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Effect of reculture of the thawed slowly developing embryos before transfer on implantation and pregnancy; a Randomized Controlled Study

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Abstract

Background: Embryos resulting from assisted reproduction techniques and cultured to reach the day 5 Fully Expanded Blastocyst stage (FEB). For some reasons, embryos may have delayed division and development. In a fresh embryo transfer cycles using these embryos, pregnancy rates are expected to drop significantly due to defective embryo-endometrial synchronization. A proposed approach is to freeze these embryos then thaw and reculture for 24 hours before embryo transfer as an attempt to enhance embryo development to reach FEB before embryo transfer. *Methods*: It is a prospective randomized controlled study, including 60 women undergoing FET in Shatby university hospital and one private IVF center. Endometrial preparation was done using estradiol valerate supplementation and luteal phase support using a combination of vaginal and intramuscular progesterone. Then women were randomized to FET either after 24 hours of reculturing of thawed embryos (group 1, n = 30) or on the same day of embryo thawing (group 2, n =30). *Results*: Out of 54 embryo transferred in 30 FET cycles in group 1, 18 intrauterine gestational sacs with pulsating fetal poles were observed using transvaginal ultrasound (33% implantation rate) and 13 of these progressed till beyond 12 weeks of gestation (24.1% ongoing pregnancy rate). However, a total of 84 embryo were transferred in 30 FET cycles in group 2, of which 16 intrauterine gestational sacs with pulsating fetal poles were observed using transvaginal ultrasound (19.3% implantation rate) and 10 of these progressed till beyond 12 weeks of gestation (12 % ongoing pregnancy rate). No statistically significant difference was observed (P 0.063, 0.066). *Conclusion*: Deferring embryo transfer and reculturing till reaching a fully expanded embryos for

later vitrified-warmed transfer may improve the LBRs, although further studies are needed to clarify this hypothesis.

Keywords: Reculture; slowly developing embryos; Frozen embryo transfer; implantation rate; Randomized Controlled Trial.

Introduction

Many studies used to compare fresh and frozen-thawed embryo transfer (FET) cycles in normal responders. The results of these studies showed a significantly higher clinical pregnancy rate per transfer in the Frozen embryo transfer (FET) cycles versus the fresh cycles.(1-3). Impaired endometrial receptivity in the fresh cycles as a consequence of stimulation was believed to be the cause of this improvement in pregnancy rate, (1-3) and therefore, there has been a trend toward FET cycles in the last several years.

There is also a recent trend toward blastocyst culture and single embryo transfer (ET) as a trial to reduce the risk of multiple pregnancies.(4, 5). However, several factors that may affect the pregnancy rate that needs to be taken into account before determining the number of embryos to transfer. These factors include the age of the patient, blastocyst quality, and the number of failed IVF cycles in the past(6, 7).

Outcomes of FET cycles with blastocysts have been examined in many studies. Some reports showed comparable outcomes between blastocysts formed and cryopreserved on day 5 (D5 blastocysts) and blastocysts formed and cryopreserved on day 6 (D6 blastocysts)(1, 8). Meanwhile, evidence showing that the CPR, IR, and live birth rate (LBR) with D5 blastocysts are higher than with D6 blastocysts in FET cycles (9-11).

Several studies have provided evidence that in fresh cycles blastocysts transfer on day 6 should be avoided, and it should be performed on day 5(12, 13). However, the suitable timing to transfer D6 blastocysts in FET cycles is yet to be determined.

Even when using the most advanced culture media you cannot guarantee that 100% of the embryos will reach the blastocyst stage. There has been a debate for the last few years over whether to prefer embryo transfer at the cleavage stage or to culture the embryos to the blastocyst stage and transfer them at day 5 (14). Some of the embryos do not develop to the blastocyst stage on day 5, some of them are not viable, and some of them are slow-developing embryos at the morula or cavitating morula (CAVM) stage. Normally, the embryo is at the morula stage on day 4, but there is insufficient evidence regarding the etiology, prognosis, and how to proceed when the embryo is still at the morula stage on day 5. Good pregnancy results have been elaborated in studies that included fresh transfers of morulae on day 4,(15, 16) but pregnancy outcomes with embryos at the morula stage on day 5 need further studying.

Effect of reculture of the thawed slowly developing embryos before transfer on implantation and pregnancy

Objective

The objective of the study is to compare delayed embryo transfer in cases of slowing dividing embryos, to same-day transfer regarding implantation rate and pregnancy rate using frozen embryos.

Materials and Methods

Study design, settings and participants

El Shatby University hospital and one private ICSI centers in Alexandria, Egypt.

The present study was a registered prospective randomized controlled study conducted between October 2021 and October 2022. The Institutional ethical review board approved the study protocol and informed written consent was obtained from all participants after discussing the nature of the study.
Couples presented for frozen embryo transfer cycles (FET) for various indications were invited to participate in the study if they met our inclusion criteria. We included in:

Patients with slowly dividing embryos (day 5 morulae and early blastocyst) and at the sametime having FEB (fully expanded blastocysts) when continuing culture till day 6 attending for ICSI for various indications. The study participants were entered the screening phase of the study included history taking (age, gravity, parity, previous miscarriages, factor of infertility and history of previous ICSI cycles)

We excluded patients with:

1. expected poor ovarian responders,
2. repeated implantation failure,
3. recurrent pregnancy loss,
4. previous uterine surgery (example; hysteroscopic myomectomy, septum resection, Asherman syndrome) .
5. patients with comorbidities (renal, hepatic, cardiac diseases).
6. patients undergoing preimplantation genetic screening/diagnosis (PGS/PGD) have been excluded.

The eligible women were randomly distributed using computer based randomization (Random Digit Software) into the two study groups:

1. **Group I** (reincubation for 24 hours after thawing, day 6 embryo transfer),
2. **Group II** (thawing and transfer on same day, day 5 embryo transfer).

Sample size

Sample size calculation was performed for the primary outcome of the study (positive pregnancy test rate and implantation rate), based on prior studies (17). A minimal total sample size of 40 women was calculated to achieves 80 % study power and 95% confidence limits to detect a significant effect size in the positive pregnancy test rates and implantation rates in 2 different timings of embryo transfer using Epi Info 7 software.

Intervention

Endometrial preparation for frozen embryo transfer cycle (FET)

Endometrial priming will be performed with daily oral estradiol valerate (8 mg) beginning on cycle day 2 for 12 days to achieve an endometrial thickness of ≥7.5 mm. If this thickness is not reached, the dose of estradiol valerate was increased by 2 mg/day (until a maximum dose of 12 mg/day) for 3 days and endometrial thickness is checked again. After reaching the target endometrial thickness (≥7.5 mm), embryo transfer is scheduled, luteal phase support is started using vaginal progesterone suppository (400 mg) twice daily plus intramuscular progesterone 100mg once daily, and embryo transfer is performed on the 6th day of progesterone supplementation. In cases of endometrial thickness <7.5 mm, the cycle is canceled. In this study, all the cycles reached an endometrial thickness ≥7.5 mm. Luteal support will be continued until 10 weeks of gestation.

Embryo thawing and preparation:

Group 1: 30 patients with slowly dividing embryos will be thawed on the 5th day of progesterone supplementation. After warming, the embryos will be cultured in the incubator for 24 hours. The embryos are checked and reassessed on the day of embryo transfer (6th day of progesterone supplementation) followed by embryo transfer. Embryo transfer will be done in patients having full bladder using transabdominal ultrasound guidance and a transcervical catheter is introduced. The embryos are placed intracavitary 1 cm below the fundus (2 embryos will be transferred per cycle). The serum pregnancy test will be done 11 days after embryo transfer (ET). In the case of a positive pregnancy test, transvaginal ultrasound is done at 5 weeks gestational age to check for intrauterine pregnancy.

Group 2: 30 patients had a same day embryo transfer of slowly dividing embryos (day 5 morulae and stage I and II blastocysts).

With the second day of menses, the endometrium will be artificially prepared using the same protocol as group 1.

Embryo thawing and preparation:

Slowly dividing embryos will be thawed on the 6th day of progesterone supplementation. After warming, the embryos will be checked then followed by embryo transfer on the same day. Embryo transfer is done in patients having full bladder using transabdominal ultrasound guidance and a transcervical catheter is introduced. The embryos are placed intracavitary 1 cm below the fundus (2 embryos will be transferred per cycle). The serum pregnancy test will be done 11 days after embryo transfer (ET). In the case of a positive pregnancy test, transvaginal ultrasound is done at 5 weeks gestational age to check for intrauterine pregnancy.

Figure 1: Flowchart of the study

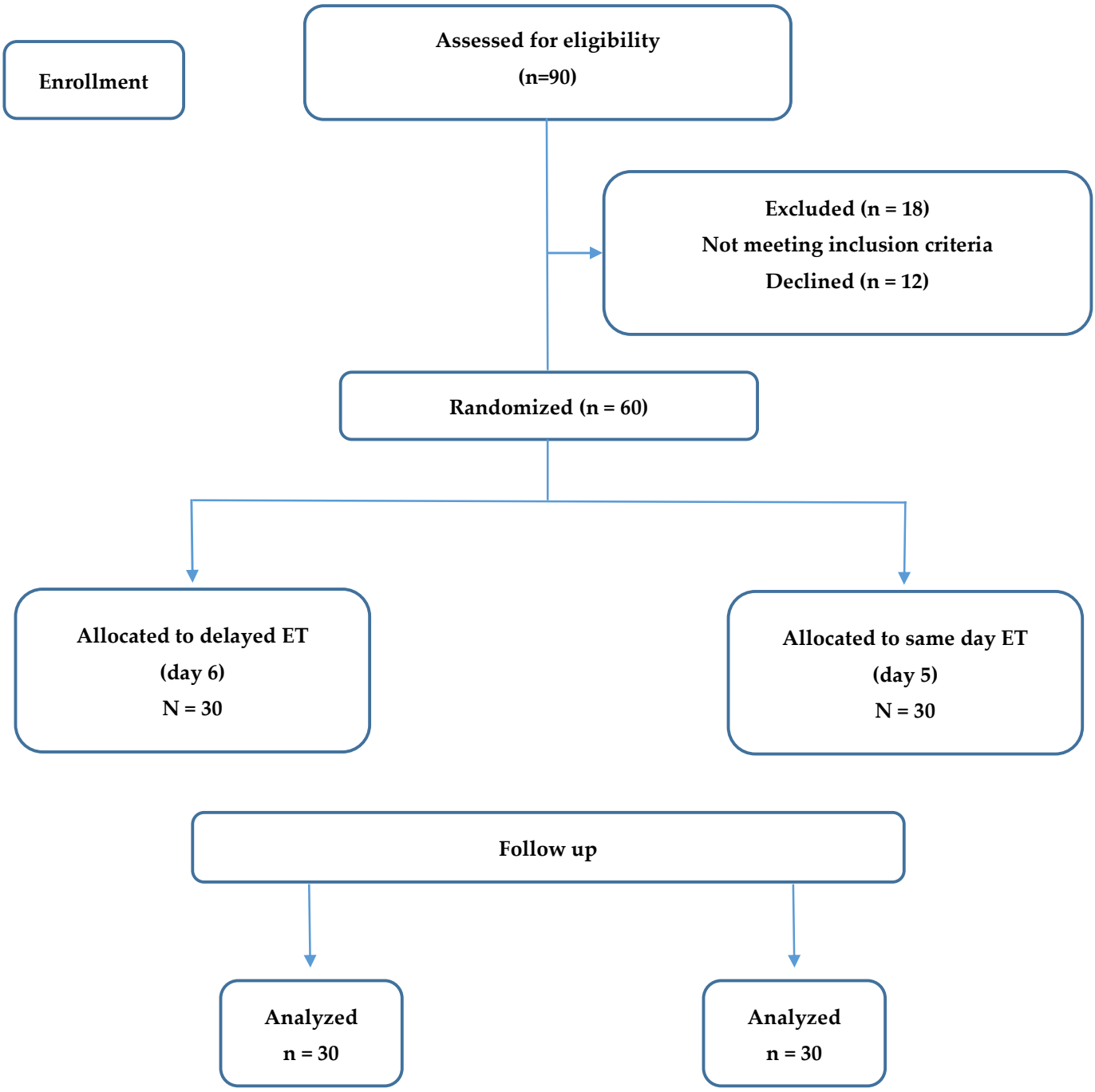


Table (1):Comparison between the two studied groups according to different parameters

	Group 1 (n=30)	Group (n=30)	Test of sig.	p
Age (years)				
Median (Min. – Max.)	32 (23 – 42)	31 (19 – 44)	t=0.532	0.597
Mean ± SD.	32 ± 4.6	31.4 ± 6.5		
Gravida				
Primi gravida	24 (80%)	28 (93.3%)	c^2=	^{FE}p=
Multi gravida	6 (20%)	2 (6.7%)	2.308	0.254
Median (Min. – Max.)	0 (0 – 4)	0 (0 – 2)	U=	0.378
Mean ± SD.	0.8 ± 1.2	0.4 ± 0.6	399.0	
Parity				
Nullipara	24 (80%)	28 (93.3%)	c^2=	^{MC}p=
Primi	5 (16.7%)	2 (6.7%)	2.456	0.261
Multipara	1 (3.3%)	0 (0%)		
Median (Min. – Max.)	0 (0 – 3)	0 (0 – 1)	U=	0.126
Mean ± SD.	0.3 ± 0.6	0.1 ± 0.3	389.0	
Abortion				
<1	21 (70%)	22 (73.3%)	c^2=	0.774
≥1	9 (30%)	8 (26.7%)	0.082	
Median (Min. – Max.)	0 (0 – 3)	0 (0 – 2)	U=	0.654
Mean ± SD.	0.5 ± 0.9	0.3 ± 0.6	426.0	
Factor of infertility				
Ovarian	10 (33.3%)	0 (0%)	c^2=12.0*	0.001*
Unexplained	1 (3.3%)	1 (3.3%)	c^2=0.0	^{FE}p=1.000
Male factor	16 (53.3%)	18 (60%)	c^2=0.271	0.602
Anovulation	11 (36.7%)	8 (26.7%)	c^2=0.693	0.405
PCO	1 (3.3%)	1 (3.3%)	c^2=0.0	^{FE}p=1.000
Tubal factor	1 (3.3%)	13 (43.3%)	c^2=13.416*	<0.001*

Study outcomes

Our primary outcomes are positive pregnancy test rate and implantation rate and the secondary outcome is clinical pregnancy rate. Positive pregnancy test rate is the number of patients having positive beta human chorionic gonadotropin (BHCG) serum test 11 days after embryo transfer divided by the total number of patients in each group. Implantation rate (calculated as the number of intrauterine gestational sac(s) observed through trans- vaginal ultrasound divided by the number of transferred embryos). Clinical

pregnancy rate (calculated by considering clinical pregnancy, determined by the visualization of a viable gestational sac within the uterine cavity by ultrasound 3– 4 weeks after embryo transfer), Ongoing pregnancy rate (defined as pregnancy progressing beyond 12 weeks gestation).

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. **(**Armonk, NY: IBM Corp**)**. Categorical data were represented as numbers and percentages. **Chi-square test** was applied to compare between two groups. Alternatively, **Fisher Exact correction** test was applied when more than 20% of the cells have expected count less than 5**. Monte Carlo correction** test was applied when more than 20% of the cells have expected count less than 5. For continuous data, they were tested for normality by **the Shapiro-Wilk test**. Quantitative data were expressed as range (minimum and maximum), mean, standard deviation and median **Student t-test** was used to compare two groups for normally distributed quantitative variables. **Mann Whitney test** was used to compare two groups for not normally distributed quantitative variables. Significance of the obtained results was judged at the 5% level.

Results

Table 1 shows the mean age, previous obstetric history and the factor of infertility for the sixty patients enrolled in the study. The mean age of the women was 32 ± 4.6 years in group 1 and 31.4 ± 6.5 years in group 2. No significant differences could be observed regarding age in the two groups. Also, no significant differences were seen in terms of gravity, parity and abortion status between women of both groups as seen in **table 1**.

All patients underwent embryo vitrification (either with or without fresh embryo transfer). The mean number of embryos vitrified in group 1 per cycle was 4.1 ± 2.5, while in group 2 was 3.2 ± 1, showing no significant difference was shown in table 2. Number of embryos transferred in group 1 was significantly less than the number of embryos transferred in group 2 (1.8 ± 0.6, 2.8 ± 1 respectively; $P < 0.001$).

Table (2): Comparison between the two studied groups according to number of embryos

	Group 1 (n=30)	Group 2 (n=30)	Test of sig.	p
Number of embryos vitrified				
Median (Min. – Max.)	4 (1 – 10)	2 – 5	U=	0.389
Mean ± SD.	4.1 ± 2.5	3.2 ± 1	393.0	
Number of embryos thawed				
Median (Min. – Max.)	2 (1 – 6)	3 (2 – 5)	U=	0.065
Mean ± SD.	2.5 ± 1.2	2.9 ± 0.9	333.0	
Number of embryos transferred				
Median (Min. – Max.)	2 (1 – 3)	3 (1 – 5)	U=	<0.001*
Mean ± SD.	1.8 ± 0.6	2.8 ± 1	198.0*	
Number of remaining embryos				
Median (Min. – Max.)	0 (0 – 8)	3 (2 – 3)	U=	0.146
Mean ± SD.	1.6 ± 2.2	2.7 ± 0.6	23.50	
Improvement of embryos				
No	0 (0%)	21 (70%)	c^2=	<0.001*
Yes	30 (100%)	9 (30%)	32.308*	

In addition group 1 showed significantly higher rate of embryo advancement after reculturing the embryos 24 hours after thawing when compared with the same day embryo transfer protocol used in group 2. A total of 30 frozen embryo transfer cycles in each group, of which 9 cycles showed embryo advancement (30%), while in group 1 100% of embryos were advanced on day 6 (P <0.001). When comparing both arms of our study in terms of implantation rate and ongoing pregnancy rates as shown in table 3, out of 54 embryo transferred in 30 FET cycles in group 1, 18 intrauterine gestational sacs with pulsating fetal poles were observed using transvaginal ultrasound (33% implantation rate) and 13 of these progressed till beyond 12 weeks of gestation (24.1% ongoing pregnancy rate). On the other, a total of 84 embryo were transferred in 30 FET cycles in group 2, of which 16 intrauterine gestational sacs with pulsating fetal poles were observed using transvaginal ultrasound (% 19.3 implantation rate) and 10 of these progressed till beyond 12 weeks of gestation (12 % ongoing pregnancy rate). The increase is not statistically significant (P 0.063, 0.066).

Discussion

The present study reported no significant difference between group 1 and group 2 in terms of positive BHCG, implantation rate and ongoing pregnancy rates per FET cycle, moreover without compromising the effectiveness of technique of embryo transfer.

Table (3): Comparison between the two studied groups according to implantation and ongoing pregnancy rates

	Group 1 No. (%)	Group 2 No. (%)	c^2	p
Embryo transferred	**54**	**83**		
Implantation rate	18/54 (33.3%)	16/83 (19.3%)	3.464	0.063
Ongoing pregnancy	13/54 (24.1%)	10/83 (12.0%)	3.387	0.066

However, it was shown in our study that there is an increase in implantation and ongoing pregnancy in group 1 compared with group 2 in terms of embryos transferred, but also this difference was not statistically significant. In our words, this study shows that reculturing of slowly developing embryos for further 24 hours after day 5 vitrification will improve the developmental stage of the embryo and may improve its implantation potential and consumption of less number of embryos per FET cycle with comparable pregnancy rates. These slightly improved outcomes could result from better embryo-endometrium synchronization or better embryo selection.

Several studies used to comparing Days 5 and 6 embryos in frozen embryo transfer (FET) cycles showed contradictory results. There is still a lack of evidence regarding the best approach, performing fresh transfer or deferring transfer and continuing culture until fully developed blastocysts are achieved, when the entire cohort of embryos is slow growing.

Tannus et al (18) study included cycles in which the embryos either began blastulation by Day 5 of culture but did not reach the fully expanded stage (Gardner Stage III) or had delayed blastulation with only morula embryos present by Day 5 of culture. All of the subjects in the study underwent elective, single embryo transfer (slow or delayed blastocysts) on Day 5 and had at least one embryo that developed into a FEB on extended culture Day 6 that was suitable for vitrification. All subjects, regardless of the outcome of the fresh transfer, returned for at least one subsequent FET cycle of Day 6 embryos.

Our study showed similar results to a particular part of Tannus et al study in that embryos that started blastulation but did not reach full expansion on Day 5 (early blastocysts) had similar outcomes to transferring fully expanded Day 6 blastocysts in subsequent FET cycle.

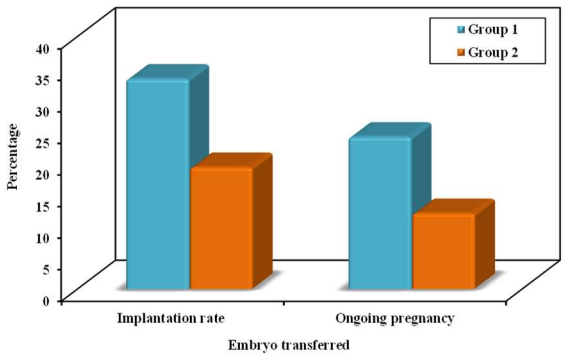
On the other hand, We had some contradicting results to Tannus et al when there study mentioned that cycles in which the embryos did not start blastulation by Day 5 resulted in significantly lower

LBRs compared to embryos from the same cohort that reached full expansion on Day 6 and were then transferred in subsequent FET cycles.

Our metholodolgy differs than the previously mentioned study in that we did not perform fresh embryo transfer cycles for all patients in the study. Secondly, We considered both embryos that started blastulation on day 5 or not (as morulae) as a single entity and not two different qualities of embryos as they did. Lastly, in our study the number of embryos transferred per cycle ranged from 2-4 embryos, with a significantly higher number of embryos transferred per cycle in group 2.

Kaye et al (8) retrospectively evaluated a total of 261 day 5 blastocysts and 207 day 6 blastocysts for frozen-thawed SET between 2010 and 2016. They concluded, similar to our study, that day 6

Figure (1): Comparison between the two studied groups according to embryo transferred



cryopreserved blastocysts resulted in similar implantation and ongoing pregnancy rates compared to day 5, in FET cycles. In our study we exclusively used vitrification technique for embryo freezing, however Kaye et al used slow freezing protocol using gradual exposure from 5% glycerol to 9% glycerol and 0.2 M sucrose was used prior to January 2013, and embryos were frozen in vials. After January 2013, vitrification with rapid exposure to a cryoprotectant solution.

Unlike our study results, Dowling et al. (17) retrospectively studied the impact of delayed embryo transfer (day 6) in case of slowly developing embryos in terms of implantation rates and found that these embryos elaborated significantly higher implantation rates if grown to advanced developmental stage on Day 6, vitrified and transferred in FET cycle.

Conclusion

Embryos having delayed blastulation (morula embryo transfer) or started blastulation but fail to reach full expansion by Day 5 (early blastocysts) may have better implantation and ongoing pregnancy rates if thawed and recultured for additional 24 hours to reach an advanced blastocyst stage (day 6 embryo transfer) than being thawed and transferred on the same day (day 5 embryo transfer). In this case, deferring embryo transfer in favor of extending embryo culture till reaching a

fully expanded embryos for later vitrified-warmed transfer may improve the LBRs, although further studies are needed to clarify this hypothesis.

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