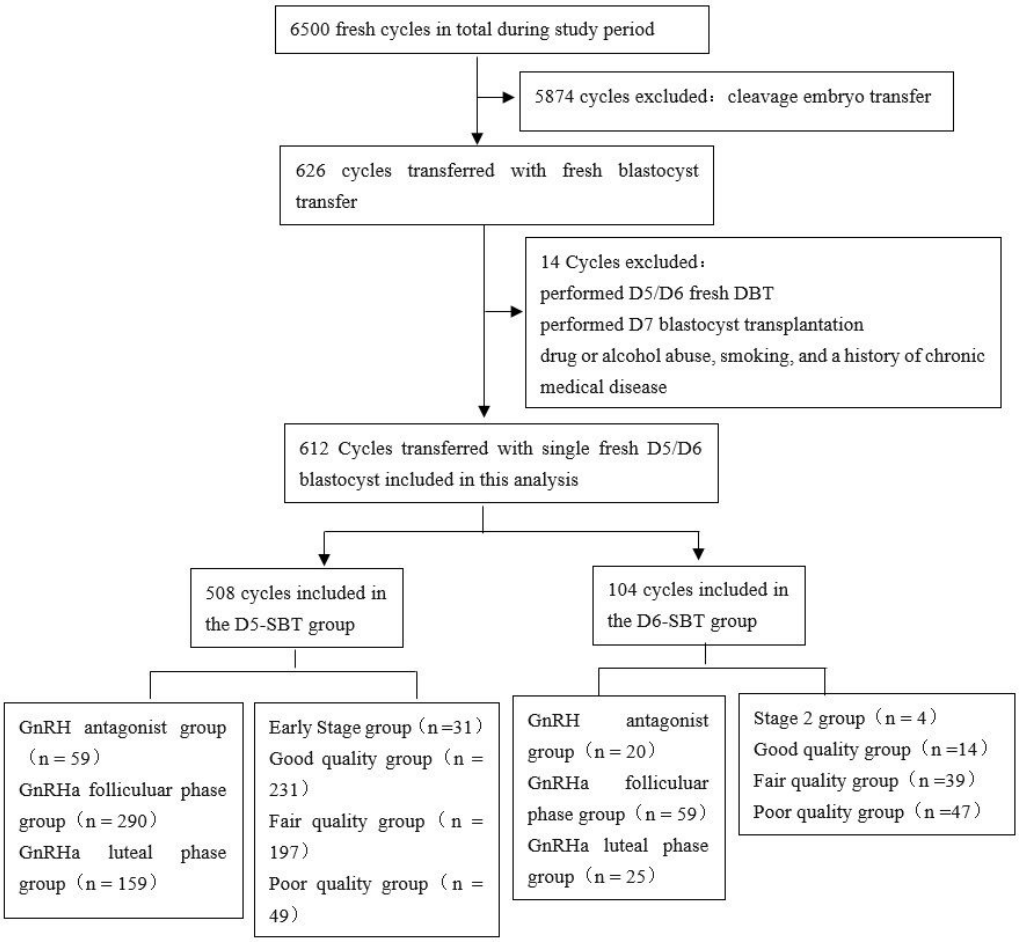

Figure 1

Flowchart of eligibility criteria

Supplementary Files

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