IJHSRP

International Journal of Health Services Research and Policy

www.dergipark.org.tr/ijhsrp

e-ISSN: 2602-3482

Research Article

REPRODUCTIVE OUTCOMES FOLLOWING FROZEN-THAWED EMBRYO TRANSFER IS SUPERIOR WITH THE TRANSFER OF BLASTOCYSTS EXPANDED ON DAY 5 THAN ON DAY 6

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Abstract: *To compare pregnancy and live birth rates between D5 and D6 transfer of frozen-thawed blastocysts in women undergoing 'freeze-all' cycles. Our study, designed as a retrospective cohort study, evaluates single frozen thaw embryo transfer in 209 patients. The cohort group included in the study was divided into two according to the day of becoming blastocyst: (i) D5 blastocysts and (ii) D6 blastocysts. A 'freeze-all' strategy was adopted using GnRH antagonist cycles and vitrification as the method of freezing. The pregnancy rate was higher in the D5 group than the D6 group, although it was not statistically significant (72.6% vs. 59.6%, respectively, p=0.078). Live birth rate was significantly higher in the D5 group than in the D6 group (66.9% vs. 48.1%, respectively, p=0.015). The rates of abortus, biochemical pregnancy, and preterm birth were comparable between the groups. Live birth rate is superior when blastocysts expanded on D5 are used in frozen-thawed cycles, compared to those expanded on D6. The day of the blastocyst expansion appears to be an important predictor of pregnancy outcome and, thus, taken into account as well as D5 embryos should be given the priority in frozen-thawed transfer cycles.*

Keywords: *frozen embryo transfer; live birth rate; day 5 versus day 6; blastocyst vitrification.*

Received: January 24, 2021 Accepted: June 10, 2021

1. Introduction

A conventional in vitro fertilization (IVF) cycle involves several consecutive steps, starting from the controlled ovarian stimulation (COS) with gonadotropins and ending with the transfer of the best quality embryo(s) at the cleavage or blastocyst stage. However, in certain circumstances, a fresh embryo transfer cannot be completed due to either some medical concerns such as premature progesterone (P4) elevation at the time of hCG triggering or increased risk of ovarian hyperstimulation syndrome (OHSS) or parental desire to pursue preimplantation genetic testing for aneuploidy (PGT-A). Thus, the entire cohort of viable embryos is cryopreserved, a strategy referred to as 'freeze-all' [1-4].

On the other hand, COS aims to obtain an appropriate number of competent oocytes with the minimum risks for the woman [5]. Many cohort studies have shown the relationship between the number of oocytes collected and live birth rates, and it has been proven that the higher the number, the higher

the live birth rate [6-7]. However, as the national reproductive health care policies continue to endorse limiting the number of embryos transferred and single embryo transfer has been adopted as the standard care in many countries, freezing more surplus embryos for use in subsequent cycles has become a standard approach [8-9].

Several achievements in the optimal embryo culture, laboratory procedures, and imaging systems allowed ART physicians to prefer embryo transfers at the blastocyst stage. A substantial number of clinical studies have shown that the likelihood of live birth after fresh blastocyst stage (Day 5-6; D5-6) embryo transfer is higher than after fresh cleavage stage (Day 2-3; D2-3) transfer [10-12]. Moreover, a recent Cochrane review has revealed that fresh D5-6 embryo transfer is associated with superior reproductive outcomes compared to D2-3 [13]. However, the rate of blastulation is not the same for all embryo cohort. As some embryos reach blastulation by D5 and others not until D6 or even D7, comparison of embryos with different blastulation rates has been performed in several studies, and the chance of pregnancy was reported to be significantly reduced if a fresh embryo transfer is performed on D6 compared to D5 [14-17], a result largely attributed to endometrial-embryonic asynchrony [18]

The hormonal preparation of endometrium in frozen embryo transfer (FET) cycles aims to optimize pregnancy rates by synchronizing the ET stage with that of endometrial receptivity [19]; therefore, no difference in pregnancy rates is expected between D5 and D6 blastocysts in FET cycles. However, there exists inconsistency concerning reproductive outcomes for D5 versus D6 cryopreserved blastocyst transfers, as some studies reported that D6 embryos are outperformed by D5 embryos [20-23], while others revealed similar ART outcomes for D5 and D6 thawed blastocyst transfers [16, 24-27]. It is noteworthy to express that not all these studies report live birth rates and are heterogeneous in terms of the number of blastocysts transferred.

Thus, in the present study, we aimed to present our results and compare reproductive outcomes in pregnancy and live birth rates between D5 and D6 transfer of frozen-thawed blastocysts in women undergoing ‘freeze-all’ cycles.

2. Materials and Methods

The present is a retrospective cohort follow-up study that included all autologous frozen-thawed blastocyst transfers performed after ovarian stimulation between January 2014 and December 2017 in the ART unit of our institution.

Ethical Considerations: The study was approved by the local ethic committee of Istanbul Yeni Yuzyil University (07 May 2018; 2018/5).

2.1. Patient Selection

In our study, 209 patients who were applied only all freezing protocols were included. According to our inclusion criteria; Patients aged 40 years and younger who underwent ICSI and subsequently underwent autologous frozen-thawed embryo transfer. Our exclusion criteria are poor-responder patients, patients with preimplantation genetic screening or preimplantation genetic diagnosis, and natural endometrial preparation cycles. Reproductive outcomes of groups were compared based on the development day of blastocyst expansion (i) D5 blastocysts (157) and (ii) D6 blastocysts (52). A total of 209 blastocyst transfers were analyzed during the study period. Only one cycle of the patients included in the study was included in the study.

2.2. Stimulation Methods

Recombinant FSH was used in all patients for controlled ovarian hyperstimulation (Gonal-F, Merck Serono, Germany), and treatment was started on the second day of the menstrual cycle.

Gonadotropin dose was adjusted according to the patient's age, body mass index, and antral follicle count. GnRH antagonist (Cetrotide; Merck-Serono, Switzerland) was used for pituitary down-regulation. The decision to start an antagonist was made when the leading follicle size reached 12-13 mm. Trigger decision was made for final maturation when the leading follicle reached 17 mm in patients with a low ovarian reserve and when at least three follicles reached 17 mm in patients with normal or high reserve. For this, highly purified hCG (Choriomon 5000 IU, IBSA) was used in patients with a low ovarian reserve and normal reserve, GnRH agonist, triptorelin acetate, and 0.2 mg Gonapeptyl were used to prevent OHSS in cases with high ovarian reserve for final maturation. (A serum E2 level above 3000 pg/dl was adopted as high-risk for OHSS, and, in this case, oocyte maturation was triggered using GnRH agonist). Oocyte retrieval was performed 35-35.5 hours after the trigger. After all the collected oocytes were applied to intracytoplasmic sperm injection (ICSI), pronucleus control was performed at the post-OPU 20th hour. All blastocysts formed after embryo culture were frozen by the vitrification method.

2.3. Insemination, Embryo Quality Assessment, and Cryopreservation

Single-step media was used as culture media for the incubation of all embryos. (LifeGlobal®). The embryos in the group called Group 1 reached full blast characteristics on the 5th day and were vitrified on the 5th day. However, nonexpanded embryos, those in morula or cavitating morula stages, are kept until the 6th day and vitrified if they gain full blast characteristics, which was named Group 2, and embryos that did not develop blastocysts were discarded. All embryos in both groups were monitored under the same conditions and using the same media until the day of vitrification. One hundred fifty-seven blastocysts evaluated in group 1 were vitrified in D5, and 52 blasts in group 2 were vitrified in D6. The embryo scoring was conducted according to the grading scale proposed by Gardner & Schoolcraft (28). Embryos were graded from 1 to 6 according to the level of expansion and hatching. The inner cell mass (ICM) was scored according to the number of cells as follows; It was accepted as best grade (A) if the ICM cells were large in number and closely monitored as a package. If some of the ICM cells were observed in loose groups, it was considered grade B. Those with the worst grade (C) were those with few and loose ICM cells.

Similarly, in trophectoderm (TE) scoring, it was considered the best grade (A) if most TE cells were in the form of multiple epithelial layers. Few TE cells consisting of a loose epithelium were defined as grade (B); if very few TE cells were represented, it was the worst grade (C). From blastocysts expanded both on D5 and D6, only those with quality ≥3BB, namely 'good quality,' were selected and transferred.

According to the manufacturer's instructions, the vitrification method used was the Irvine Scientific Freeze Kit (Cat. 90133-SO; Irvine Scientific, Santa Ana, CA, USA) with HSV straws.

2.4. FET Endometrial Preparation and Embryo Transfer

In all patients included in the study, the artificial cycle was preferred as the endometrial preparation method. Ovarian suppression and possible pathologies (ovarian cyst, fibroid, polyp) were eliminated with transvaginal ultrasonography and baseline hormonal measurements performed in the second menstruation period after OPU. E2, P4, and LH levels were measured in all patients at the beginning of the endometrial preparation. E2 levels >80 pg/ml, P4 levels >1.2 ng/ml or sonographic evidence of ovarian cysts, ET was canceled, and the patient was re-evaluated for the onset of the following menstrual cycle.

Endometrial preparation is initiated on the 2nd or 3rd day of the FET cycle with oral estrogen (Estrofem 2 mg tablet, Novo Nordisk, Bagsvaerd, Denmark) supplementation at a dose of 2 mg daily and titrated to 10 mg daily to allow for endometrial development. In addition to P4 and E2

measurements, a transvaginal ultrasound assessment was made on the 12th day of the cycle. Once the endometrium reached a thickness >7 mm, vaginal progesterone (Crinone 8% vaginal gel; Merck Serono, Bedford, England) supplementation began on the 15th day to achieve endometrial differentiation. Endometrial thickness <7 mm was counted as an exclusion criterion from the study. Embryos (day 5/6) in both groups were thawed and transferred on the 6th day of progesterone addition. Embryos in both groups were thawed 2-4 hours before the transfer, and survival was checked after 30 minutes. After 2 hours of the thawing process, a second control was made, and they were evaluated for hatching, re-expansion, and extensive cytoplasmic granulation. Eligible blastocysts were transferred on the same day. The embryo transfer was performed under abdominal ultrasound guidance using a full bladder and a 5 MHz transabdominal probe (GE Voluson S6 General Electric, Wauwatosa, USA). Cook transfer catheter (Cook Medical, Indiana, and USA) was used as the transfer catheter. In the dorsal lithotomy position, the external catheter was inserted into the cervix after gentle cleansing of the cervical mucus with saline solution. Embryos were then transferred to approximately 1-1.5 cm from the surface of the fundal endometrium in the upper part of the endometrial cavity. The catheter was checked under a microscope for embryo retention or the presence of blood.

Vaginal progesterone gel and estradiol tablets were used to support the luteal phase until the day of the BhCG test. Biochemical pregnancy was determined by measuring serum β-hCG levels 12 days after the transfer. Estradiol supplementation was discontinued when the pregnancy test result became positive, while vaginal progesterone supplementation was not stopped until the 9th gestational week. Spontaneous abortus cases were also recorded and indicated with the term 'abortus'. The detection made the clinical diagnosis of pregnancy of the gestational sac in the transvaginal ultrasonography performed five weeks after the transfer.

2.5. Statistical Analysis

Data analysis was performed using the NCSS (Number Cruncher Statistical System, 2007, Kaysville, UT, USA). Continuous data were reported as mean+ standard deviation (SD) or median (range) as appropriate. Student t-test, Pearson's chi-square test, and Fisher's exact test were used for continuous or categorical variables, respectively. Non-parametric comparisons were performed using the Mann-Whitney U test, and categorical data were evaluated using the $\chi 2$ test. A p-value <0.05 was considered statistically significant.

3. Results

A total of 209 blastocysts ETs were analyzed, as 157 (75.1%) and 52 (24.9%) embryos were vitrified on D5 and D6, respectively. Serum E2 levels on the 2nd day of the FET cycle did not significantly differ between the groups (p=0.953). Serum P4 levels on the 2nd day of the FET cycle did not significantly differ between the groups (p=0.426).

Basic demographic characteristics and cycle outcomes are presented in Table 1. Mean patient ages and infertility periods did not significantly differ between D5 and D6 groups (p=0.104 and 0.192, respectively). Mean endometrial thickness by transvaginal sonography on the 12th day of the FET cycle was comparable between the groups (p=0.541).

Table 1. Comparison of the demographic and clinical characteristics between the groups

	Group 1 (n=157)	Group 2 (n=52)	Overall (n=209)	p-value
Age at retrieval	3.50±4.94	32.83±5.43	31.83±5.09	0.104[a]
BMI, kg/m^2	2.96±5.04	24.60±3.62	24.87±4.72	0.578[a]
Smoking, n(%)	48 (30.6)	17 (32.7)	65 (31.1)	0.775[b]
Infertility period, y	4.49±3.38	4.67±2.50	4.54±3.17	0.192[c]
Endometrial thickness at 12th day, mm	9.32±1.68	9.36±1.72	9.20±1.54	0.541[a]
Estradiol, pg/ml	42.51±24.29	39.75±15.27	41.13±19.78	0.953[c]
Progesterone, ng/ml	0.29±0.20	0.26±0.18	0.27±0.19	0.426[c]

Data are presented as mean±standard deviation (SD), [a]Student's t-test, [b]Chi-square test, [c]Mann Whitney U test. BMI: Body Mass Index.

In the overall patient cohort, in 45.5% of the ET procedures, a single embryo was transferred, whereas two embryos were transferred in 54.5% of the cycles. The number of transferred embryos, 1 or 2, was not statistically significantly different between D5 and D6 groups (p=0.397). The pregnancy rate was 69.4% in the total cohort. This rate was higher in the D5 group than the D6 group, although not statistically significant (72.6% *vs.* 59.6%, respectively, p=0.078). Live birth rate was 62.2% in the overall cohort and was significantly higher in the D5 group than in the D6 group (66.9% vs. 48.1%, respectively, p=0.015). However, the rates of abortus, biochemical pregnancy, and preterm birth were comparable between the groups. The number of pregnancies and the number of live birth rates, either 1 or 2 newborns, were comparable between the groups (p=0.408 and 0.244, respectively) (Table 2).

Table 2. Comparison of the reproductive outcomes between the groups

	Group 1 (n=157)	Group 2 (n=52)	Overall (n=209)	p-value
Transferred embryo number, n (%)				0,397[d]
1 embryo	74 (4.1)	21 (40.4)	95 (45.5)	
2 embryos	83 (52.9)	31 (59.6)	114 (54.5)	
Pregnancy, n (%)	114 (72.6)	31 (59.6)	145 (69,4)	0.078[d]
Live birth, n (%)	105 (66.9)	25 (48.1)	130 (62.2)	0.015[d]
Abortus, n (%)	6 (3.8)	3 (5.8)	9 (4.3)	0.693[e]
Biochemical pregnancy, n (%)	3 (1.9)	3 (5.8)	6 (2.9)	0.165[e]
Preterm birth, n (%)	1 (0.6)	1 (1.9)	2 (1.0)	0.437[e]
Pregnancy outcome n(%)				0.408[d]
1 baby	89 (78.1)	22 (71.0)	111 (76.6)	
2 baby	25 (21.9)	9 (29.0)	34 (23.4)	
Live birth number n (%)				0.244[e]
1 baby	85 (81.0)	23 (92.0)	108 (83.1)	
2 baby	20 (19.0)	2 (8.0)	22 (16.9)	

Data are presented as numbers and percentages, n (%). [d]Chi-square test, [e]Fisher's Exact Test

4. Discussion

IVF treatment methods have improved significantly since Louise Brown in 1978, but infertile couples with previous unsuccessful treatments are still among the main challenges facing IVF centers [28]. Also, several studies have shown a decrease in implantation rates after repeated unsuccessful IVF

treatments [29]. One of the main reasons for this is that embryos are transferred to the uterus on day 2 or 3 of the cleavage stage [28]. Two ways have been proposed to treat low implantation rates among couples having multiple previous failed IVF treatment attempts: Placing more than two embryos in the endometrium during the cleavage stage or transferring the embryos at a stage other than the cleavage stage.

Improvements in embryo culture media, promoting embryonic growth through genome activation, blastocele development, and embryonic expansion, allowed selection of embryos with the best implantation potential and led to an increase in the practice of embryo transfer at the blastocyst stage [29–31]. With blastocyst transfer, higher pregnancy, implantation, and live birth rates have been achieved than embryo transfers in the early cleavage stage [32–33]. In previous studies [29, 30, 32, 33], embryo transfer has been recommended on the 5th day after egg retrieval, as the higher implantation associated with blastocyst transfer has a pregnancy live birth rate. The possible cause of these high pregnancy rates may be the more advanced developmental stage of the blastocyst [30, 34, 35].

Multiple pregnancies have been a major concern for infertile couples, physicians, and public health. Maternal mortality and morbidity are important in infant mortality, morbidity, and costs on the community health system. Blastocyst transfer allows the selection of better quality embryos and has been proposed as a method that reduces the possibility of multiple pregnancies [36].

Reducing the number of transferred embryos to prevent multiple pregnancies and complications caused by multiple pregnancies should be the most important goal of assisted reproductive technology (ART) programs. The best way to achieve this is to select and transfer the single embryo with the highest implantation success [33].

According to the results of our cohort study, live birth rates are significantly higher when blastocysts expanded on Day 5 (D5) are transferred in FET cycles compared to those expanded on Day 6 (D6). However, although higher in cycles that employ blastocysts vitrified on D5, the difference in pregnancy rates does not reach statistical significance.

Previous studies reported compromised reproductive outcomes when embryos reached blastulation by day 6 compared to day 5 in fresh transfer cycles [15, 37]. However, literature data regarding the clinical implications of the transfer of embryos with a delayed rate of blastulation in vitrified-thawed blastocyst transfer cycles is controversial. Some studies concluded that delayed blastocyst development has no impact on the pregnancy outcomes of the FET cycle [16, 38]. In support of this, Capalbo et al. [39] demonstrated that euploidy rates in faster-growing blastocysts (D5) were similar to those in slower-growing ones (D6). In 2010, Sunkara et al. [40] published a meta-analysis involving 15 studies and reported a significantly higher clinical pregnancy rate and ongoing pregnancy and live birth rate with D5 than D6 frozen-thawed blastocyst transfers. However, sensitivity analysis revealed no differences in ongoing pregnancy and LBR after D5 versus D6 thawed blastocyst transfer with the same morphological blastocyst quality on the day of the cryopreservation. Kaye et al. [27] compared clinical and ongoing pregnancy rates in cycles with single embryo transfer of blastocysts cryopreserved on D5 or D6. However, their cohort comprised cryopreservation by both slow freezing and vitrification. Their results suggested comparable outcomes between D5 and D6 blastocyst groups for both the overall and vitrified blastocyst cohorts.

Conversely, Haas et al. [22] compared the pregnancy outcomes of 537 and 254 cycles, including blastocysts vitrified on D5 and D6, and, consistent with our results, reported significantly lower clinical pregnancy rates with blastocysts vitrified on D6 compared to blastocysts vitrified on D5, even when the D6 vitrified blastocyst morphology was at least as good as that of blastocysts vitrified on D5. Their thawing protocol differs from ours. They thawed D5 blastocysts 20–24 hours before the embryo transfer, whereas D6 blastocysts were thawed 2–4 hours before the transfer, which confers the heterogeneity between the cohorts thawed on different periods, and this is in contrast to our protocol, in which both

D5 and D6 vitrified blastocysts were thawed 2–4 hours before the transfer. Tubbing et al. [41] compared clinical outcomes between D5 and D6 vitrified blastocysts, and their results favored D5. In 2018, another study compared Live birth Rate **(**LBR) after frozen-thawed D5 and D6 blastocyst transfers in 1347 single autologous cycles [22]. Their results suggested that LBR following thawed blastocyst transfer was significantly lower when D6 vitrified blastocysts were used than D5 blastocysts, regardless of their quality. Our results are consistent with those reported by Ferreux et al., as we demonstrated the superiority of D5 blastocyst transfer in terms of live birth rates. Also, Ferreux et al. [23] evaluated potential confounders and reported that blastocyst expansion at D6 was independently associated with a significant decrease in LBR compared to D5 expanded-blastocysts.

Our study has some limitations. Clinical outcomes, such as pregnancy rates and live birth rates, might be influenced by many factors in freezing and thawing and the transfer process. These factors are not adjusted in the study. Another limitation might be morphological assessment in the embryo selection in both blastocyst groups, as this approach has been associated with poor predictive value [42]. Moreover, a pre-implantation genetic screening has not been performed in our study, although the significant difference between the two groups might be conceivably attributed to the embryo aneuploidies. One of the study's strengths is that all included cycles employed vitrification as the method of freezing. Thus, our data exclude the possible impact of the freezing method on the outcomes as it does not contain slow-freeze cycles. The thawing process also was performed 2–4 hours before the embryo transfer, which excludes any possible methodological heterogeneity between embryo cohorts thawed on different periods. Another strength of this study is the use of artificial endometrial preparation in both D5 and D6 blastocyst cohorts, eliminating the possible endometrial receptivity factor.

5. Conclusion

Based on the present study results, we suggest that transferring blastocysts expanding at D5 is associated with a significantly higher likelihood of pregnancy and live birth than those expanding at D6 in frozen-thawed embryo transfer cycles, even when D6 blastocysts are good-quality. Thus, the day of the blastocyst expansion appears to be an important predictor of pregnancy outcome and, thus, should be taken into account as well as D5 embryos should be given priority in frozen-thawed transfer cycles.

Conflict of Interest: The authors confirm that they have no interests that might be perceived as posing a conflict or bias.
Ethical Consideration: The study was approved by the local ethic committee of Istanbul Yeni Yuzyil University (07 May 2018; 2018/5).
Research and Publication Ethics: This work was carried out by obeying research and ethics rules.
Author Contributions: All of the authors declare that they all have participated in the design, execution, and analysis of the paper and approved the final version.

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