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ASSISTED REPRODUCTION TECHNOLOGIES

Thawing day 3 embryos and culturing to day 5 may be a better method for frozen embryo transfer

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Abstract

Purpose Does thawing cleavage embryos and culturing them for transfer as blastocysts improve pregnancy and perinatal outcomes compared to transferring thawed blastocysts?

Methods Retrospective, observational cohort study performed at two assisted reproductive technology centers, 2014 to 2020. A total of 450 patients with 463 thawed embryo transfer cycles were divided into 2 groups according to the embryonic developmental stage at cryopreservation and transfer: 231 thawed blastocysts (day 5 group) and 232 thawed cleavage embryos that were cultured for 2 days and transferred as blastocysts (day 3–5 group). The two groups were compared for demographics, routine parameters of IVF treatment, pregnancy rates, and perinatal outcomes.

Results Multivariable logistic regression analysis for ongoing pregnancy and delivery demonstrated that the day 3–5 group had a greater likelihood of achieving ongoing pregnancy and delivery compared to the day 5 group (OR 1.58, 95%CI 1.062–2.361, $p = 0.024$). Perinatal outcomes were comparable between the three groups.

Conclusion Our results support culturing post-thaw cleavage embryos for 2 days and transferring them as blastocysts to increase chances of ongoing pregnancy and delivery.

Keywords Cleavage · Blastocyst · Vitrification · Thawed embryo transfer

Introduction

Cryopreservation of embryos and oocytes has become routine in assisted reproduction technology (ART). Live birth rates following frozen embryo transfers have increased significantly. Data show that cryopreservation has significantly

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improved survival rates and cumulative pregnancy rates, as well as the safety of ART [1]. Other studies show equal or even superior outcomes regarding pregnancy and live birth rates with frozen versus fresh embryo transfers [2, 3].

Cryopreservation has several advantages. It maintains supernumerary embryos not used for fresh transfer; allows single-embryo transfer, thus reducing multiple gestations; enables a freeze-all strategy to prevent ovarian hyperstimulation syndrome; is useful for social or medical fertility preservation; and allows embryo biopsy for preimplantation genetic testing, oocyte donation (OD), luteal phase stimulation, and dual stimulation protocols.

A recent trend is to perform blastocyst fresh/frozen single-embryo transfers. The advantages include exposing the embryo to a more natural uterine environment. Also, by extending the duration of culture, embryo self-selection will occur and may enable the highest chance of implantation [1, 4]. Due to a potential damage to the expanded blastocyst during vitrification procedure [5, 6], an emerging clinical question is whether cryopreserving cleavage stage embryos, then thawing and culturing to blastocysts will achieve better outcomes, as compared to transfer of a thawed blastocyst.

To the best of our knowledge, no previous study has evaluated this question. Thus, the current study evaluated pregnancy rates and perinatal outcomes of cleavage stage embryos thawed and cultured to blastocysts, as compared to thawed blastocyst embryos.

Materials and methods

Study design

This was a retrospective cohort study of FET cycles of patients with primary or secondary infertility, who had at least one embryo that underwent cryopreservation on day 3 or day 5. The clinical and laboratory data were collected between 2014 and 2020 from the electronic records of 2 outpatient centers: Assuta Medical Center, Rishon Letzion, Israel, and the IVF Department of Meir Medical Center, Kfar Saba, Israel.

Patients who were older than 45 years of age, who underwent IVF for fertility preservation or for surrogacy and cycles with egg donation, were excluded. Also, women with recurrent implantation failure, defined as 3 failed in vitro fertilization (IVF) attempts with good quality embryos [7], were excluded to avoid other confounders that might affect the results.

Embryo transfer protocols

Among the embryo transfer (ET) protocols used, spontaneous or letrozole was performed in 387 of the thawed embryo transfers. Estradiol, progesterone, luteinizing hormone, and ultrasound monitoring were used to determine the day of ovulation. An artificial endometrial preparation protocol was performed in 63 embryo transfers, using estradiol and progesterone.

Group selection

The patients were divided into 2 groups according to the developmental stage of embryos at cryopreservation and FET: thawed blastocysts, which were transferred 5 days after ovulation and thawed cleavage stage embryos, designated as day 3–5 group, were cultured for 2 additional days and transferred as blastocysts, 5 days after ovulation.

The cryopreservation groups were determined randomly according to the day of the week. Our units are closed on Saturdays and except for embryo thawing or fertilization assessment, oocyte pick-up (OPU) and embryo transfers are not performed. For this reason, cryopreservation on day 3 or day 5 occurred randomly, according to the day of the week: following a fresh embryo transfer, surplus embryos that developed from oocytes retrieved on Mondays were cryopreserved on Thursdays, as there is no option for day 5 cryopreservation. Since the majority of FET was performed on spontaneous cycles, cleavage-stage embryo transfers that should have occurred on a Saturday were postponed and took place 2 days later, corresponding to day 3–5 group, considering the risk of cycle cancellation. Following fresh embryo transfer, surplus embryos developed from oocytes that were retrieved on Wednesdays were cryopreserved on day 5 (a Monday). We prefer not to transfer 2-day embryos because it is too early to know which will develop adequately. According to this unintentional randomization, we established the 2 study groups as follows: For the day 5 group, we analyzed all thawed embryo transfers for which OPU occurred on Wednesdays. For the day 3–5 group, we analyzed all thawed embryo transfers that occurred on Mondays and grouped the cases that were thawed on Saturdays at the cleavage stage, cultured in fresh new media for 48 h, and transferred as blastocysts since embryo transfers do not take place on Saturdays, as noted above.

Vitrification and embryo selection

For both ART centers, vitrification was used as an embryo cryopreservation technique for cleavage stage embryos and for blastocysts, with a post-thawing survival rate of 91%. The same single-step culture medium was used for both groups. The freezing device used was either Cryotop (Kitazato, Japan) or Cryolock (Biotec, Alpharetta GA, USA). The blastocyst embryo transfer was performed at least 2 h after warming. Due to an approximately 55% blastulation rate, cleavage stage embryos were cryopreserved in pairs, so two

embryos were usually thawed in the day 3–5 group. When supernumerary blastocysts were obtained from culturing thawed cleavage stage embryos, they were cryopreserved again and data regarding the outcomes of those embryos is being collected.

For blastocysts, a single-embryo transfer (SET) was the policy performed at both ART centers, unless there was no contraindication, and the patient was willing to undergo multiple embryo transfers. However, there was no difference in the number of embryos transferred between the 2 groups. In the day 3–5 group, 232 embryos were transferred among 448 thawed embryos from 224 patients, and in the day 5 group, 231 embryos from 226 patients were transferred. Although not compulsory, SET was performed in all cases except for 5 cases of double embryo transfer in the day 5 group and 8 cases in the day 3–5 group. These resulted in 7 twin gestations in the entire cohort: 5 in the day 3 to 5 group and 2 in the day 5 group. For both ART centers, only top quality embryos were cryopreserved. These top quality embryos were vitrified from best quality to least quality, and the best available embryo was thawed. For blastocysts, we used the Gardner and Schoolcraft grading system [8] and top quality embryos were defined as grade > 3BB, as described in the literature [9]. Blastocysts derived from thawed cleavage embryos were graded again according to the Gardner and Schoolcraft system and only top quality blastocysts (> 3BB) were transferred. Top quality cleavage embryos were defined as those having 6–8 cells on day 3, < 10% fragmentation, cellular symmetry (cells are all the same size), and no multinucleation.

Study outcomes

The following information was obtained from the medical records of each patient: (1) age, BMI, and smoking status; (2) fertility information, including primary/secondary infertility, infertility cause (male or other), number of retrieved oocytes, cycle outcomes including ongoing pregnancy (beyond 20 weeks of gestation) and delivery, miscarriage, ectopic pregnancy, or no pregnancy; and (3) obstetric information (gestational week at delivery, birthweight, and neonatal sex). These data were compared between the 2 groups.

The primary outcome was ongoing pregnancy, defined as pregnancy lasting beyond 20 weeks of gestation or delivery. The secondary outcomes included overall pregnancy rate, live-born infants per embryo fertilized, miscarriage rate, and obstetric outcomes, as described above.

Ethical approval

The study was approved by the Institutional Ethical Review Board. Informed consent was not required due to the retrospective nature of the study.

Statistical analysis

Sample size calculation

Epi Info software was used for sample size calculation. Based on the assumption that we accepted a 13% difference in clinical pregnancy rates among the groups, we calculated that 220 embryo transfers were required in each group to provide a power of 80%, at a 2-sided alpha level of 5%.

Data are presented as mean and standard deviation for continuous variables and as numbers and percentage for nominal parameters. For continuous data, a univariate analysis was done with one-way ANOVA or Mann–Whitney test, according to the Shapiro–Wilk test of normality, to find differences among the embryo transfer groups. Nominal variables were tested with the chi-square test.

For results that were significant or showed a statistical trend in univariate analysis, multivariable analysis was performed with a multiple logistic regression model to assess the potential impact of those parameters on ongoing pregnancy and delivery. $p < 0.05$ was the border for statistical significance. All analyses were performed with SPSS-25 software (IBM, Armonk, NY, USA).

Data regarding number of retrieved oocytes were missing for 13 patients in the entire cohort (2.8%), who underwent OPU at a different ART center. However, embryo transfers for those cases were performed at the study ART centers and information regarding fertility and obstetric outcomes was available. Therefore, these cases were included in the study. Despite efforts, it was impossible to obtain the missing data.

Results

The study population consisted of 463 FET from 450 patients and included 2 groups of frozen-thawed embryo transfers. The day 5 group refers to 231 blastocyst embryos of 226 patients that were thawed and transferred at the blastocyst stage. Days 3–5, the study group, refer to 232 frozen cleavage stage embryos of 224 patients that were thawed and cultured for 2 days and transferred as blastocysts.

The sample is described in Table 1. The mean age of the patients was similar between the groups (32.8 ± 5.1 years in the day 3–5 group vs. 32.3 ± 5.6 years in the day 5 group, $p = 0.45$). The groups had comparable BMI (24.3 ± 5.3 vs. 23.6 ± 4.8, $p = 0.21$) and similar numbers of retrieved oocytes (12.9 ± 5.3 vs. 12.8 ± 6, $p = 0.83$ for day 3–5 and day 5 groups, respectively). The etiologies of infertility (male factor vs. nonmale factor) and infertility status (primary vs. secondary) were comparable, as well. More patients in the day 5 group smoked, as compared to the day 3–5 group (34.3% vs. 8%, respectively; $p < 0.0001$).

Table 1 Description of the study sample

Variable	Days 3–5	Day 5	*P*-value
Age, years (mean ± SD)	32.8 ± 5.1	32.3 ± 5.6	0.45
Body mass index, kg/m^2 (mean ± SD)	24.3 ± 5.3	23.6 ± 4.8	0.21
Smoking rate, *n* (%)	16 (8)	73 (34.3)	< 0.0001
Primary infertility, *n* (%)	136 (60.7)	137 (61.2)	0.92
Male factor, *n* (%)	78 (34.8)	76 (33.8)	0.81
Retrieved oocytes (mean ± SD)[a]	12.9 ± 5.3	12.8 ± 6	0.83

SD, standard deviation

[a]Data are available for 218/226 ET of day 5 group and 219/224 of day 3–5 group

Table 2 Univariate analysis for ongoing pregnancy and delivery

Variable	Ongoing pregnancy and delivery		*P*-value
	Yes	No	
Age, years (mean ± SD)	31.4 ± 4.8	33.2 ± 5.6	0.001
Body mass index, kg/m^2 (mean ± SD)	23.7 ± 4.8	24.1 ± 5.2	0.56
Smoking rate, *N* (%)	32 (20.4)	57 (22.4)	0.63
Primary infertility rate, *N* (%)	103 (62)	170 (60.5)	0.74
Male factor rate, *N* (%)	53 (31.7)	101 (35.9)	0.36
Retrieved oocytes (mean ± SD)	13.7 ± 5.7	12.3 ± 5.6	0.01

SD, standard deviation

Table 3 Multivariable logistic regression analysis for ongoing pregnancy and delivery

Variable	OR	95% CI for OR		*P*-value
		Lower	Upper	
Day-of-transfer 3–5 vs. day 5	1.584	1.062	2.361	0.024
Age	1.056	1.016	1.098	0.005
Number of retrieved oocytes	0.967	0.934	1.002	0.062

OR, odds ratio; *CI*, confidence interval

Table 2 shows the univariate analysis for ongoing pregnancy beyond 20 weeks of gestation and delivery. Younger age and higher number of retrieved oocytes were found to be significant parameters for achieving ongoing pregnancy and delivery (mean age 31.4 ± 4.8 vs. 33.2 ± 5.6; $p = 0.001$ and mean number of retrieved oocytes 13.7 ± 5.7 vs.12.3 ± 5.6; $p = 0.01$). BMI, smoking status, infertility status, and etiology of infertility were not related to achieving ongoing pregnancy and delivery (Table 2).

Multivariable logistic regression analysis for ongoing pregnancy and delivery (Table 3) demonstrated that the day-of-transfer group and patient age were significant variables. Analyzing which day-of-transfer group had the highest impact on the primary outcome demonstrated that the day 3–5 group had a significantly greater likelihood of achieving ongoing pregnancy and delivery compared to the day 5 group (OR 1.58, 95%CI 1.062–2.361, $p = 0.024$).

Pregnancy rate and obstetric outcomes are depicted in Table 4. Overall pregnancy rate and the rate of spontaneous abortion were comparable between the day 3–5 and day 5 groups (50.7% vs. 43.6%, $p = 0.13$ and 15.9% vs. 21.4%, $p = 0.3$, respectively). Comparing ongoing pregnancy beyond 20 weeks of gestation and delivery, a trend toward more ongoing pregnancies was seen in the day 3–5 group compared to the day 5 group (41.7% vs. 32.9%, $p = 0.05$, respectively). Analysis of live-born infants per embryo fertilized demonstrated a higher rate among day the 3–5 group, as compared to the day 5 group (42.2% vs. 32.9%, $p = 0.03$; respectively). The 2 groups had comparable obstetrical outcomes, including term delivery, neonatal birthweight, and sex (Table 4).

Discussion

This study reports on different thawing strategies for FET, comparing day 5 to day 3–5. Our findings demonstrate that thawing cleavage embryos, then culturing and transferring them as blastocysts, yields improved pregnancy rates and

Table 4 Pregnancy rate and obstetric outcomes per patient

Variable	Days 3–5	Day 5	*P*-value
Overall pregnancy rate, *n* (%)	113 (50.7)	98 (43.6)	0.13
Ongoing pregnancy and delivery rate, *n* (%)	93 (41.7)	74 (32.9)	0.05
Live born infant per embryo fertilized, *n* (%)	98 (42.2)	76 (32.9)	0.03
Spontaneous abortion rate, *n* (%)	18 (15.9)	21 (21.4)	0.3
Term delivery rate, *n* (%)	29 (85.3)	55 (88.7)	0.62
Neonatal birthweight, g (mean ± SD)	3209.5 ± 639.9	3181.2 ± 578.1	0.43
Male sex, *n* (%)	12 (36.4)	25 (41)	0.11

SD, standard deviation

perinatal outcomes compared to thawed blastocyst embryo transfers.

Embryo cryopreservation has become a cornerstone in ART. With improved vitrification techniques, FET show equal or even higher implantation and pregnancy rates than do fresh embryo transfers [1, 2, 10].

A recent worldwide trend is to perform a fresh/frozen blastocyst single-embryo transfer. Studies suggest that the blastocyst stage is perhaps the most efficient for cryopreservation, with better reproductive outcomes. This allows for the self-selection of embryos, while reducing the multiple gestation rate [1, 11, 12].

The current study compared 2 groups of frozen embryo transfers at different stages of embryonic development. The day 5 group included thawed blastocyst transfers and the day 3–5 group consisted of thawed cleavage embryos that were cultured in fresh media and transferred as blastocysts.

Multivariable regression analysis demonstrated a clear benefit for culturing thawed cleavage embryos for two additional days and transferred as blastocysts, as compared to blastocyst FET. This could be explained by overcoming the potential damage to the blastocyst during cryopreservation [5, 6]. Although vitrification should avoid ice crystals, potential damage due to insufficient permeation of cryoprotectant inside the blastocoel, resulting in some ice crystal formation and ultrastructural cellular damage to expanded blastocysts might occur, possibly resulting in lower survival, transfer, and implantation rates [6, 13]. To overcome this obstacle, a new method, in which the blastocoelic cavity is reduced prior to vitrification, helps avoid ice crystal formation and cellular damage [5, 6, 14]. In our opinion, the process of cryopreservation at a cleavage stage, thawing and culturing for two additional days in fresh media might avoid this potential expanded blastocoelic damage prior to vitrification, with post-thaw survival rates of 91%. Furthermore, the extended culture in fresh media for 48 h may also improve this outcome.

In addition to the day-of-transfer, younger patient age was also a significant factor for achieving ongoing pregnancy and delivery. This result is consistent with reports in the literature [15]. Despite the results of multivariable regression analysis for the primary outcome, univariate analysis for secondary outcomes did not show a difference in the overall pregnancy rates between the day 3–5 and day 5 groups, although analysis of live-born infants per embryo fertilized demonstrated a higher rate among the day 3–5 group. The spontaneous abortion rate was comparable as well.

To our knowledge, this is the first study to compare two groups of frozen embryos that were randomly allocated according to the embryonic developmental stage at cryopreservation and transfer, according to the day of the week. Also, to limit confounding factors, only the first embryo transfer for each patient was considered. The 2 groups of women were homogenous regarding age, BMI, and infertility parameters, including ovarian reserve, as demonstrated by similar numbers of retrieved oocytes. The smoking rate was significantly higher among patients in the day 5 group compared to the day 3–5 group. We consider this finding to be incidental. Moreover, smoking was not a significant factor influencing the primary outcome of ongoing pregnancy and delivery.

This study had some limitations. Since it was performed at two ART centers, the IVF site could be a possible confounder. Also, as this was a retrospective study, 63 transfers of the entire cohort were performed after artificial endometrial preparation using estradiol and progesterone, while all other transfers were performed with spontaneous or letrozole cycles. To rule out this bias, we omitted the estradiol-progesterone cycles and reanalyzed the data. A multivariable regression analysis for the primary outcome of ongoing pregnancy and delivery demonstrated a significant difference between day 3–5 and day 5 groups, favoring the former.

In conclusion, as the indications to cryopreserve all embryos have increased substantially in recent years, and according to the recent trend of blastocyst SET, this study aimed to answer a clinical question encountered in daily practice regarding at what stage should embryos be frozen. Our results support development after thawing, by culturing these embryos for 2 additional days and then transferring them as blastocysts, thereby increasing the rates of ongoing pregnancies and deliveries. Ideally, randomized controlled trials should be conducted to address this question, as well as the effect of re-freezing a thawed cleavage embryo that was cultured to blastocyst, in comparison to blastocyst cryopreservation.

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Data availability Data are available on request from the corresponding author.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

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