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Comparison of day 2 and overnight day 3 frozen embryo transfers: A prospective randomized controlled trial

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ABSTRACT

In certain patients cleavage stage embryos may be preferred. The relationship between an additional day in culture and pregnancy outcomes is not well established. We aimed to compare outcomes of day 2 versus overnight day 3 frozen embryo transfer (FET). In this randomized controlled trial, patients with day 2 cryopreserved embryos were allocated to two groups. In group A embryos were transferred on day 2, the same day of thawing. In group B embryos were transferred one day after thawing, on day 3 after overnight incubation. Out of 410 patients eligible, 92 were recruited. Finally, 72 patients participated, 39 in group A and 33 in group B. No significant difference in implantation (11 % in group A and 14 % in group B, p = 0.81), clinical pregnancy (18 % in group A and 21 % in group B, p = 0.73) or live birth rates (13 % in group A and 18 % in group B, p = 0.53) was found. To conclude, no significant difference in reproductive outcomes was found when comparing patients with day 2 or overnight day 3 FET. Considering published data on blastocyst transfer, cleavage stage ET may still be a relevant option and the decision between day 2 or overnight day 3 ET depends on patients' and physicians' preference and recommendation.

1. Introduction

Frozen embryo transfer (FET) has become standard of care and an important part of in vitro fertilization (IVF) treatments. Since the introduction of vitrification methods as the technique of choice for embryos cryopreservation, an increase of successful pregnancy rates of FET has been reported worldwide [1,2]. Similar results in live birth rates were found when comparing frozen and fresh embryo transfers (ET) [3]. Other studies reported lower rates of preterm labor, low birth weight and perinatal mortality in FET cycles [4,5]. The freezing approach of remaining embryos or oocytes enables single ET in the fresh cycle, and by so, decreases the previously high multiple pregnancy rates, together with all their complications as a high-risk pregnancy [6]. The successful reproductive outcomes of FET cycles lead many IVF clinics to embrace an approach of freezing all embryos [1].

Timing of ET has also been a matter of research. IVF embryos are usually transferred to the uterus in either cleavage (day 2–3 embryos) or blastocyst stage (day 5–7 embryos). Originally, most embryos were transferred at cleavage stage due to relatively less advanced culture media and lower percentage of embryos reaching the blastocyst stage [7]. Since the improvements in culture conditions lead to the ability of culturing good quality blastocytes, many studies were published on the blastocyst transfer as compared with embryos in cleavage stage [8–10].

A recently published Cochrane meta-analysis found higher live birth rates in fresh blastocyst transfer cycles compared to cleavage stage transfer cycles, with no difference in miscarriage or multiple pregnancy rates [10]. However, when comparing fresh and frozen-thawed transfer of cleavage stage embryos and blastocysts, no difference in cumulative pregnancy following was found.

Moreover, in vitro incubation may lead to an arrest in embryo development, leaving the patient with no viable embryos for transfer and cycle cancellation, with a chance that these embryos could have survived in the physiological environment of the uterus [7,10]. According to the above-mentioned Cochrane meta-analysis, failure of transferring any embryo in the blastocyst group was higher compared to the cleavage stage group. This is particularly relevant in poor prognosis patients, who often have only few zygotes after fertilization.

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Looking into cleavage stage embryos, day 2 ET and day 3 ET may potentially present different reproductive outcomes. As the first wave of zygotic genome activation occurs at the 4–8 cell stage [11], and may result in the arrest of many embryos, later ET may allow embryologists to select the most propitious embryo [12,13]. The relationship between an additional day in culture and pregnancy outcomes is not well established. Studies comparing day 2 and day 3 ET showed inconsistent results regarding the impact on reproductive outcomes [14–19]. In a prospective randomized controlled study (RCT) by Laverge et al., implantation and pregnancy rates did not differ between day 2 and day 3 ET, yet, more embryos of moderate to poor quality were found in day 3 embryos [20]. This study was in line with a previous prospective randomized study by Ertzeid et al. concluding that extending the embryo culture from 2 to 3 days resulted in better embryo selection, yet had no effect on implantation and live birth rates [12]. A Cochrane review published in 2016, comparing day 2 and day 3 fresh ET, consisted of 15 RCTs, found no difference in live birth, pregnancy, or miscarriage rates. The quality of evidence was graded from moderate to very low due to methodological issues. As only fresh ET cycles were included no conclusion can may made on FET [21].

Many patients have stored cryopreserved cleavage stage embryos and the possibility of transferring day 2 versus overnight thawed embryos has not been widely studied.

Transfer of cleavage stage embryos is also relevant in poor prognosis patients, having only few zygotes after fertilization. When deciding on cleavage ET, incubation of day 2 frozen – thawed embryos to day 3 may enable evaluation of their further development potential and possibly better selection of embryos for transfer.

On the other hand, in vitro incubation may eventually lead to an arrest in embryo development, leaving the patient with no viable embryos that could have survived in the physiological environment of the uterus transfer and cycle cancellation.

The aim of the present RCT was to compare day 2 vs overnight day 3 FET and investigate whether frozen-thawed cleavage stage embryos transferred after overnight incubation have a clinical advantage on reproductive outcomes. Our hypothesis is that no significant difference exists between day 2 and overnight day 3 FET.

2. Material and methods

2.1. Study design and participants

This prospective randomized controlled study was conducted at the Reproductive Endocrinology and IVF unit of Carmel Medical Center, in Israel, during the period of June 2011 to June 2018. The study was approved by the local Ethical Committee.

The study was prospectively registered in the National Library of Medicine, accessible at www.clinicaltrials.gov, registration number NCT01287273.

Day 2 freezing of embryo was offered to patients in whom less than 4 zygotes were available post fertilization, no blastocysts were developed in culture in a previous IVF cycle or in who had previously conceived and delivered a baby with a day 2 embryo transfer. These patients were considered for inclusion. In these patients, either a freeze all approach or cryopreservation of supernumerary embryos approach was applied.

Excluded were patients who preferred to have same day FET (day 2).

All patients included signed an informed consent form which was conducted according to the Declaration of Helsinki and Good Clinical Practice on the day of their day 2 cryopreservation.

2.2. Randomization

After consent, on the day of their day 2 embryo cryopreservation, random numbers table was used to produce sealed opaque envelopes, which were used to allocate patients to one of two treatments on the day of their embryo freeze.

Randomization timeline and patients' follow-up is described in Fig. 1.

Physicians, nurses, and embryologists as well as patients were not aware of which group they were allocated to until the start of endometrial preparation FET protocol. At this point blinding was impossible to maintain as endometrial preparation differed between the two groups.

Embryo thawing and transfer was performed according to the patients' group allocation. All embryos were frozen on day 2. Group A was consisted of patients with embryos thawed and transferred on the same day, thus embryos were transferred on day 2. Group B was consisted of patients with embryos transferred one day after thawing, thus embryos were transferred on day 3 after overnight incubation.

2.3. Ovarian stimulation protocols

In their stimulation cycle, patients in both groups underwent ovarian stimulation with standard protocols with a personal adjustment according to patient age, ovarian reserve, and previous response to controlled ovarian hyperstimulation (COH). Final oocyte maturation was triggered when two or more follicles reached a diameter of 18 mm. Oocyte retrievals were performed under transvaginal ultrasound guidance 35−37 h after triggering.

2.4. Cryopreservation and embryo transfer (ET)

All embryos, from patients in both groups, were incubated in a time lapse system (EmbryoScope). Cryopreservation was performed on Day 2, most of them by vitrification (77 %) and the minority by slow freezing. Cryopreserved were embryos of two to four cells with up to 50 % fragmentation. Survival of 50 % was

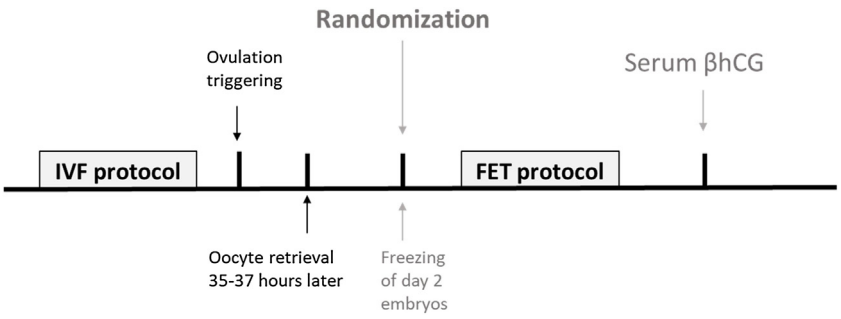

Fig. 1. Randomization timeline.

sufficient for transfer. Endometrial preparation was performed using Estradiol Valerate (Bayer, Israel) or Estrofem (Novo nordisk, Denmark) 6 mg daily starting from day 3 of the cycle. When endometrial thickness reached > 7 mm, vaginal application of Uterogestan (CTS Ltd, Israel) 800 mg/day was added.

Frozen–thawed embryo transfer was performed after 2 or 3 days of progesterone treatment for day 2 or day 3 embryos, respectively. Progesterone treatment was continued to support the endometrium until the pregnancy test results. If results were positive treatment was continued until positive fetal heart beats and then until week 9 of pregnancy.

Slow freezing and thawing were performed using SAGE Quinn's advantage kits by the Planer KRYO-10. Vitrification and thawing were performed using SAGE kits. Vitrified embryos were loaded on cryotop (Kitazato Japan) or on Cryolock (biotech INC USA).

2.5. Outcome variables

The primary outcome of our study was positive beta hCG defined as 1 value of > 10 mIU two weeks after ET.

The study's secondary outcome variables included implantation rate, clinical pregnancy rate, live birth rate, multiple pregnancy rate and miscarriage rate.

Implantation rate was defined as the number of sacs divided by the number of embryos returned. Clinical pregnancy was defined by visualization of a gestational sac with a positive fetal heartbeat. Live birth was defined as a pregnancy that resulted in delivery of live infant(s) beyond the 24th gestational week. Multiple pregnancy was defined as the presence of more than one gestational sac on transvaginal ultrasound. Miscarriage was defined as fetal loss prior to the 20th week of gestation per clinical pregnancy [24].

2.6. Statistical analysis

Data was analysed using the SPSS software version 26 (SPSS Inc., Chicago, IL). Categorical data were expressed as numbers and compared using the Chi-square test presented with Pearson Chi-Square and p. value. If a count less than five was expected, a Fisher's Exact Test was conducted. $P < 0.05$ was considered statistically significant. Data was analysed per protocol.

A sample size of 150 patients was calculated as needed for appropriate power by using anticipated live birth rate. However, the recruitment was relatively slow mainly due patients' requested blastocyst transfer. Despite not achieving the sample size required, after 8 years of recruitment, and with decreasing rates of cleavage stage ET, we decided to analyze the results in a time frame reasonable for pregnancy outcomes in the IVF center. Post-hoc power calculation reveled a power of 8.7 %.

3. Results

3.1. Participants

Out of 410 patients with day 2 frozen embryos, 92 patients agreed to participate and were eventually recruited to our study. Patients were recruited from June 2011 to June 2018 and followed up until April 2019. The patients' flow diagram is presented in

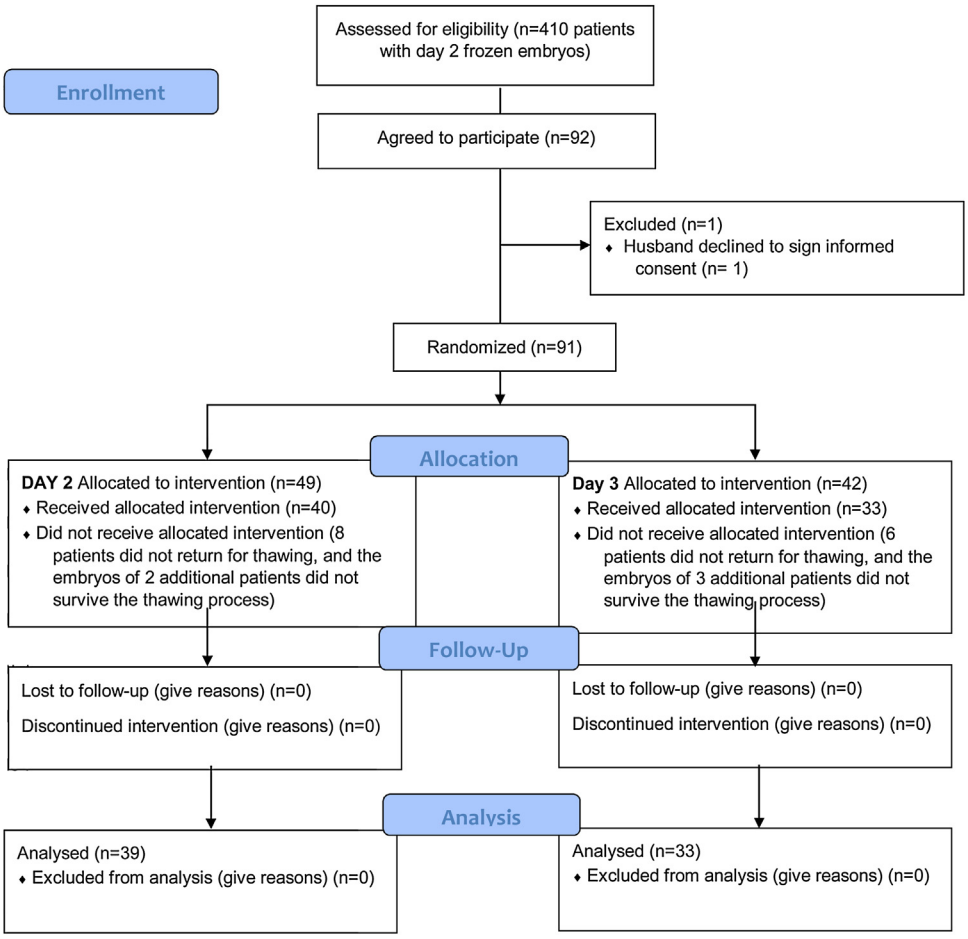

Fig. 2. Patients' flow diagram.

Fig. 2. 49 patients were allocated to group A (embryos transferred on day 2) and 42 patients were allocated to group B (embryos transferred on day 3 after overnight incubation). In group A, 8 patients did not return for thawing, and the embryos of 2 additional patients did not survive the thawing process. In group B, 6 patients did not return for thawing, and the embryos of 3 additional patients did not survive the thawing process. All embryos incubated overnight survived. One cycle was excluded as the husband who agreed to participate, didn't sign the informed consent.

Finally analyzed were 39 patients in group A and 33 patients in group B. Patients' characteristics are presented in Table 1, no baseline differences were detected between the two groups. 24 patients were fertilized by IVF, while 48 patients underwent intracytoplasmic sperm injection (ICSI). The average current ET cycle number was 3.9, no patient underwent their first ET in our study. Patients were at average 33 years old.

3.2. Outcomes

Reproductive outcomes are presented in Table 2. Overall clinical pregnancy rate was 14/72 (19 %). Clinical pregnancy was achieved in 7/39 (18 %) patients in group A and in 7/33 (21 %) with no statistical significance between the groups (p = 0.73).

Live birth rate also did not differ between the two groups. Overall, 11 (15 %) live births were observed, 5/39 (13 %) in group A and 6/33 (18 %) in group B (p = 0.53).

Two patients had a twin pregnancy, in group A and 1 in group B.

Analyzing the reproductive outcomes according to fertilization type (IVF versus ICSI) also yielded similar results between groups.

4. Discussion

4.1. Main findings

In the present RCT, no significant difference was found in terms of positive β-hCG, clinical pregnancy, live birth, miscarriage, and multiple pregnancy rates when comparing patients with day 2 frozen embryos transferred with overnight day 3 frozen embryos transfers.

Our results are in line with the Cochrane review and meta-analysis comparing reproductive outcomes in women who underwent fresh day 2 or day 3 ET [21]. This meta-analysis of 15 RCTs did not find a difference in reproductive outcomes when comparing day 2 or 3 ET, however, it did not focus on FETs.

In view of these results, is offering cleavage stage embryos still a viable option?

A certain proportion of genetically normal embryos may be unable to develop to blastocysts stage due to suboptimal culture media conditions in the IVF lab. Consequently, during prolonged culture, extra potentially viable embryos eligible for cryopreservation may be lost [25].

For poor prognosis patients with a low number of embryos this may be of importance and they may benefit from a day 2 ET [18,26]. In these patients, better pregnancy rates and lower miscarriage rates were observed, possibly due to better conditions in the uterus compared to in vitro culture conditions [25].

Cleavage stage embryos present several advantages as compared to blastocyst ET. Recently, blastocyst transfer, especially in FET cycles, was shown to result in pregnancies with increased rates of preeclampsia, placental perinatal mortality, preterm labor, and large for gestational age babies [27–31]. The observed effects mentioned could possibly be explained by epigenetic changes during prolonged embryo culture. In vitro culture may potentially alter epigenetic programming of embryos due to the culture media and oxygen tension, influencing reproductive success rates and having implications on neonatal outcomes [32].

As cleavage stage ET remains a relevant option, especially in poor prognosis patients, the question whether to further incubate day 2 thawed embryos arises.

The answer to this question has not been well studied. The present RCT found no difference in positive β-hCG, clinical pregnancy, live birth, miscarriage, and multiple pregnancy rates when transferring day 2 frozen embryos or overnight day 3 frozen embryos transfers.

The potential development and further selection of embryos assumed to be present after overnight incubation was not found clinically significant. Thus, especially in poor prognosis patients, where every embryo may implant in the physiological uterine environment, clinicians may transfer either day 2 or overnight day 3 embryos with similar outcomes and choose between the two options according to patients' preference and physicians' individual recommendation.

4.2. Strengths and limitations

This RCT was conducted meticulously, and although the study population is small, our analysis is of higher accuracy and assurance. Mostly included in our study were embryo frozen by vitrification rather than slow freezing, which has been shown to have superior clinical outcomes [36].

A limitation of our trial is that despite the long term of patient enrollment, only 92 patients were eventually enrolled to the study. Although a sample size of 150 patients was calculated as needed for appropriate power, the recruitment was relatively slow. This may be a result of several reasons, including patients who did not give consent to participate orpatients who conceived and didn't plan or deferred another pregnancy. Furthermore, over the years, patients preferred blastocyst stage ET and vitrification of day 2 embryos was performed less frequently.

Between 2011 and 2019, a significant decrease in the number of day 2 ETs was recorded. Whereas 70 % of transfers were day 2 in 2011, in 2014 this number decreased to 38 % and in 2019 only 11 % of transfers were of day 2 embryos.

Table 1
Baseline characteristics of groups.

Characteristic		Returned on day 2 (n = 39)	Returned on overnight day 3 (n = 33)	P. value
Age (mean ± SD)		33.15 ± 5.37	32.33 ± 5.49	0.96
Fertilization type	IVF (%)	13 (33)	11 (33)	1.0
	ICSI (%)	26 (67)	22 (67)	
Thawing type	Slow freezing (%)	9 (23)	8 (24)	0.91
	Vitrification (%)	30 (77)	25 (76)	
Number of embryos thawed (mean ± SD)		2.54 ± 1.19	3.09 ± 1.07	0.34
Number of embryos returned (mean ± SD)		1.92 ± 0.84	1.79 ± 0.86	0.80
Current embryo transfer cycle number (mean ± SD)		4.05 ± 2.28	3.76 ± 2.08	0.54

Table 2
Outcomes by Day of embryo transfer.

Outcome	Returned on day 2 (n = 39)	Returned on overnight day 3 (n = 33)	Pearson Chi-Square	P. value
Implantation	11 %	14 %	0.06	0.81
Positive β-HCG	13 (33 %)	10 (30 %)	0.08	0.78
Clinical Pregnancy	7 (18 %)	7 (21 %)	0.12	0.73
Live birth	5 (13 %)	6 (18 %)	0.40	0.53
Multiple pregnancy	1(14 %)	1(14 %)	0.00	1.00
Miscarriage	2 (29 %)	1(14 %)	0.42	0.51

In the included population study, no baseline differences were detected, however not all confounding factors were eliminated (e.g. infertility cause). Worth mentioning is the relatively low live birth rate found in the present study, may be a result of the patients' characteristics, such as multiple previous IVF cycles (average current cycle attempt 3.9 that may imply that the population consisted of patients with repeated implantation failure patients. These patients referred to frozen day 2 ET were usually patients with less oocytes retrieved and therefore less embryos, leading eventually to lower live birth rates.

5. Conclusion

Considering the recently published pregnancy and postnatal complications of frozen blastocysts transfers, and the results of other studies and the present RCT results we believe it may be time to re-examine the advantages of cleavage stage embryo transfer.

As it appears no difference was found between day 2 and overnight day 3 ET, any strategy of cleavage stage embryo transfer may be acceptable and should be tailored for each patient individually.

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Human rights statements and informed consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients for being included in the study.

Animal studies

Not applicable.

Ethical consideration

The protocol for the research project including human subjects has been approved by the local Ethics Committee.

Clinical trial registration

Prospectively registered in the National Library of Medicine, accessible at www.clinicaltrials.gov, registration number NCT 01287273.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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