Original Article

Effect of Long-Term Embryo Cryopreservation on Subsequent Frozen Embryo Transfer Outcomes: A Retrospective Cohort Study

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Abstract

Background: The duration of cryopreservation of embryos and its effect on the subsequent pregnancy outcomes, when they have been frozen for a longer duration remains a matter of concern. There is a continuous debate among studies comparing different durations of embryo cryopreservation as the results are contradictory. **Aims:** This study aims to find out if long-term cryopreservation of embryos has any effect on pregnancy and perinatal outcomes. **Settings and Design:** Retrospective cohort study was conducted in the department of reproductive medicine and surgery in a university-level teaching hospital. **Materials and Methods:** The study included women who underwent frozen embryo transfer (FET) from autologous *in vitro* fertilisation between January 2012 and December 2020 with the duration of cryopreservation of more than 5 years as one group and 3–5 years as another group. Pregnancy and perinatal outcomes were analysed. **Statistical Analysis Used:** Regression analysis was performed using logistic regression by entering clinically important variables associated with pregnancy outcome, and the results were expressed as odds ratio with a 95% confidence interval (CI). All statistical analysis was performed with SPSS (version 21.0, IBM, USA). **Results:** A total of 1680 FET cycles were carried out during the study period. Among these, 75 cycles with a duration of 3–5 years and 20 cycles with a duration of more than 5 years were included. Live birth rate (LBR) was 40.8% in the 3–5 years group and 35% in the more than 5 years group. After adjusting for important confounders, the LBR has no significant association in the more than 5 years group (adjusted odds ratio 1.07; 95% CI 0.34–3.32; $P = 0.913$) compared to the 3–5 years group. **Conclusion:** The duration of cryopreservation of embryos has no statistically significant effect on the pregnancy and perinatal outcomes.

Keywords: *Cryopreservation, embryos, frozen embryo transfer, live birth rates, long term, vitrification*

Introduction

Pregnancy following frozen embryo transfer (FET) is dated back to the 80s with the first pregnancy reported in 1983;[1] since then, cryopreservation of embryos has become an essential part of assisted reproductive technology (ART). Over the past three decades, there is a tremendous improvement in the cryopreservation techniques and an increase in the number of FETs. According to the International Committee for Monitoring Assisted Reproductive Technologies world report, the global trend of FET cycles increased by 67.5% from 2010 to 2014.[2] Reasons for the increase in FET cycles in recent years are the

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adoption of the freeze-all strategy to prevent ovarian hyperstimulation syndrome (OHSS), policy towards single embryo transfer to minimise the risk of multiple pregnancies, cryopreservation of supernumerary embryos after the fresh transfer and premature progesterone elevation in the late follicular phase.[3-5] Following embryo cryopreservation, they can be transferred immediately in the next cycle after the oocyte pick up to a few years later, depending on the couple's choice and other reasons. The duration of the cryopreservation and its effect on the subsequent pregnancy outcomes, when they have been frozen for a longer duration remains a matter of concern.

Two basic methods of embryo cryopreservation have been used slow freezing and vitrification. Over the past two decades, there has been a dramatic shift in practice from slow freezing to vitrification. The development of vitrification was a milestone in ART. In contrast to slow freezing, vitrification is an ultra-rapid cooling procedure that prevents intracellular ice formation and converts it to a glass-like solid.[6] High concentrations of cryoprotectants and rapid cooling rates are essential to vitrify embryos. Vitrification had less impact on the metabolic rate of the embryo, resulting in increased cryosurvival rate and further development than slow freezing.[7,8] Vitrification has become a well-established and routine method of embryo cryopreservation.

There are gaps in the current literature about how long a human embryo can safely be cryopreserved and transferred without compromising the outcome. There is a continuous debate among studies comparing different durations of embryo cryopreservation as the results are contradictory. While few studies showed no difference in pregnancy rates between the duration of cryopreservation of embryos and subsequent FET,[9,10] other studies have reported that pregnancy rates decrease as the duration of embryo cryopreservation increases.[11] There is a lack of reassuring data regarding pregnancy and neonatal outcomes which is essential in counselling the couple. The majority of the available studies in the literature have reported outcomes for cryopreservation of fewer than 5 years.[12] Few case reports are available for long-term cryopreservation for more than 5 years.[13,14] There is a need to have effectiveness and safety data based on the duration of cryopreservation of embryos. In the present study, we aim to observe if long-term cryopreservation of embryos has any effect on pregnancy and perinatal outcomes.

Materials and Methods

Study population

This is a retrospective cohort study conducted in a university-level teaching hospital. Women who underwent FET from autologous *in vitro* fertilisation (IVF) between January 2012 and December 2020 with a duration of cryopreservation of more than 3 years were included. Baseline characteristics, cycle and outcome details were obtained from the hospital's electronic medical records. These FET cycles were divided into two cohorts depending on the duration of embryo cryopreservation. These FET cycles were divided into two cohorts depending on the duration of embryo cryopreservation as three to five years and more than 5 years. Cryopreservation of all the embryos was done by vitrification. Women who underwent FET with a duration of embryo cryopreservation of fewer than 3 years were excluded from the study. We also excluded fresh embryo transfers during the study period. Only data from those women who allowed the use of anonymous data for retrospective studies and gave written informed consent were included in the present study. The study was conducted in accordance with the guidelines laid down by the Declaration of Helsinki. Ethics approval was obtained from the institutional review board (IRB No. 14148 dated 28.07.2021).

In vitro fertilisation/intracytoplasmic sperm injection protocol

Women underwent IVF with antagonist or agonist protocol. Following controlled ovarian hyperstimulation, the oocyte retrieval was done after giving the trigger. The oocytes were fertilised by *in vitro* insemination or intracytoplasmic sperm injection depending on the sperm and oocyte quality. Oocytes were incubated in benchtop incubators (MINC; Cook IVF, Australia) with a triple gas mixture (5% oxygen, 6% carbon dioxide and 89% nitrogen). Sequential culture media was used. The decision to do a fresh transfer either at cleavage or blastocyst stage or go for a 'freeze all' was taken depending on the clinical situation and OHSS risk.

Vitrification

Vitrification of embryo was done using the solid surface method which involves contact of carrier fibre plug (CryoLogic, Victoria, Australia) loaded with embryo within media droplet with cryoprotectants ethylene glycol and Dimethyl sulfoxide (DMSO) in cryobuffer over precooled metal block (CryoLogic, Victoria, Australia). The metal block is placed inside the cryobath (CryoLogic, Victoria, Australia) with liquid nitrogen. The loaded fibre plug was transferred into a labelled goblet and canes were stored in a liquid nitrogen cryobank.[15]

Frozen embryo transfer

Women who were planned for a FET cycle with a hormonal replacement cycle were started on an increasing dose of estradiol valerate (Progynova, Schering AG, Germany) from day 1 of the spontaneous

or withdrawal cycles. On day 15, transvaginal ultrasound was done. If the endometrial pattern was normal, administration of progesterone was initiated. Few women planned for a natural cycle and stimulated protocol, follicular growth was monitored with ultrasonography, serum luteinising hormone and serum progesterone. Micronised progesterone 400 mg twice daily (Naturogest vaginal Pessaries, Zydus Healthcare Limited, India) and parenteral progesterone (Gestone, Ferring pharmaceuticals, Switzerland) 100 mg twice weekly were administered for luteal support, and the transfer was planned after 3–5 days of starting progesterone depending on the stage of the embryo. On the day of embryo transfer, embryos were warmed and assessed for cryosurvival. One to three embryos (Grade 1 or 2) were transferred depending on the age, previous cycle performance and stage of the embryo. Serum β-human chorionic gonadotropin (β-hCG) was done 2 weeks after embryo transfer to detect pregnancy. Women with serum β-hCG positive were followed up till delivery. Pregnancy and perinatal outcomes were recorded.

Definitions

Embryo cryosurvival is the proportion of embryos with >50% of the blastomeres intact post-cryopreservation. Blastocyst cryosurvival is defined as the proportion of blastocysts with at least 75% of cells perceived to be intact after warming.[16] Implantation rate was defined as the number of gestational sacs observed divided by the number of embryos transferred. Clinical pregnancy rate is defined as pregnancy diagnosed by ultrasonographic visualisation of one or more gestational sacs and expressed per embryo transfer. The miscarriage rate was defined as the spontaneous loss of a pregnancy before 22 completed weeks of gestational age. The miscarriage rate was expressed as miscarriage per clinical pregnancy. The multiple pregnancy rates are defined as multiple pregnancies (more than one gestational sac on ultrasonography) per clinical pregnancy. Live birth rate (LBR) is defined as a foetus showing any sign of life beyond 22 weeks gestational age. The LBR was expressed per embryo transfer.[6]

Statistical analysis

Sample size calculation was not done as it was a retrospective study. Continuous variables are expressed as mean ± standard deviation and median with interquartile range (IQR) and compared with Student's *t*-test and Mann–Whitney *U* test as per the normality assumption. Categorical variables are expressed as frequency and percentage and compared using the Chi-square test and Fisher's exact test (less cell count). Further, regression analysis was performed using logistic regression by entering clinically important variables associated with clinical pregnancy, live births and miscarriage rates, and the results were expressed as odds ratio (OR) with a 95% confidence interval (CI). A two-sided $P < 0.05$ was considered statistically significant. All statistical analysis was performed with SPSS (version 21.0, IBM, USA).

Results

Baseline and frozen embryo transfer cycle characteristics

A total of 1680 FET cycles were carried out during the study period. Among these, 75 cycles with a duration of 3–5 years and 20 FET cycles with a duration of more than 5 years were included. In the 75 cycles with a duration of 3–5 years, two women did not undergo embryo transfer as the embryos did not survive post-warming and two cases were lost to follow-up after clinical pregnancy [Figure 1]. Mean female age at the time of cryopreservation was 29.4 ± 3.6 years in the 3–5 years group and 27.6 ± 2.9 years in the more than 5 years group. In the 3–5 years group, 97.3% cycles and in more than 5 years 100% cycles were women with secondary infertility. The most common cause of infertility was the combined factor (32%) in the 3–5 years group and the male factor (45%) in the more than 5 years group. Blastocyst transfer was done in 89.3% and 100% cycles in the 3–5 years group and more than 5 years group, respectively. The median duration of cryopreservation was 3.82 years in the 3–5 years group and 6.02 in the more than 5 years group [Table 1]. Majority of the endometrial preparation cycles were hormone replacement cycles in both the 3–5 years

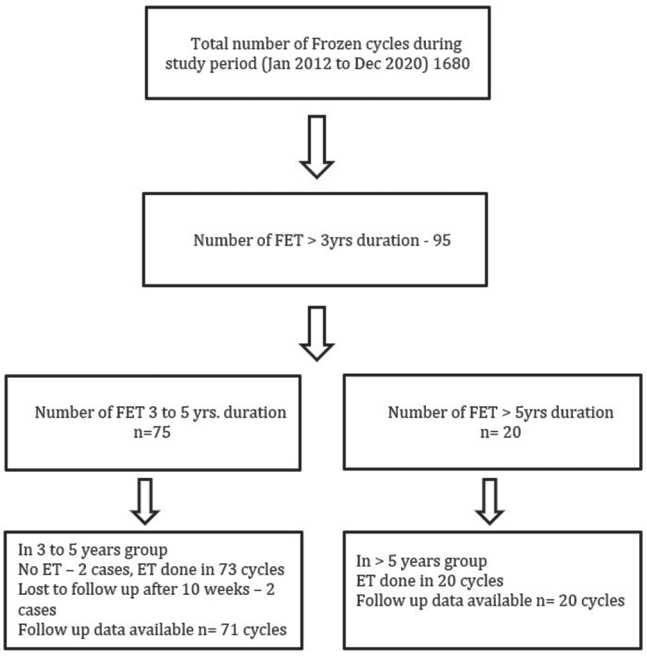

Figure 1: Data flow of the women undergoing FET. FET = Frozen embryo transfer

group (96%) and the more than 5 years group (95%). Cryosurvival was 88% in the 3–5 years group and 86% in the more than 5 years group [Table 2]. The median number of embryos transferred was 2 (IQR: 1–2) in the 3–5 years group and 1.5 (IQR: 1–2) in the more than 5 years group [Table 2].

Outcomes

The clinical pregnancy rate per transfer was 40/73 (54.8%) in the 3–5 years group and 10/20 (50%) in the more than 5 years group. Among 40 clinical pregnancies in the 3–5 years group, 7 had a miscarriage, 2 women had a stillbirth, 2 women lost to follow-up till delivery and 29 had live births. LBR was 29/71 (40.8%) and 7/20 (35%) in the 3–5 years group and more than 5 years group, respectively [Table 3]. Mean gestational age was 36.0 ± 4.3 and 37.0 ± 0.6 weeks and mean birth weight was 2.7 ± 0.6 and 2.9 ± 0.4 kg in the 3–5 years group and more than 5 years group, respectively [Table 3].

For the LBR, no significant association was observed with more than 5 years group (OR 0.78; 95% CI 0.28–2.19; P = 0.637 compared to 3–5 years group [Table 4]. After adjusting for important confounders, i.e., female age at vitrification, body mass index, cause of infertility and number of embryos transferred, no significant association was observed in the more than 5 years group (adjusted OR [aOR] 1.07; 95% CI 0.34–3.32; P = 0.913) compared to 3–5 years group.

For clinical pregnancy, no significant association was observed with more than 5 years group (OR 0.83; 95% CI 0.31–2.22; P = 0.703) compared to 3–5 years group. The aOR for clinical pregnancy also showed no significant difference [Table 4]. For miscarriage, no significant association was observed with more than 5 years group (OR 1.90; 95% CI 0.39–9.23; P = 0.427) compared to the 3–5 years group [Table 4]. After adjusting for important confounders, no significant association was observed in the more than 5 years group (aOR 1.57; 95% CI 0.27–9.23; P = 0.620) compared to the 3–5 years group [Table 4].

Discussion

The current study showed no statistically significant difference in the pregnancy and LBRs following frozen transfer among women between 3 and 5 years and beyond 5 years duration of embryo cryopreservation. No statistically significant difference was noted in the perinatal outcomes.

In the early days close to the inception of embryo cryopreservation, studies reported negative effects on

Table 1: Baseline characteristics of women undergoing frozen embryo transfer

	3-5 years (*n*=75), *n* (%)	>5 years (*n*=20), *n* (%)	Total (*n*=95), *n* (%)	*P*
Female age at vitrification (years)*	29.4±3.6	27.6±2.9	29.1±3.5	0.043
Female BMI*	24.4±4.6	24.2±4.2	24.4±4.4	0.843
Type of infertility, *n* (%)				
Primary	2 (2.7)	0 (0)	2 (2.1)	1.000
Secondary	73 (97.3)	20 (100)	93 (97.9)	
Previous live birth, *n* (%)				
Yes	64 (85.3)	18 (90)	82 (86.3)	0.729
No	11 (14.7)	2 (10)	13 (13.7)	
Cause of infertility, *n* (%)				
Polycystic ovaries	13 (17.3)	2 (10)	15 (15.8)	0.045
Male	21 (28)	9 (45)	30 (31.6)	
Unexplained	5 (6.7)	1 (5)	6 (6.3)	
Endometriosis	1 (1.3)	1 (5)	2 (2.1)	
Tubal	11 (14.7)	6 (30)	17 (17.9)	
Combination	24 (32)	1 (5)	25 (26.3)	
Nature of freezing, *n* (%)				
Freeze all	26 (34.7)	7 (35)	33 (34.7)	0.978
Supernumerary	49 (65.3)	13 (65)	62 (65.3)	
Stage of an embryo, *n* (%)				
Cleavage	8 (10.7)	0 (0)	8 (8.4)	0.197
Blastocyst	67 (89.3)	20 (100)	87 (91.6)	
Duration of cryopreservation (years)†	3.82 (3.37-4.15)	6.02 (5.18-6.77)	4.00 (3.47-4.60)	<0.001

*Mean±SD and *P* value is obtained from *t*-test, †median with IQR and *P* value is obtained from Mann-Whitney test. *n* (%) and *P* value is obtained from Chi-square and Fisher's exact test. *P*<0.05 is considered a statistically significant difference. BMI=Body mass index, SD=Standard deviation, IQR=Interquartile range

Table 2: Frozen embryo transfer cycle characteristics of women undergoing frozen embryo transfer

	3-5 years (*n*=75), *n* (%)	>5 years (*n*=20), *n* (%)	Total (*n*=95), *n* (%)	*P*
Endometrial preparation				
HRT	72 (96)	19 (95)	91 (95.8)	0.618
Natural cycle	2 (2.7)	1 (5)	3 (3.1)	
Stimulated cycle	1 (1.3)	0	1 (1)	
Number of warm cycles, *n*	75	20	95	-
Number of transfer cycles	73 (97.3)	20 (100)	93 (97.9)	-
Cryosurvival	140/159 (88)	37/43 (86)	177/202 (87.6)	0.7233
Number of embryos transferred*	2.0 (1.0-2.0)	1.5 (1.0-2.0)	2.0 (1.0-2.0)	0.918

*Median with IQR and *P* value is obtained from Mann-Whitney test. *n* (%) frequency with percentage and *P* value is obtained from Chi-square test. *P*<0.05 is considered a statistically significant difference. HRT=Hormone replacement cycles, IQR=Interquartile range

Table 3: Pregnancy and neonatal outcomes of women undergoing frozen embryo transfer

	3-5 years (*n*=75), *n* (%)	>5 years (*n*=20), *n* (%)	Total (*n*=95), *n* (%)	*P*
Pregnancy rate (test positive)	46/73 (63)	11/20 (55)	57/93 (61.3)	0.514
Implantation rate	50/114 (43.8)	11/30 (36.7)	61/144 (42.4)	0.478
Clinical pregnancy rate (per cycle)	40/75 (53.3)	10/20 (50)	50/95 (52.6)	0.791
Clinical pregnancy rate (per transfer)	40/73 (54.8)	10/20 (50)	50/93 (53.8)	0.703
Multiple pregnancy rate	8/40 (20)	1/10 (10)	9/50 (18)	0.665
Miscarriage rate*	7/38 (18.4)	3/10 (30)	10/48 (20.8)	0.414
Live birth rate*	29/71 (40.8)	7/20 (35)	36/91 (39.6)	0.637
Gestational age†	36.0±4.3	37.0±0.6	36.2±3.9	0.563
Birth weight†	2.7±0.6	2.9±0.4	2.8±0.6	0.500

†Mean±SD and *P* value is obtained from *t*-test, *In 3-5 years group, 2 out of 73 transfers lost to follow-up till live birth (these two cases removed from the denominator for miscarriage and live birth rate). Values are *n* (%) and *P* value is obtained from Chi-square test. *P*<0.05 is considered a statistically significant difference. Clinical pregnancies (40)=29 live births +7 miscarriages +2 stillbirths +2 lost to follow-up. SD=Standard deviation

Table 4: Logistic regression analysis

	No live birth (*n*=55), *n* (%)	Live birth (*n*=36), *n* (%)	Unadjusted OR (95% CI)	*P*	Adjusted OR* (95% CI)	*P*
3-5 years	42 (76.4)	29 (80.6)	Reference			
>5 years	13 (23.6)	7 (19.4)	0.78 (0.28-2.19)	0.637	1.07 (0.34-3.32)	0.913
	No clinical pregnancy (*n*=43), *n* (%)	**Clinical pregnancy (*n*=50), *n* (%)**	**Unadjusted OR (95% CI)**	***P***	**Adjusted OR* (95% CI)**	***P***
3-5 years	33 (76.7)	40 (80.0)	Reference			
>5 years	10 (23.3)	10 (20.0)	0.83 (0.31-2.22)	0.703	1.06 (0.36-3.14)	0.910
	No miscarriage (*n*=38), *n* (%)	**Miscarriage (*n*=10), *n* (%)**	**Unadjusted OR (95% CI)**	***P***	**Adjusted OR* (95% CI)**	***P***
3-5 years	31 (81.6)	7 (70.0)	Reference			
>5 years	7 (18.4)	3 (30.0)	1.90 (0.39-9.23)	0.427	1.57 (0.27-9.23)	0.620

*Adjusted for female age at vitrification, BMI, cause of infertility and number of embryos transferred. *P*<0.05 is considered a statistically significant difference. OR=Odds ratio, BMI=Body mass index

the embryos with prolonged storage. In 1987, Testart *et al*. found that the cryosurvival decreases as the duration of cryostorage increases with 70.6% survival at 1 month and 52.6% survival at 6–15 months.[17] Later, Riggs *et al*. retrospectively analysed 1927 IVF and 490 oocyte donation cycles and showed that there was no significant impact of the duration of cryopreservation of embryos on pregnancy outcomes, which was similar to our study, although their method of cryopreservation was slow freezing, stage of embryos was pronuclear and cleavage stage.[18] Yuan *et al*., in a retrospective study, included 20 patients with more than 12 years of storage duration by slow freezing and reported live births even after long-term cryopreservation.[19] A few case reports have reported live birth following 12 and 20 years of cryopreservation.[13,14] The above-mentioned studies used slow freezing as a method of cryopreservation.

Wirleitner *et al*. retrospectively studied 603 FET cycles and found that there was no negative impact on blastocyst cryosurvival and the pregnancy rates

of embryos up to 6 years after vitrification. The cryosurvival rate in our study was 88% and 86% for 3–5 years and more than 5 years group was similar to the Wirleitner study, 83% during the 1st year and 83.1% after 5–6 years for blastocyst vitrification.[9] Li *et al*. did a retrospective study of 786 vitrified-warmed cycles that showed no significant differences in the cryosurvival rate, clinical pregnancy, LBR, gestational age and birth weights at different storage times up to 5 years.[20] Ueno *et al*. in a retrospective study of 8736 cycles with single blastocyst transfer after vitrification showed no statistically significant difference in the pregnancy and neonatal outcomes with a maximum storage duration of 8 years. However, they included only women with age 35–39 years at oocyte retrieval which could lead to selection bias.[10]

Li *et al*. in a retrospective study of 24,698 patients with the first FET following the freeze-all strategy after vitrification showed that as the duration of cryopreservation increases the LBRs decreased (3–6 months: aOR 0.89 with 95% CI 0.85–0.95 and 12–24 months: aOR 0.59 with 95% CI 0.48–0.72 $P < 0.001$) but did not affect the miscarriage rates and neonatal outcomes. The findings of this study were in disagreement with the current study. They included women undergoing their first FET following freeze all, and those women with previous fresh or FETs were excluded.[12] Zhang *et al*. retrospectively studied 17,826 women who underwent their first frozen transfer following the freeze-all strategy and found that cryostorage duration was negatively associated with pregnancy and live birth ($P < 0.001$), but did not influence miscarriage, and the duration of cryopreservation was up to 4 years.[11] Cui *et al*. studied retrospectively 9806 frozen-thawed single-embryo transfer cycles and reported that when the duration of cryopreservation was <5 years, there was no significant difference in the pregnancy outcomes; however, when the duration of vitrification exceeded 5 years, the clinical pregnancy and live births decreased significantly, which is in contrast to our study finding.[21]

The strength of the current study is the inclusion of storage duration of more than 5 years because the evidence about more than 5 years of duration of cryopreservation is scarce. No major modifications were done in the procedures involved in our ART laboratory during this study period which could have excluded the confounding effect of time on the outcomes. The limitation of the current study is the retrospective nature and small sample size. Due to the retrospective nature, the effect of unknown confounders on the outcomes cannot be ruled out. Long-term follow-up of children was not done which is also an important outcome.

Conclusion

The duration of cryopreservation has no significant effect on the pregnancy and perinatal outcomes between 3 and 5 years and more than 5 years duration. However, studies with a large sample size and long-term follow-up are needed to support these findings.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement

The data supporting the results presented in this article are available from the corresponding author upon reasonable request.

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