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Comparison of Pregnancy Outcomes Between Single-Morula Embryo Transfer and Single-Blastocyst Transfer in Fresh IVF/ICSI Cycles

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Background: This study investigated the effectiveness and feasibility of day 4 (D4) morula embryo transfer (ET) in comparison with day 5 (D5) blastocyst ET, with regards to their clinical data, laboratory test results, and pregnancy outcomes.

Material/Methods: This retrospective cohort study enrolled 1070 patients, including 178 cases in group D4 and 892 cases in group D5. The endpoint was live birth rate after fresh embryo transfer. Furthermore, the clinical outcomes of D4 embryos with different morphology were compared and assigned to 3 groups: in group 1 (n=66) the embryos were compacted but not expanded, in group 2 (n=102) the embryos were compacted and expanded (early blastocyst), and in group 3 (n=10) the embryos were not compacted.

Results: Groups D4 and D5 had comparable clinical pregnancy rates (53.37% vs 59.97%) and live birth rates (43.25% vs 50.89%), and there were no significant differences between the 2 groups. In group 3, there was only 1 clinical pregnancy and no live birth. In comparison between group 1 and group 2, the clinical pregnancy rate of group 2 showed an upward trend (48.48% vs 60.78%), but there was no significant difference. There was also no statistically significant difference in the live birth rate between the 2 groups (42.42% vs 49.01%).

Conclusions: Transferring of compacted embryos or early blastocysts can result in high clinical pregnancy rates and live birth rates. In addition to the cleavage and blastocyst ET, morula ET may serve as an alternative option for the clinician.

Keywords: Blastocyst • Live Birth • Morula

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Background

At present, cleavage-stage embryo transfer (ET) and blastocyst stage ET are commonly applied in many reproductive centers during fresh IVF/ICSI (in vitro fertilization/intracytoplasmic sperm injection) cycles. However, day 4 (D4) ET or single-morula embryo transfer (SMET) is often neglected. Previous studies have suggested that day 5 (D5) ET has a higher clinical pregnancy rate compared to day 3 (D3) ET. Elective single-blastocyst embryo transfer (SBET) can effectively lower the rate of multiple pregnancies, thereby reducing the risk of obstetric and neonatal complications [1,2]. However, contradictory findings have been reported. A recent study has demonstrated that fresh blastocysts offer no obvious advantage compared to cleavage-stage embryo transfer in women under 39 years old in terms of implantation and pregnancy rate [3], but the study involved transferring 2 cleavage embryos or blastocysts. According to both the National Institute for Health and Clinical Excellence (NICE) 2013 fertility guidelines and the Practice Committees of the American Society for Reproductive Medicine, the extended embryo culture into blastocyst stage during IVF/ICSI cycles can reduce the number of embryos that can be frozen, and there is a small increased the risk of adverse neonatal outcomes, but there is not enough evidence to prove a causal relationship [4,5].

Although the embryo culture system is constantly improving, it is different from the environment in the human body. There are potential increased risks of developmental arrest and reduced embryo quality after prolonged culture in vitro [6]. Hence, the balance between the risks and pregnancy rate remains a necessity. In addition, D5 ET is not always feasible for those clinics that are operate only on weekdays. Therefore, this study aimed to compare the clinical pregnancy and live birth rates between D4 and D5 ET, and the findings may provide evidence for the selection of D4 ET.

Material and Methods

Study Objects

This retrospective cohort study was conducted at the Reproductive Center of the Second Affiliated Hospital of Wenzhou Medical University. Female infertile patients who received single-morula embryo transfer (SMET) were selected, total of 178 cases from January 1, 2016 to August 31, 2019. There were a total of 892 cases of D5 transfer during January 1 2018 to December 31,2018.

Ovarian Stimulation Protocol

All patients received a long-term protocol of pituitary down-regulation with gonadotrophin-releasing hormone (GnRH) agonist for controlled ovarian hyper-stimulation (COH). Firstly, the patients were treated with 3.75 mg of GnRH agonist on the 2nd to 4th day of menstruation, and luteal-phase ovarian stimulation was initiated 30 to 40 days later. The stimulation was adjusted according to the size of antral follicles and the serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), and progesterone (P). In addition, gonadotropins (Gn), such as recombinant FSH and human menopausal gonadotropins (hMG), were used for COH. The starting dose of Gn ranged from 75 to 300 IU, depending on patient age and ovarian function, as well as their responses to previous ovarian stimulation. Gn doses were based on the growth of ovarian follicles. When at least 2 follicles had a mean diameter of 18 mm, the final oocyte maturation was triggered by 4000 to 10 000 IU of human chorionic gonadotropin (hCG). Lastly, transvaginal ovum retrieval was carried out 34 to 36 hours after hCG administration. Fertilization was observed 20 hours after insemination based on the appearance of 2PN. The embryos were cultured in G-1 and G-2 medium (Vitrolife Co., Ltd., Australia) at 37°C, under 6% CO_2, 5% O_2, and 89% N_2.

Embryo Grading

Grading of D4 embryos was conducted according to the 2011 ESHRE Istanbul Consensus and Ryh-sheng Li's system for day 4 embryos [7,8]. On day 5, blastocyst stage embryo grading was conducted according to Gardner's embryo grading system [9], by taking into account of the degree of expansion and the development of inner cell mass and trophectoderm. Based on this grading system, the ideal embryos were categorized into 3AA, 4AA, 5AA, 3AB/BA, 4AB/BA, and 5AB/BA. Meanwhile, cleavage-stage embryo grading was carried out by referring to the 2011 ESHRE Istanbul Consensus for D3 embryo [7], which involved the amount of cell fragmentation (%), cell multi-nucleation, cell symmetry, vacuoles, and zona pellucida. Based on this grading system, the optimal embryos were categorized into 7A, 8A, 9A, 7B, 8B, and 9B.

Embryo Transfer and Luteal Phase Support

Embryo transfer (ET) was performed under the guidance of transabdominal ultrasound. The starting time of luteal phase support depended on serum P level on the day of hCG trigger. When P<1.2 ng/ml, luteal support was initiated 1 day after oocyte retrieval. When Pg≥1.2 ng/ml, luteal support was initiated 2 days after ovulation. If Pg≥1.5 ng/ml, ET was canceled. Crinone gel and dydrogesterone tablets were used for luteal phase support until day 13-14 after ET. Luteal support was continued until week 10-12 of pregnancy.

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Table 1. Comparison of demographic and clinical data between D4 and D5 group.

Characteristic	D4 group	D5 group	*P* value
Number of cycles	178	892	
Number of transferred	1	1	
Age (years)	32.04±4.56	31.02±4.08	0.003*
Infertility duration (years)	3.17±2.17	3.30±2.55	0.506
Basic FSH	7.62±3.02	7.22±1.91	0.021*
BMI	21.84±3.11	21.76±3.25	0.77
AFC	15.28±7.68	15.49±5.52	0.670
Days of Gn	11.48±2.45	11.42±2.05	0.758
Doses of Gn	2606.19±1094.98	2457.14±833.96	0.004*
E2 on trigger day (pg/ml)	2051.19±1187.65	2426.51±1130.23	<0.0001*
Diagnosis			
PCOS (%)	14/178 (7.86%)	104/892 (11.65%)	0.140
Endometriosis (%)	15/178 (8.42%)	51/892 (5.71%)	0.170

Data are presented as mean±SD or n (%). * Indicate statistical significance.

Outcome Measurement

The primary endpoint of this study was the live birth rate, while the secondary endpoints were clinical pregnancy rate, early spontaneous abortion rate, ectopic pregnancy rate, multiplets pregnancy rate, preterm delivery rate, and neonate weight.

We defined the pregnancy outcomes in this study according to "CSRM consensus on key indicators for quality control in ART clinical operation" [10].

1. Clinical pregnancy rate: Clinical pregnancy cycle number/fresh transfer cycle number×100%. Clinical pregnancy was diagnosed when 1 or more pregnancy sacs were observed on ultrasound; we could only see the gestational sac, not the fetal heart. The rate included normal intrauterine pregnancy, ectopic pregnancy, and heterotopic pregnancy.
2. Early spontaneous abortion rate: The number of spontaneous abortion cycles within 12 weeks of pregnancy/number of clinical pregnancy cycles ×100%.
3. Ectopic pregnancy rate: The number of ectopic pregnancy cycles/clinical pregnancy cycles ×100%.
4. Multiplets pregnancy rate: Multiple pregnancy cycles/clinical pregnancy cycles ×100%.
5. Live birth rate: The number of live births/transfer cycles ×100%.
6. Preterm delivery rate: The number of live births before 37 weeks/number of live births ×100%.

Statistical Analysis

Statistical analysis was performed using SPSS software version 23.0 (IBM Corp, Armonk, NY, USA). The *t* test was used to analyze continuous variables and the chi-square test was used for non-continuous variables. Univariate variance analysis was carried out to compare differences between the 3 groups. All data are presented with mean±standard deviation (SD). *P* values of less than 0.05 were regarded as statistically significant.

Results

We compared the patient characteristics and laboratory test results between the D4 group and D5 group. There were 178 cycles in the D4 group and 892 cycles in the D5 group. **Table 1** summarizes the demographic data of patients in the D4 and D5 groups. Compared with those in the D5 group, patients in the D4 group were older (32.04±4.56 vs 31.02±4.08 P=0.003). Ages of patients in the D4 group ranged from 22.5 to 44.0 years and those in the D5 group ranged from 20.0 to 43.3 years. In the D4 group, the basic FSH was higher (7.62±3.02 vs 7.22±1.91, *P*=0.021), the total Gn used was higher (2606.19±1094.98 vs 2457.14±833.96, *P*=0.004), and trigger day E2 was lower (2051.19±1187.65 vs 2426.51±1130.23, *P*=0.000). There was no statistically significant difference in BMI, AFC, infertility duration, or days of Gn between the 2 groups.

The laboratory data of patients in the D4 and D5 groups are summarized in **Table 2**. Comparing group D4 with group D5, the number of retrieved oocytes, number of MII oocytes, number of D3 optimal embryos, number of frozen high-quality

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Table 2. Comparison of laboratory data between D4 and D5 group.

Laboratory data	D4 group	D5 group	*P* value
Number of cycles	178	892	
Number of retrieved oocytes	11.46±6.52	13.16±4.82	<0.0001*
Number of MII oocytes	10.28±6.24	12.09±4.81	<0.0001*
Number of fertilized oocytes	8.44±5.55	9.31±4.03	0.003*
Number of D3	7.08±4.85	8.04±3.81	0.004*
Number of D3 optimal embryos	3.96±3.06	4.57±2.77	0.008*
Number of frozen high-quality blastocysts	1.51±1.81	2.05±2.01	0.001*
Number of frozen blastocysts	3.42±3.21	3.96±2.71	0.019*

Data are presented as mean±SD or n (%). * Indicate statistical significance.

Table 3. Comparison of pregnancy outcomes between D4 and D5 groups.

Outcome	D4 group	D5 group	*P* value
Clinical pregnancy rate	95/178 (53.37%)	535/892 (59.97%)	0.102
Early spontaneous abortion rate	14/95 (16.84%)	57/535 (10.6%)	0.246
Live birth rate	77/178 (43.25%)	454/892 (50.89%)	0.063
Ectopic pregnancy rate	2/95 (2.10%)	4/535 (0.74%)	0.209
Monozygotic twins rate	1/95 (1.05%)	12/535 (2.24%)	0.452
Preterm delivery rate	4/77 (5.19%)	33/454 (7.26%)	0.509
Neonate weight	3258.68±577.23	3207.27±470.95	0.456

Data are presented as mean±SD or n (%).

blastocysts, number of total frozen blastocysts of D4 group are all lower than those of D5. There was a statistically significant difference.

Pregnancy Outcomes Between D4 and D5 Groups

As shown in **Table 3**, the D4 group and D5 group had similar clinical pregnancy rates (53.37% vs 59.97%, *P*>0.05), early spontaneous abortion rate (16.84% vs 10.6%), live birth rate (43.25% vs 50.89%), ectopic pregnancy rate (2.10% vs 0.74%), monozygotic twins rate (2.1% vs 0.74%), preterm delivery rate (5.19% vs 7.26%), and neonate weight (3258.68±577.23 vs 3207.27±470.95). There was no statistically significant difference between the 2 groups (*P*>0.05).

Among the 178 patients in the D4 group, there were 10 cases without compaction, including 1 case of clinical pregnancy but early abortion. There were 66 patients with compaction but without expanding (group 1), and 102 cycles with early blastocyst formation (group 2). The clinical data and pregnancy outcomes of the 2 groups were compared. Patient characteristics and laboratory data between group 1 and group 2 are summarized in T**ables 4 and 5**. There were no statistically significant differences in age, infertility duration, basic FSH, AFC, days of Gn, doses of Gn, E2 on trigger day, number of retrieved oocytes, number of MII oocytes, number of fertilized oocytes, number of D3, or number of D3 optimal embryos, between the 2 groups (*P*>0.05). However, there were significantly fewer frozen high-quality blastocysts and total frozen blastocysts in group 1 than in group 2.

Pregnancy Outcomes in Group 1 and Group 2

The clinical pregnancy rate of group 2 was higher than in group 1 (48.48% vs 60.78%, *P*>0 0.05), but the difference was not statistically significant, nor was there any statistically significant difference in the live birth rate (42.42% vs 49.01%, *P*>0.05), as shown in **Table 6**.

Discussion

The findings of many studies indicated that blastocyst ET had a higher clinical pregnancy rate compared to cleavage ET [11,12]. This is probably because the blastocyst transfer can improve synchronization between the endometrium and embryo, and

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Table 4. Comparison of demographic and clinical data between group 1 and group 2.

Characteristic	Group 1	Group 2	*P* value
Number of cycles	66	102	
Age (years)	31.92±4.75	31.75±4.35	0.812
Infertility duration (years)	3.14±2.30	3.17±1.94	0.927
Basic FSH	7.68±3.57	7.42±2.55	0.741
AFC	13.33±7.21	17.27±7.34	0.001
Days of Gn	11.14±2.03	11.50±2.57	0.335
Doses of Gn	2617.89±965.69	2468.83±1113.438	0.374
E2 on trigger day (pg/ml)	2000.86±1291.26	2158.12±1089.62	0.399

Data are presented as mean±SD or n (%).

Table 5. Comparison of laboratory data between group 1 and group 2.

Laboratory data	Group 1	Group 2	*P* value
Number of cycle	66	102	
Number of retrieved oocytes	11.06±6.95	12.51±5.81	0.146
Number of MII oocytes	10.02±6.82	11.17±5.57	0.233
Number of fertilized oocytes	8.09±6.13	9.30±4.90	0.158
Number ofD3	6.85±5.25	7.66±4.44	0.286
Number of D3 optimalembryos	3.79±3.16	4.29±2.94	0.292
Number offrozenhigh-quality blastocysts	1.00±1.34	1.96±2.00	0.001*
Number offrozen frozenblastocysts	2.86±3.23	4.04±3.13	0.002*

Data are presented as mean±SD or n (%). * Indicate statistical significance.

Table 6. Comparison of pregnancy outcomes between group 1 and group 2.

Outcome	Group 1	Group 2	*P* value
Clinical pregnancy rate	32/66 (48.48%)	62/102 (60.78%)	0.117
Early spontaneous abortion rate	5/33 (15.15%)	10/62 (16.12%)	0.901
Live birth rate	28/66 (42.42%)	50/102 (49.01%)	0.618
Ectopic pregnancy rate	0/32	2/62	
Monozygotic twin rate	0/32	1/62	
Neonate weight	3088.33±418.70	3270.24±488.59	0.105

Data are presented as mean±SD or n (%).

due to the reduced proportion of chromosomal abnormalities in blastocysts, which increased the selection of suitable embryos for transfer [13,14]. However, several studies have shown that the cumulative pregnancy rate of blastocyst transplantation is not significantly different from or is even lower than that of cleavage embryos [15,16]. A recent meta-analysis also concluded that D5 transfer is not superior to D3 transfer, although the current evidence is low-quality or moderate-quality [17]. In addition, it has been reported that the blastocyst formation rate of D3 optimal embryos is approximately 30% to 50% [18,19]. Although this proportion is increasing with the development of embryo culture technology, the blastocyst formation rate of D3 optimal embryos in our study was more than 50%. If all patients receive blastocyst culture, the total number of transplantable embryos will inevitably decrease, and there may even be no embryos for transfer. In this study, 44 cases in the D4

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group had no blastocysts frozen (including D5 and D6) after D4 transfer; 11 of them had no high-quality D3 embryos. Of the 11 cases, 4 had clinical pregnancy, and 3 had live births. Therefore, compared with D5 transfer, D4 transfer appears to have higher embryo utilization but similar clinical pregnancy rate and live birth rate. However, this study did not compare the embryo transfer cycle cancelation rate between the 2 groups because it was only a retrospective study. Because the patients who cancel the transfer due to embryo problems on D4 may have to cancel the transfer again on D5 because no cultured embryos are available then, we need to design a stricter RCT to compare the embryo transfer cycle cancelation rate between the 2 groups. Meanwhile, we would study more about the blastocyst formation rates of different grades of embryos in D4. In this study, it was also found that the basic conditions of the D4 and D5 groups were different, whether from age or number of retrieved oocytes, D3 number of embryos, D3 number of total embryos, D3 number of high-quality blastocysts, and total blastocysts. The D5 group was superior to the D4 group. It may be related to our transfer strategy. If there are few retrieved oocytes, or if the embryo compaction was not good on D4, we ask the patient's opinion about whether to transfer. The other cycles of day 4 transfer were mainly because of organizational reasons; for example, when day 5 was a Sunday or public holiday.

Clinical pregnancy rates and live birth rates were similar to those in the D5 group, even though the age and embryo conditions of patients in the D4 group were not as good as in the D5 group. This is consistent with previous studies [20-22]. Therefore, it is suggested that blastocyst transfer can increase the pregnancy rate in each ET cycle, but limit the number of viable embryos transferred. D4 has a higher transfer rate than D5, and has a comparable clinical pregnancy rate and live birth rate. Additionally, prolonged culture in vitro may be detrimental to embryos. Research has shown that the risk of preterm birth and congenital anomalies may be higher in blastocyst transfer than in cleavage transfer [23]. In this study, the D4 prematurity rate was 5.19% and the D5 rate was 7.26%, and there was no significant difference between the 2 groups. Other potential risks of D5 ET have been reported previously, such as the higher risk of monozygous twins, large for gestational age babies, altered sex ratio, and higher birthweight [24-27], but the mechanism needs to be further studied. There are only animal experiments that shows differences in the expression of several genes, particularly of imprinted genes involved in apoptosis, oxidative stress, and gap junction formation in prolonged cultured embryos [28]. In our study there was no significant difference in the rate of monozygotic twins or birth weight between the 2 groups, but our sample size was small.

Compared to cleavage-stage ET, morula-stage ET had the same or higher implantation and pregnancy rates, but with fewer embryos transferred [29]. There are 2 possible reasons underlying the increased pregnancy rate among patients treated with D4 ET compared to D3 ET. Firstly, the expression of embryonic genes is initiated on D4, and thus the embryos with higher developmental potential can be selected after D4. As a consequence, D4 morula-stage embryos confer a greater selection value than cleavage-stage embryos [29,30]. Secondly, the uterine contractility is reduced at D4, and the embryos demonstrate improved synchronicity with the receptive endometrium [31].

To assess which D4 embryo is more conducive to good clinical outcomes, we further divided the D4 group into 3 groups: in group 1 the embryo was compacted but not expanded, in group 2 the embryo was compacted and expanded, and in group 3 the embryo was not compacted. We found that there were no live births in group 3. The clinical pregnancy rate of group 2 showed an upward trend compared with group 1 (48.48% vs 60.78%, $P>0$ 0.05), but the difference was not significant, and there was no significant difference in live birth rates (42.42% vs 49.01%). Therefore, it appears that as long as the embryo is compacted, there will be a high clinical pregnancy rate and live birth rate. Of course, more cases are needed for further studies, and larger sample sizes are needed to explore the relationship between degree of compaction and clinical pregnancy rate.

Conclusions

Our results suggest that D4 ET can allow a more flexible ET scheduling between clinicians and patients and avoid ET during weekends and holidays, without affecting IVF success rates. It offers an easier, flexible, and valuable method for routine ET. D4 transfer could be complementary to our current transfer strategy. In this way, patients, clinicians, and laboratorians would have more choices on the transfer day.

Due to the relatively small sample size in this study, further investigations with larger sample sizes are warranted.

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