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Does Culture of Post-Thawed Cleavage-Stage Embryos to Blastocysts Improve Infertility Treatment Outcomes of Frozen-Thawed Embryo Transfer Cycles? A Randomised Clinical Trial

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Abstract

Background: There is a definite shift in assisted reproductive centres from cleavage-stage embryo transfer (ET) to blastocyst transfer that is attributed to improvements in laboratory environments and advances in the development of embryo culture media. The aim of the study was to investigate the reproductive outcomes of thawed cleavage-stage ET versus blastocysts derived from an extended culture of these embryos.

Materials and Methods: This open-label, randomised, parallel group clinical trial study enrolled 182 women aged ≤37 years who underwent frozen-thawed ET from November 2015 to June 2020 at Royan Institute Research Centre, Tehran, Iran. The women were randomly assigned to either the thawed cleavage ET group (n=110) or the post-thaw extended culture blastocysts group (n=72). The primary outcome measure was the clinical pregnancy rate. Secondary outcome measures were implantation rate, live birth rate (LBR), and miscarriage rate. A P<0.05 indicated statistical significance.

Results: There were no significant differences between the two groups in terms of demographic characteristics. Both the mean numbers of embryos transferred and good quality embryos transferred were significantly lower in the post-thaw extended culture blastocysts group compared to thawed cleavage-stage ET cycles. However, the post-thaw extended culture blastocysts group had higher clinical pregnancy (56.94 vs. 40.91%, P=0.034), implantation (34.43 vs. 19.84%, P=0.001) and live birth (49.3 vs. 33.63%, P=0.036) rates compared to the thawed cleavage-stage ET group. Miscarriage and multiple gestations rates were comparable between the groups.

Conclusion: These results allow us to take a position in favour of post-thaw extended culture blastocysts; thus, it is important to improve the post-thawing extended culture technique (registration number: NCT02681029).

Keywords: Blastocyst, Cleavage-Stage, Cryopreservation, Culture, Embryo Transfer

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Introduction

Since the first live birth of a "test tube baby" in 1978 (1), assisted reproductive technology (ART) has been used worldwide for treatment of infertile couples (2) through conventional *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) with fresh embryo transfer (ET) or frozen-thawed ET. There are reports of negative effects of fresh ET cycles on early pregnancy with subsequent effects on perinatal outcomes in terms of hormone pre-treatment, including controlled FSH ovarian stimulation, anaesthesia and surgery for IVF oocyte retrieval (3). Frozen-thawed ET allows the extra embryos produced by IVF/ICSI to be stored and transferred later; therefore, it increases cumulative pregnancy rates and decreases the economic burden placed on the family and society (4).

Preventive measures should be considered by using a freeze-all strategy (5) with transfer in subsequent cycles in patients who undergo IVF/ICSI with a high risk of developing ovarian hyperstimulation syndrome (OHSS). This method does not pose a risk to patient safety and provides an opportunity for positive pregnancy outcomes (6). There has been a general shift in ART from cleavage-stage ET to blastocyst transfer attributed to improvements in laboratory environments and advances in the development

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of embryo culture media (7). Although blastocyst culture offers advantages such as self-selection and better growth potential for chromosomally normal embryos (8), in addition to improvements in live birth rate (LBR) (9, 10), recent systematic reviews and a meta-analysis (11, 12) show no additional benefits for blastocyst transfer compared with cleavage-stage ET in clinical practice.

A literature search of the freeze-all policy, which is commonly applied for young patients at risk of OHSS or higher progesterone levels on the day of triggering (13), indicates that evaluating the influence of diverse embryo stages on the reproductive results will be serious and important to advance the success of frozen-thawed ET. Selection of cleavage-stage ET versus blastocyst transfer for frozen-thawed ET cycles is a debatable topic (4). Many studies, systematic reviews, and meta-analyses (11, 14) have compared outcomes between blastocysts and cleavage-stage ET in ART in fresh cycles. However, the few studies that have examined the superiority of frozen-thawed blastocyst transfer in frozen-thawed ET cycles reported mixed results. To the best of our knowledge, only two studies (9, 15) focused on post-thaw extended culture blastocysts in frozen-thawed ET cycles. Therefore, the purpose of the current study is to examine reproductive outcomes of thawed cleavage-stage ET versus blastocysts derived from an extended culture of these embryos.

Materials and Methods

Participants

This open-label randomised, parallel group clinical trial was approved by the Institutional Review Board and Ethics Committee of Royan Institute, Tehran, Iran (IR.ACECR.ROYAN.1394.44), and conducted in compliance with the Declaration of Helsinki and its subsequent versions. All participants provided informed consent prior to enrolment. A total of 182 women aged ≤37 years who underwent frozen-thawed ET from November 2015 to June 2020 at Royan Institute Research Centre, Tehran, Iran enrolled in this study.

Eligibility criteria consisted of: primary type of infertility; age ≤37 years; enrolled in the gonadotropin hormone-releasing hormone (GnRH) agonist long protocol; and having at least four good quality frozen embryos. Patients were excluded if they had any of the following: surgical history on the uterus and ovaries; uterine factor infertility; severe male factor infertility (TESE, TESA, severe oligoteratozoospermia); history of recurrent abortion (≥2 abortions); and poor ovarian reserve.

The eligible women were randomly assigned (1:1) to two groups-a control group that received thawed cleavage embryos (n=110) or the study group that received post-thaw extended culture blastocysts (n=72). Block randomisation was conducted in equal block sizes of four. Randomisation was performed by a third party with the aid of computer-generated random numbers (SPSS version 18.0; IBM, Armonk, NY, USA) prepared by a statistician.

Sample size was calculated by PASS version 11 (NCSS, Kaysville, UT, USA) on the basis of our pilot study data (n=20). The sample size required for this study was estimated to be 97 patients in each group in order to detect a between-group difference of 0.2 (0.6-0.4) in clinical pregnancy, taking into consideration an alpha value of 0.05 and statistical power of 80% by the two-sided test. We enrolled 110 patients in each group, taking into account a dropout rate of 10%.

Stimulated cycles with IVF/ICSI

Controlled ovarian stimulation was performed with the standard long protocol using a gonadotropin-releasing hormone agonist (Suprefact; Hoechst, Frankfurt, Germany) and recombinant follicle-stimulating hormone (Gonal-F; Serono Laboratories Ltd., Geneva, Switzerland) or human menopausal gonadotropin (Menopur; Ferring GmbH, Kiel, Germany). An intramuscular injection of 10 000 IU human chorionic gonadotropin (Choriomon; IBSA, Lugano, Switzerland) was performed once at least one follicle reached 17-18 mm in diameter. Transvaginal oocyte retrieval was performed 34-36 hours later via vaginal ultrasound guidance; the oocytes were subsequently incubated for insemination or sperm injection.

Endometrial preparation and embryo transfer

For endometrial preparation, all patients received oral contraceptive pill-low dose before treatment beginning from the fifth day of their previous menstrual cycle in addition to a daily dose of GnRH agonist (0.5 mg/day, Suprefact; Hoechst, Frankfurt, Germany) from the 17th day of the cycle until pituitary down regulation, which was confirmed by serum estradiol (E2) <50 pg/mL, luteinizing hormone (LH)<5 IU/L, and basal ultrasonography. Then, 4 mg oral oestradiol valerate was started daily from the second day of the menstrual cycle; the dosage was adjusted according to the thickness of the endometrium. After ultrasound confirmation of an endometrial thickness of at least 7 mm, 100 mg of progesterone in oil (Aburaihan Pharmaceutical Co., Iran) was administered intramuscularly or 400 mg of vaginal progesterone (Cyclogest®, Actavis, Barnstaple, EX32 8NS, UK) twice a day. Luteal support was continued for two weeks. Serum β-hCG levels were measured on the 14th day after ET.

Patients were scheduled for thawed cleavage-stage ET or post-thaw extended culture blastocysts on the first day of progesterone administration based on the initial random allocation. ET was performed using a soft ET catheter (Labotect Labor-Technik, Göttingen GmbH, Germany) on day 3 or day 5 after initiation of progesterone.

Embryo vitrification-warming method and grading

All excellent and good quality embryos that were at the cleavage and blastocyst stages, as assessed by morphological scoring, were frozen by the vitrification method. The vitrification/warming method was performed according to a Royan protocol (16, 17). For this aim, the cryotop carrier system (Kitazato Biopharma Co., Ltd.,

Japan) was used for vitrification; 7.5% ethylene glycol and 7.5% dimethyl sulphoxide (equilibration solution) followed by 15% (v/v) ethylene glycol, 15% (v/v) dimethyl sulphoxide and 0.5 mol/l sucrose (vitrification solution) were used as the cryoprotectant. The 1.0, 0.5, and 0.0 mol/l sucrose solutions were used in warming the stepwise cryoprotectant dilution. With the exception of the first warming step, all steps were carried out at room temperature; the first warming step was carried out at 37°C.

Excellent and good quality cleavage stage embryos had four blastomeres on day 2 or six to eight cells on day 3, an equal blastomere size, less than 20% fragmentation, and absence of clear morphological abnormalities (18, 19). On day 5, blastocyst grading was performed according to the Gardner scoring system (20). Briefly, blastocysts were scored based on the level of cavitation or blastocoel expansion, and the number and quality of the inner cell mass and trophectoderm (TE).

Outcome measures

The primary outcome measure was clinical pregnancy rate. Secondary outcome measures were the implantation rate, LBR, and miscarriage rate. Clinical pregnancy was defined as the detection of at least one gestational sac by ultrasound examination over the number of ET cycles. Implantation rate was defined as the number of gestational sacs seen on transvaginal ultrasonography divided by the total number of embryos transferred. Live birth was defined as the number of deliveries that resulted in at least one live-born baby. Miscarriage rate was defined as a pregnancy loss before gestational week 20 per the total number of clinical pregnancies. Twin birth rate reflected the number of twin births per total number of clinical pregnancies. Blastocyst formation rate was defined as the total number of blastocysts formed in a cycle by the total number of thawed cleavage-stage embryos.

Statistical analysis

Data analysis was performed with SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). We used per-protocol analysis to test our hypothesis. Data for continuous variables are written as mean ± standard deviation (SD) and the Student's t test was used for comparison between groups. Categorical data are presented as frequencies and percentages; the chi-square or Fisher's exact tests were used for comparison between groups. $P<0.05$ indicated statistical significance.

Results

A total of 220 patients were randomised to each group, with 110 patients per group. Of the 110 participants in the blastocyst group, 38 women were excluded from the analysis. In two cases, the treatment cycle was cancelled before ET due to an inadequate endometrial thickness and menstruation, three cases had a change in their treatment protocols, and there were no blastocysts available for transfer in 33 cases. The number of cycles in each group was as follows: 110 cycles in the thawed cleavage-stage and 72 cycles in blastocysts from the thawed cleavage stage. Figure 1 summarises participants' recruitment, intervention allocation, follow-up, and analysis.

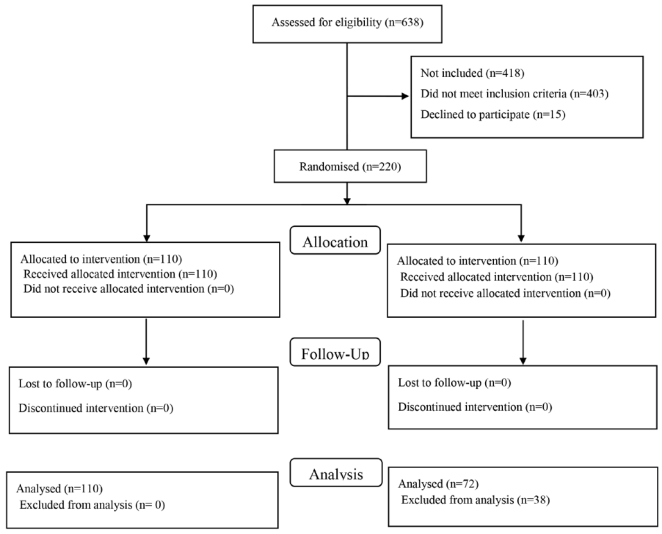

Fig.1: Flow chart of patient enrolment, allocation, follow-up, and analysis.

Table 1 provides baseline data on demographic and clinical characteristics between the thawed cleavage-stage ET and post-thaw extended culture blastocysts. There were no statistically significant differences between the two groups in regards to mean age, body mass index, infertility duration, and cause of infertility. The mean number of previous IVF/ICSI cycles, history of fresh ET, cause for freeze-all strategy, mean number of retrieved oocytes, mean number of MII oocytes, fertilisation rate, and mean number of retrieved embryos were similar in both groups.

Table 2 shows the clinical outcomes of patients in the frozen-thawed ET cycle. The two groups did not differ significantly in duration of cryostorage, basal hormone levels on day 3, maximum oestradiol dose, endometrial thickness on progesterone day injection, endometrial thickness on ET day, and difficulty of ET. However, there were statistically significant increases in the mean number of embryos transferred (2.38 ± 0.05 vs. 2.21 ± 0.05, P=0.016) and mean number of good quality embryos transferred (1.05 ± 0.09 vs. 0.71 ± 0.09, P=0.009) between the thawed cleavage-stage ET cycles compared to the post-thaw extended culture blastocysts cycles. The blastocyst formation rate in the post-thaw extended culture blastocysts cycles was 49.46% (138/279).

Reproductive outcomes of frozen-thawed ET cycles indicated that the post-thaw extended culture blastocysts group had significantly higher rates for clinical pregnancy (56.94 vs. 40.91%, P=0.034), implantation (34.43 vs. 19.84%, P=0.001), and live birth (49.29 vs. 33.63%, P=0.036) compared to the thawed cleavage-stage ET group. There were no significant differences in rates of miscarriage, twin birth, and other outcomes between both groups (P>0.05, Table 3). There were no reported birth defects in either group.

Table 1: Baseline characteristics and clinical history of ovarian stimulation cycle

Variables	Thawed cleavage-stage group (n=110)	Post-thaw extended culture blastocysts group (n=110)	P value
Age (Y)	29.96 ± 4.09	29.99 ± 4.09	0.961
Body mass index (kg/m^2)	25.28 ± 3.83	25.45 ± 4.08	0.755
Infertility duration (Y)	6.41 ± 3.78	6.19 ± 3.45	0.648
Cause of infertility			
Ovulatory	12 (10.91)	17 (15.45)	0.397
Tuboperitoneal	7 (6.36)	5 (4.55)	
Unexplained	16 (14.55)	23 (20.91)	
Male	67 (60.91)	61 (55.45)	
>1 factor	8 (7.27)	4 (3.64)	
No. of previous IVF/ICSI cycles	1.80 ± 0.96	1.96 ± 0.86	0.210
History of fresh embryo transfer			
Yes	36 (32.73)	27 (24.55)	0.180
No (freeze-all strategy)	74 (67.27)	83 (75.45)	
No. of retrieved oocytes	15.15 ± 5.97	16.02 ± 5.88	0.281
No. of MII oocytes	11.67 ± 0.48	12.55 ± 0.47	0.197
Fertilisation rate	1015/1540 (65.9)	1065/1616 (65.9)	0.997
No. of embryos	9.84 ± 3.67	10.28 ± 3.85	0.381

Data are written as mean ± SD or n (%). P value was obtained by the independent sample t test and chi square test. Statistically significant level was 0.05. IVF; *In vitro* fertilization and ICSI; Intracytoplasmic sperm injection.

Table 2: Clinical outcomes of patients in frozen-thawed ET cycles

Variables	Thawed cleavage-stage group (n=110)	Post-thaw extended culture blastocysts group (n=72)	P value
Duration of cryostorage (days)	316.85 ± 39.59	371.79 ± 43.69	0.352
Basal hormone levels on day 3			
LH (mIU/mL)	5.00 ± 0.23	5.75 ± 0.4	0.244
FSH (mIU/mL)	5.90 ± 0.2	5.95 ± 0.22	0.848
No. of embryos transferred	2.38 ± 0.05	2.21 ± 0.05	0.016
Quality of embryos transferred			
Excellent	1.08 ± 0.09	1.28 ± 0.08	0.126
Good	1.05 ± 0.09	0.71 ± 0.09	0.009
Fair	0.28 ± 0.05	0.21 ± 0.05	0.341
Assisted hatching			
Yes	23 (20.91)	54 (75.00)	<0.001
No	87(79.09)	18 (25.00)	
Maximum oestradiol dose			
4–8 mg	109 (99.09)	72 (100.00)	0.417
>8 mg	1 (0.91)	0 (0.0)	
Endometrial thickness: progesterone day injection (mm)	9.45 ± 1.27	9.42 ± 1.62	0.859
Endometrial thickness: ET day (mm)	9.74 ± 1.65	9.57 ± 1.52	0.569
Difficulty of ET			
Easy	104 (94.55)	70 (97.22)	0.506
Difficult	6 (5.45)	2 (2.78)	

Data are written as mean ± SD or n (%). P value was obtained by the independent sample t test and chi-square test or Fisher's exact test. Statistically significant level was 0.05. LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, and ET; Embryo transfer.

The results revealed that the laser-assisted hatching procedure was statistically higher in the post-thaw extended culture blastocyst cycles compared to the thawed cleavage-stage ET cycles (P<0.001, Table 2). Reproductive outcomes according to the laser-assisted hatching protocol are shown in Table 4. There were no significant differences in clinical pregnancy rates between those cycles with assisted hatching and unhatched cycles in the post-thaw extended culture blastocyst cycles and thawed cleavage-stage ET cycles (Table 4).

Table 3: Reproductive outcomes of frozen-thawed ET cycles

Variables	Thawed cleavage-stage group (n=110)	Post-thaw extended culture blastocysts group (n=72)	P value
Clinical pregnancies/ET cycle	45/110 (40.91)	41/72 (56.94)	0.034
Implantation/ET	52/262 (19.84)	52/151 (34.43)	0.001
Blighted	5/110 (4.54)	4/72 (5.55)	0.508
EP	0/110 (0)	1/72 (1.38)	0.396
Live birth delivery/ET cycle	37/110 (33.63)	35/72 (49.29)	0.043
Miscarriage/clinical pregnancy	3/110 (2.72)	1/72 (1.38)	<0.999
Twin birth/clinical pregnancy	7/110 (6.36)	10/72 (13.9)	0.088
Birth weight of first born	3012.08 ± 823.17	2964.57 ± 763.96	0.803
Birth weight of second born	1891.80 ± 554.18	2181.90 ± 594.15	0.379

Data are written as mean ± SD or n (%). P value obtained by the independent sample t test and chi square test. Statistically significant level was 0.05. ET; Embryo transfer and EP; Ectopic pregnancy.

Table 4: Clinical pregnancy and LBR of frozen-thawed ET cycle according to assisted hatching protocol

Outcomes	Thawed cleavage-stage group (n=110)		P value	Post-thaw extended culture blastocysts group (n=72)		P value
	Assisted hatching	No assisted hatching		Assisted hatching	No assisted hatching	
Clinical pregnancy rate	9/23 (39.13)	36/87 (41.37)	0.845	29/54 (53.7)	12/18 (66.6)	0.336
LBR	8/23 (34.78)	29/87 (33.33)	0.896	26/54 (48.15)	9/18 (50.00)	0.892

Data are presented as n (%). P value was obtained by the chi square test. Statistically significant level was 0.05. LBR; Live birth rate and ET; Embryo transfer.

Discussion

There is a recent, increasing trend towards a freeze-all approach. This trend will certainly impact decisions about the cleavage or blastocyst stages of embryo development for ET (7). Studies are being conducted to determine the key indicators that can affect the process of managing patients who benefit most from blastocyst culture. Approximately half of the trial results have shown higher success rates in the blastocyst transfer cycles. The trials can be separated into those that evaluated the superiority of blastocyst culture over standard cleavage-stage transfer in unselected patient populations or those that investigated the application of blastocyst culture in the clinical setting for enhanced success in specific patient subgroups (21). Here, we compared reproductive outcomes following transfer of post-thaw extended culture blastocysts and thawed cleavage-stage ET in patients who underwent frozen-thawed ET cycles.

We observed that the transfer of post-thaw extended culture blastocysts significantly improved rates for implantation, clinical pregnancy and live birth compared with thawed cleavage-stage ET. Enhanced pregnancy outcomes for blastocyst transfer compared with other stages of embryo development in frozen-thawed ET cycles have been reported (22, 23). To the best of our knowledge, only two studies (9, 15) assessed post-thaw extended culture blastocysts with conflicting results. A large retrospective population-based study (9) (n=150367) in Australia reported that the cycles with transfer of post-thaw extended culture blastocysts had significantly improved outcomes compared to thawed cleavage-stage ET cycles or thawed blastocyst transfer cycles. Remarkably, both LBR and a healthy baby delivery rate following transfer of post-thaw extended culture blastocysts were statistically comparable to those with fresh cleavage-stage transfer despite the physical damages to embryos from the freezing and thawing processes. The findings of this study indicate that the transfers of fresh blastocyst culture in fresh cycles and post-thaw extended culture blastocysts in frozen-thawed ET cycles improve the rate of healthy babies. The strong point of this retrospective cohort study was the use of population-based data extracted from all ET cycles performed in Australia during four years. In contrast, in a retrospective comparative study (15) that assessed the clinical outcomes of frozen-thawed ET with blastocysts found no benefit in pregnancy outcomes following frozen-thawed blastocyst transfer compared with frozen-thawed cleavage-stage or post-thaw extended culture blastocysts. They reported an extremely high multiple pregnancy rate (62.5%) in women who underwent post-thaw extended culture blastocyst transfer. A possible explanation for these results may be that the blastocysts in this study were cryopreserved by vitrification and the pronuclei were cryopreserved by the slow-freezing method, which might have impacted the results. An improved survival rate and similar or even better pregnancy rate for vitrification compared with the slow freezing method has been reported in numerous literature (24, 25). In line with these results, the data on transfer of post-thaw extended culture blastocysts is low; therefore, there is a need for further studies in this area.

Other studies sought to determine whether patients who underwent frozen-thawed ET cycles could benefit from the transfer of thawed blastocysts. The findings indicated significantly higher pregnancy rates (23) or

insignificantly higher cumulative ongoing pregnancy rates (26) in favour of thawed blastocyst transfer with fewer ETs and comparable multiple pregnancy rates as compared to thawed cleavage-stage ET.

The results of the present study show the benefits of transfer of post-thaw extended culture blastocysts. This higher success rate with blastocyst transfer might be attributed to the embryo selection process. It is reported that 59% of day 3 high-quality embryos are chromosomally abnormal, whilst only 35% of high-quality blastocysts have chromosomal anomalies (27). Even in cases where the blastocyst stage development has not been prevented by genetic abnormalities, the incidence of chromosomal abnormalities in blastocysts would be lower than those seen in cleavage-stage embryos. Blastocyst transfer would have a smaller risk of aneuploidy than embryo cleavage-stage embryos. This increases the chances for pregnancy (23). In addition, asynchrony between the developmental stage of transferred cleavage-stage embryos and the counterpart of the reproductive tract (28) may compromise embryo viability because the nutritional environments provided by the oviduct and uterus do not match with the developing embryo; thus, cleavage-stage ET might undergo metabolic stress (27, 29). However, blastocyst-stage embryos are better synchronised with the female reproductive tract during natural pregnancy and, therefore, are protected from this environmental stress (23).

Although our results indicated that the mean number of ETs was significantly lower in post-thaw extended culture blastocysts compared with cleavage-stage ET group, we observed a better outcome in the post-thaw extended culture blastocysts group. This finding was also reported in another large retrospective study. Those authors found a similar LBR with the transfer of frozen embryos on days 3 and 5 (30). An explanation for the fewer number of blastocysts-stage embryos than cleavage-stage embryos in different studies is the lack of options rather than policy, and the reason for the high rate of unsuccessful blastocyst transfer is mainly due to arrested embryonic development before the ET day (21). Some studies included patients that had transfer of developmentally delayed blastocyst stage embryos, whilst other studies were more selective and excluded those with transfer of embryos under stages of late morula or early blastocyst (7). The blastocyst formation rate is reported as 22.4 to 60% in different studies and may be associated with pregnancy rate per ET in each study (31, 32). This suggests that various formulations and brands of embryo culture media probably affect blastocyst formation rate and subsequent outcomes (21). We observed a blastocyst formation rate of 49.5% (138/279).

In our study, the percentage of treated post-thaw extended culture blastocysts with laser-assisted hatching was significantly higher than treated cleavage-stage embryos with assisted hatching (75 vs. 20.91%, $P<0.001$). Nonetheless, separate data analysis in thawed cleavage-stage ET cycles and post-thaw extended culture blastocysts showed no significant differences in clinical pregnancy rates between the assisted hatching and unhatched cycles. The evidence demonstrates that implantation rate of human embryos is associated with zona thickness (33), which might be related to zona pellucida hardening during vitrification (12). However, a considerable amount of meta-analysis and systematic reviews (34, 35) are uncertain about the effects of assisted hatching on LBR.

Although, there are some cost considerations associated with offering an extended culture to blastocyst stage for the patients including the cost of an additional incubator for culture, extra media costs, and increased weekend work for laboratory staff; for the patient, an increased probability of cancellation owing to the stricter selection process of the blastocyst culture might end to a lower treatment cost. The treatment cost is essential to be evaluated and compared to the chances of having a healthy baby (21).

A limitation of our study is the presence of factors that can affect pregnancy rate, such as the embryologist who performed the embryo grading, selection, and transfer.

Conclusion

The results strongly advocate in favour of using post-thaw extended culture blastocysts. It will be important to a improve the post-thawing extended culture technique before making the extra effort for transferring blastocysts in frozen-thawed ET cycles. A prospective randomised controlled trial that has a large sample size is suggested.

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Authors' Contributions

T.M.; Study conception and Design. N.J.; Methodology, Writing- review and Editing. A.Y., M.Z.; Data acquisition. S.V.; Statistical analysis. P.E.-Y.; Project development. All authors read and approved the final manuscript.

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