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Embryology

Vitrification preservation of good-quality blastocysts for more than 5 years reduces implantation and live birth rates

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ABSTRACT

STUDY QUESTION: Does vitrification cryopreservation of embryos for more than 5 years affect the pregnancy outcomes after frozen embryo transfer (FET)?

SUMMARY ANSWER: Vitrification cryopreservation of good-quality blastocysts for more than 5 years is associated with a decrease in the implantation rate (IR) and live birth rate (LBR).

WHAT IS KNOWN ALREADY: Previous studies have predominantly focused on embryos cryopreserved for relatively short durations (less than 5 years), yet the impact of extended cryopreservation duration on pregnancy outcomes remains a controversial issue. There is a relative scarcity of data regarding the efficacy and safety of storing embryos for 5 years or longer.

STUDY DESIGN, SIZE, DURATION: This retrospective study involved 36 665 eligible vitrified-thawed embryo transfer cycles from 1 January 2016 to 31 December 2022, at a single fertility center in China.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Patients were divided into three groups according to embryo storage time: Group 1 consisted of 31 565 cycles, with storage time of 0–2 years; Group 2 consisted of 4458 cycles, with a storage time of 2–5 years; and Group 3 included 642 cycles, with storage time exceeding 5 years. The main outcome measures were IR and LBR. Secondary outcome variables included rates of biochemical pregnancy, multiple pregnancy, ectopic pregnancy, and miscarriage, as well as neonatal outcomes. Reproductive outcomes were analyzed as binary variables. Multivariate logistic regression analysis was used to explore the effect of preservation time on pregnancy outcomes after correcting for confounding factors. In addition, we also assessed neonatal outcomes, such as large for gestational age (LGA) and small for gestational age (SGA).

MAIN RESULTS AND THE ROLE OF CHANCE: IRs in the three groups (0–2, 2–5, and >5 years) were 37.37%, 39.03%, and 35.78%, respectively ($P = 0.017$), and LBRs in the three groups were 37.29%, 39.09%, and 34.91%, respectively ($P = 0.028$). After adjustment for potential confounding factors, compared with the 0–2 years storage group, prolonged embryo vitrification preservation time (2–5 years or >5 years) did not affect secondary outcomes such as rates of biochemical pregnancy, multiple pregnancy, ectopic pregnancy, and miscarriage ($P > 0.05$). But cryopreservation of embryos for more than 5 years reduced the IR (adjusted odds ratio (aOR) 0.82, 95% CI 0.69–0.97, $P = 0.020$) and LBR (aOR 0.76, 95% CI 0.64–0.91, $P = 0.002$). Multivariate stratified analysis also showed that prolonging the cryopreservation time of blastocysts (>5 years) reduced the IR (aOR 0.78, 95% CI 0.62–0.98, $P = 0.033$) and LBR (aOR 0.68, 95% CI 0.53–0.87, $P = 0.002$). However, no effect on cleavage embryos was observed ($P > 0.05$). We further conducted stratified analyses based on the number and quality of frozen blastocysts transferred, and the results showed that the FET results after transfers of good-quality blastocysts in the >5 years storage group were negatively affected. However, the storage time of non-good-quality blastocysts was not significantly associated with pregnancy outcomes. Regarding the neonatal outcomes (of singletons), embryo vitrification preservation time had no effect on preterm birth rates, fetal birth weight, or neonatal sex ratios. However, as the storage time increased, rates of SGA (5.60%, 4.10%, and 1.18%) decreased, while rates of LGA (5.22%, 6.75%, and 9.47%) increased ($P < 0.05$). After adjusting for confounding factors, the increase in LGA and the decrease in SGA were significantly correlated with the duration of storage time.

LIMITATIONS, REASONS FOR CAUTION: This was a retrospective study using data from a single fertility center, even though the data had been adjusted, our findings still need to be validated in further studies.

WIDER IMPLICATIONS OF THE FINDINGS: With the full implementation of the two-child policy in China, there may be more patients whose embryos have been frozen for a longer time in the future. Patients should be aware that the IR and LBR of blastocysts

are negatively affected when the cryopreservation time is longer than 5 years. Couples may therefore consider shortening the time until FET treatment.

Introduction

IVF technology has a long developmental history, and embryo cryopreservation is an important step. Slow freezing (program freezing) technology was first used for embryo cryopreservation, while vitrification freezing was first applied in China in 2003 and fully implemented in 2012 (Zhu *et al.*, 2005; Shi *et al.*, 2012).

In 2016, China officially implemented a universal two-child policy, and so many patients wanted to transfer their embryos that had been preserved in the early years of vitrification technology. However, the high concentrations of cryoprotectants used in vitrification as well as the open system that directly exposes embryos to liquid nitrogen during storage may cause damage or osmotic toxicity in embryos that have been cryopreserved for a long time. Animal experiments on the long-term preservation of vitrified frozen embryos have shown variable results in terms of *in vitro* and *in vivo* embryo survival ability. The experimental results of mice showed that the rates of cryosurvival, fertilization, and embryonic development decreased with the extension of cryopreservation time (Yan *et al.*, 2011), but the experimental results of bovine (Fang *et al.*, 2014) or porcine (Sanchez-Osorio *et al.*, 2010) embryos showed no significant effect. Given the significant interspecies physiological differences, although these animal models are referentially valuable for human, their predictive efficacy is somewhat limited for clinical applications. Although some clinical studies have evaluated the impact of the duration of embryo vitrification cryopreservation on results of frozen embryo transfer (FET), most have focused on embryos stored for less than 5 years. Some studies have shown that the duration of embryo cryopreservation does not affect the pregnancy outcome (Li *et al.*, 2017; Ueno *et al.*, 2018); however, other studies have shown that prolonging the vitrification preservation duration has a negative impact on pregnancy outcomes (Li *et al.*, 2020; Zhang *et al.*, 2021; Mao *et al.*, 2022). A small-sample propensity score matching study suggested that if the duration of embryo cryopreservation is more than 5 years, the implantation rate (IR) and live birth rate (LBR) may be significantly reduced with the extension of freezing time (Cui *et al.*, 2021). Nearly all studies with a storage duration exceeding 5 years have small sample sizes or are case reports, which limits the availability of data on the efficiency and safety of long-term embryo preservation. The lack of clinical evidence and exact guidelines makes the issue of embryo vitrification preservation extremely controversial. Therefore, this study aimed to explore the effect of a longer vitrification embryo preservation time (>5 years) on embryo pregnancy outcomes, such as IR and LBR, and to determine a safe vitrification embryo preservation time.

Materials and methods
Study population and design
A retrospective cohort study was conducted at a single fertility center from 1 January 2016 to 31 December 2022, including all women who had experienced at least one freeze-thaw cycle. The embryos of these patients were vitrified after 2012. The exclusion criteria were non-vitrification, pre-implantation genetic testing, uterine malformation, frozen oocytes, repeated vitrification-warming embryos, two-step transfers, incomplete information, and loss to follow-up. Finally, 36 665 cycles were included in the analysis. To investigate the effect of cryopreservation time on pregnancy outcomes, we divided the 36 665 cycles into three groups based on the embryo storage time: Group 1, stored for 0–2 years; Group 2, stored for 2–5 years; and Group 3, stored for more than 5 years. The study protocol was approved by the ethics committee of the Third Affiliated Hospital of Guangzhou Medical University, and written informed consent was obtained from each participant.

ART procedures
The women were monitored and managed according to the clinical protocols of the hospital. Ovarian stimulation protocols use recombinant follicle-stimulating hormone or human menopausal gonadotropin at 150–450 IU/day, and from a variety of protocols (short-term agonist regimens, long-term agonist regimens, and gonadotropin-releasing hormone antagonist regimens), and the characteristics of each patient (age, BMI, antral follicle count, and anti-Müllerian hormone) were used to determine which regimen was selected. Transvaginal oocyte retrieval was performed 35–36 h after hCG injection. ART was performed according to standard hospital operating procedures.

The choice of conventional IVF or ICSI was determined according to semen conditions and fertilization history. Embryos were incubated individually in 25 µl droplets of G-1 Plus^{TM} medium (Vitrolife) under Ovoil (Vitrolife) and incubated at 37°C in an atmosphere of 6% CO_2 and 5% O_2. Day 3 embryos were transferred to G-2 Plus^{TM} medium (Vitrolife) until Day 5/6. Fertilization assessment was performed 16–18 h after routine fertilization/injection, and embryo quality was assessed on Days 3, 5, and 6 after insemination.

A good-quality embryo on the third day was generally defined as an embryo derived from a two pronuclei zygote, with 7–9 blastomeres of equal size, <25% fragmentation, and no multinucleation. A good-quality blastocyst was generally defined as a blastocyst of stage 3 or above in the Gardner scoring criteria, with the inner cell mass and trophectoderm scores not including a C grade.

Embryo cryopreservation techniques and transfer protocols
The selection of embryos for freezing was based on the developmental stage of the embryo as well as the embryo count. Embryo freezing and thawing were performed according to the protocol of the Vitrification Kit (Kitazato Corp., Japan). For embryo freezing, cleavage-stage embryos were exposed to equilibration solution (ES) for 5 min at room temperature or blastocysts were exposed to ES for 2 min at 37°C. Embryos were then transferred

to a vitrification solution and incubated for 45–60 s. Finally, small-volume embryos were transferred to Cryotop strips (Kitazato Corp., Tokyo, Japan) and immediately placed in liquid nitrogen. Embryos were stored in liquid nitrogen at −196°C with continuous manual monitoring. Each Cryotop strip in which embryos were stored contained 1–2 embryos.

During embryo thawing, embryos removed from the vehicle were first rapidly immersed in thawing solution (37°C) for 1 min and transferred to diluent for 3 min at room temperature and then transferred to washing solution for another 3 min (performed twice). All thawed embryos were cultured in a medium at 37°C in an incubator in the gas phase of 5% CO_2 and 5% O_2 until transfer. Embryos with less than 50% degraded cells were considered viable and could be transferred, whereas embryos with more than 50% degraded cells were considered inactive and were discarded.

The laboratory freezing and thawing procedures, embryo cryopreservation protocols, technical equipment, and storage tanks used were the same throughout the study.

Endometrial preparation for FET cycle and embryo transfer

According to the menstrual cycle and ovulation status of the patient, endometrial preparation for the FET cycle was determined based on whether the patient was going to use a natural or an artificial cycle. In patients with natural cycles, follicular development and ovulation were assessed using transvaginal ultrasonography and hormone levels. For patients with artificial cycles, estradiol valerate tablets (Progynova, Bayer, Germany) were administered orally daily, starting on Day 3 or 4 of menstruation. Once daily intramuscular progesterone (40 mg) was required when endometrial thickness was ≥7 mm. Ultrasound-guided embryo transfer was performed on Days 3 (cleavage-stage embryos) or 5 (blastocysts) after ovulation or on Days 4 (cleavage-stage embryos) or 6 (blastocysts) after progesterone exposure. All patients who underwent embryo transfer received progesterone as a post-transfer luteal support. Transvaginal ultrasound was performed 4–6 weeks after embryo transfer, and luteal support was continued until 10 weeks of gestation if the gestational sac and embryo heartbeat were detected.

Clinical outcomes

The primary outcomes were IR and LBR. The secondary outcome variables included rates of biochemical pregnancy, multiple pregnancies, ectopic pregnancy, and miscarriage, as well as neonatal outcomes. The embryo IR was calculated by dividing the number of gestational sacs detected using transvaginal ultrasonography (performed 28 days after embryo transfer) by the number of embryos transferred. A live birth was defined as a live baby delivered after 24 weeks of pregnancy. Biochemical pregnancy was defined as a diagnosis of pregnancy based on elevated serum hCG levels detected 14 days after embryo transfer but without a gestational sac. Multiple pregnancies were defined as the presence of multiple intrauterine fetuses simultaneously. Ectopic pregnancy was diagnosed using ultrasound or laparoscopic imaging of at least one ectopic pregnancy sac. Miscarriage was defined as the loss of fetal cardiac activity within 28 weeks of confirming clinical pregnancy. Only singletons were included to evaluate the relationship between storage time and neonatal outcomes. Neonatal outcomes included preterm birth (<37 weeks of gestation), small for gestational age (SGA; birth weight <10th percentile), large for gestational age (LGA; birth weight >90th percentile), appropriate for gestational age (AGA; birth weight

between 10th and 90th percentile), newborn sex (male/female), and congenital defects.

Statistical analysis

All data were statistically analyzed using SPSS 22.0 for Windows (IBM, Armonk, NY, USA). Continuous variables were presented as the mean ± SD and analyzed by ANOVA test. Categorical variables are presented as counts and percentages and were compared using the chi-square or Fisher's exact test, as appropriate. *P*-values <0.05 were considered statistically significant.

To investigate the effect of embryo storage time on pregnancy and neonatal outcomes, multivariable logistic regression analysis was conducted. Reproductive outcomes, including implantation, live birth, biochemical pregnancy, multiple pregnancies, ectopic pregnancy and miscarriage, and neonatal outcomes, including preterm birth, LGA, AGA, and SGA, were treated as binary variables. The potential confounding factors were adjusted in the models according to univariate analysis, and variables with *P*-value <0.10 were included in the multivariate regression model. Confounding factors included maternal age at oocyte retrieval, maternal BMI, type of infertility, main causes of infertility, number of oocytes retrieved, fertilization method, number of embryos transferred, development stage and quality of embryo transferred, number of previous embryos transferred, endometrial preparation method, and endometrial thickness. Considering that advanced maternal age significantly increases the risk of adverse reactions in newborns, we adjusted the age of FET. The storage duration group was included as a categorical variable, and Group 1 was used as the reference. The adjusted odds ratios (aOR) and 95% CI were calculated.

Results

A total of 36 665 cycles were included in our study, and the patients were divided into three groups according to embryo storage time: Group 1 consisted of 31 565 cycles, with storage time of 0–2 years; Group 2 consisted of 4458 cycles, with storage time of 2–5 years; and Group 3 included 642 cycles with storage time >5 years.

The demographic and clinical characteristics of the vitrified embryo transfer cycles are provided in Table 1. The mean age at oocyte retrieval among the patients included in the study was 32.31 ± 4.91 years, the mean age at FET was 33.16 ± 4.85 years, the mean BMI was 22.13 ± 3.18 kg/m^2, and the average embryo cryopreservation time was 0.85 ± 1.24 years, with the longest preservation time being 9.5 years. With the longer storage times, the maternal age at oocyte retrieval and maternal BMI decreased, the mean number of oocytes retrieved increased, and the maternal age at FET increased (*P* < 0.001). The proportion with primary infertility and female infertility also increased with longer storage times (*P* < 0.001). IVF was the main fertilization method among the three groups. With longer storage time, there was an increase in the proportion of patients who transferred non-good-quality embryos, who had more than two transfers, and who had already given birth (*P* < 0.001). There was a significant difference in the number of patients receiving transfers of blastocysts rather than cleavage stage embryos among the three groups. In addition, there were differences in the distribution of endometrial preparation methods and endometrial thickness among the three groups (*P* < 0.001). There was no significant difference in the number of transferred embryos among the three groups (*P* > 0.05). Clinical parameters, including the IR (37.37%, 39.03%, and 35.78%, *P* = 0.017), LBR (37.29%, 39.09%, and 34.91%, *P* = 0.028), and multiple pregnancy rate (18.84%, 21.54%, and

Table 1. Demographic and clinical characteristics of vitrified embryo transfer cycles.

Variable	All cycles	Group 1 0–2 years	Group 2 2–5 years	Group 3 >5 years	P-value
Number of thawing cycles, n	36 665	31 565	4458	642	
Number of frozen embryos transferred cycles, n (%)	36 432 (99.36%)	31 386 (99.43%)	4413 (98.99%)	633 (98.60%)	
Maternal age at oocyte retrieval (years)	32.31 ± 4.91	32.67 ± 4.95	30.13 ± 3.97	29.38 ± 3.62	<0.001
<35	26 432 (72.55%)	21 933 (69.88%)	3912 (88.65%)	587 (92.73%)	<0.001
35–37	5022 (13.78%)	4627 (14.74%)	359 (8.14%)	36 (5.69%)	
≥38	4978 (13.66%)	4826 (15.38%)	142 (3.22%)	10 (1.58%)	
Maternal age at FET (years)	33.16 ± 4.85	33.08 ± 4.97	33.36 ± 3.99	35.41 ± 3.69	<0.001
Storage time by vitrification (years)	0.85 ± 1.24	0.41 ± 0.39	3.23 ± 0.78	6.03 ± 0.91	<0.001
Maternal BMI (kg/m^2)	22.13 ± 3.18	22.19 ± 3.20	21.78 ± 2.99	21.69 ± 3.02	<0.001
Type of infertility, n (%)					<0.001
Primary	18 363 (50.40%)	15 470 (49.29%)	2538 (57.51%)	355 (56.08%)	
Secondary	18 069 (49.60%)	15 916 (50.71%)	1875 (42.49%)	278 (43.92%)	
Main causes of infertility, n (%)					<0.001
Female factor	19 057 (52.31%)	16 046 (51.12%)	2585 (58.58%)	426 (67.30%)	
Male factor	6204 (17.03%)	5434 (17.31%)	681 (15.43%)	89 (14.06%)	
Male and female factors	9258 (25.41%)	8113 (25.85%)	1034 (23.43%)	111 (17.54%)	
Unexplained	1913 (5.25%)	1793 (5.71%)	113 (2.56%)	7 (1.11%)	
Number of oocytes retrieved	15.15 ± 8.55	14.80 ± 8.60	17.14 ± 7.80	18.49 ± 8.04	<0.001
Fertilization method, n (%)					<0.001
IVF	28 859 (79.21%)	24 753 (78.87%)	3575 (81.01%)	531 (83.89%)	
ICSI	6486 (17.80%)	5668 (18.06%)	719 (16.29%)	99 (15.64%)	
IVF + ICSI	1087 (2.98%)	965 (3.07%)	119 (2.70%)	3 (0.47%)	
Number of embryos transferred, n (%)					0.185
1	18 620 (51.11%)	16 101 (51.30%)	2206 (49.99%)	313 (49.45%)	
≥2	17 812 (48.89%)	15 285 (48.70%)	2207 (50.01%)	320 (50.55%)	
Development stage of embryo transferred, n (%)					<0.001
Cleavage embryo	10 630 (29.18%)	9543 (30.41%)	948 (21.48%)	139 (21.96%)	
Extended culture of cleavage embryos	2388 (6.55%)	1591 (5.07%)	617 (13.98%)	180 (28.44%)	
Blastocyst	23 414 (64.27%)	20 252 (64.53%)	2848 (64.54%)	314 (49.61%)	
Good-quality embryos transferred, n (%)					<0.001
0	9408 (25.82%)	7982 (25.43%)	1226 (27.78%)	200 (31.60%)	
1	19 115 (52.47%)	16 679 (53.14%)	2159 (48.92%)	277 (43.76%)	
2	7909 (21.71%)	6725 (21.43%)	1028 (23.29%)	156 (24.64%)	
Number of previous embryo transferred, n (%)					<0.001
0	23 160 (63.57%)	21 834 (69.57%)	1171 (26.54%)	155 (24.49%)	
1	9030 (24.79%)	7039 (22.43%)	1756 (39.79%)	235 (37.12%)	
≥2	4242 (11.64%)	2513 (8.01%)	1486 (33.67%)	243 (38.39%)	
Delivery achieved during this IVF/ICSI cycle					<0.001
Yes	2366 (6.49%)	273 (0.87%)	1733 (39.27%)	360 (56.87%)	
No	34 066 (93.51%)	31 113 (99.13%)	2680 (60.73%)	273 (43.13%)	
Endometrial preparation method, n (%)					<0.001
Natural	11 038 (30.30%)	9254 (29.48%)	1568 (35.53%)	216 (34.12%)	
Artificial	22 475 (61.69%)	19 538 (62.25%)	2563 (58.08%)	374 (59.08%)	
Others	2919 (8.01%)	2594 (8.26%)	282 (6.39%)	43 (6.79%)	
Thickness of the endometrium (mm)	8.84 ± 1.60	8.81 ± 1.59	9.02 ± 1.68	8.80 ± 1.52	<0.001
Survival rate (%)	58 300/59 406 (98.14%)	49 112 (98.15%)	7859/8012 (98.09%)	1329/1358 (97.86%)	0.698
Implantation rate (%)	20 366/54 244 (37.55%)	17 441/46 671 (37.37%)	2584/6620 (39.03%)	341/953 (35.78%)	0.017
Biochemical pregnancy, n (%)	1342 (3.68%)	1154 (3.68%)	164 (3.72%)	24 (3.79%)	0.981
Live birth, n (%)	13 651 (37.47%)	11 705 (37.29%)	1725 (39.09%)	221 (34.91%)	0.028
Multiple pregnancy, n (%)	3329 (19.23%)	2802 (18.84%)	464 (21.54%)	63 (22.11%)	0.005
Ectopic pregnancy, n (%)	253 (1.46%)	213 (1.43%)	36 (1.67%)	4 (1.40%)	0.695
Miscarriage, n (%)	3235 (18.68%)	2801 (18.83%)	377 (17.50%)	57 (20.00%)	0.285

FET, frozen embryo transfer.

22.11%, $P = 0.005$) showed statistically significant differences between the three groups. However, the survival rate (98.15%, 98.09%, and 97.86%), biochemical pregnancy rate (3.68%, 3.72%, and 3.79%), ectopic pregnancy rate (1.43%, 1.67%, and 1.40%), and miscarriage rate (18.83%, 17.50%, and 20.00%) were not significantly different among the three groups ($P > 0.05$).

Multivariate logistic regression analysis was performed after controlling for confounding factors. The impact of vitrification preservation time on pregnancy outcomes is shown in Table 2. In general, compared to the 0–2 years storage group, extending the vitrification preservation time (2–5 years or >5 years) of embryos

did not influence the risk of secondary outcomes, including biochemical pregnancy, multiple pregnancy, ectopic pregnancy, and miscarriage rates ($P > 0.05$). However, compared to the 0–2 years storage group, a preservation time of more than 5 years reduced the IR (aOR 0.82, 95% CI 0.69–0.97, $P = 0.020$) and the LBR (aOR 0.76, 95% CI 0.64–0.91, $P = 0.002$).

We further conducted a stratified logistic regression analysis based on the stages of embryo transfer (Table 3), and the results showed that frozen storage time did not affect the pregnancy outcomes of cleavage stage embryos, whether the transfer was straight after thawing or after extended culture after thawing.

However, the frozen storage time of blastocysts for more than 5 years was negatively associated with the IR (aOR 0.78, 95% CI 0.62–0.98, P = 0.033) and LBR (aOR 0.68, 95% CI 0.53–0.87, P = 0.002). We further conducted a stratified analysis of blastocysts based on factors that clearly affect IR and LBR, including transfer number and blastocyst quality. Statistical differences in IR and LBR were found in some, but not all, subgroups (Table 4). In general, the stratified analysis results showed that the LBR was significantly decreased in the group with a cryopreservation time greater than 5 years, whether it was a single (aOR 0.71, 95% CI 0.50–0.99, P = 0.047) or double (aOR 0.60, 95% CI 0.42–0.85, P = 0.004) blastocyst transfer. Good-quality blastocysts stored for more than 5 years were negatively affected in terms of the IR (aOR 0.74, 95% CI 0.58–0.95, P = 0.020) and LBR (aOR 0.70, 95% CI 0.53–0.93, P = 0.013). However, for non-good-quality blastocysts, there was no significantly association between storage times and pregnancy outcomes.

The neonatal outcomes of singletons after vitrified embryo transfer are shown in Table 5. The results showed that the vitrification preservation times of the embryos did not affect the preterm birth rates, fetal birth weight, or newborn sex ratio. However, compared to the 0–2 years storage group, the

gestational age of the 2–5 years storage group and >5 years storage group was significantly reduced (38.33 ± 1.82 weeks vs 38.07 ± 1.70 weeks and 37.53 ± 2.23 weeks, P < 0.001). As the storage time increased, the incidence of LGA was significantly increased (5.22%, 6.75%, and 9.47%, respectively; P < 0.01) and the incidence of SGA was significantly decreased (5.60%, 4.10%, and 1.18%, respectively; P < 0.01). After adjusting for a number of confounding factors, the increase of LGA and the decrease of SGA were significantly associated with the duration of storage.

Figure 1 shows a line graph of storage time and clinical outcomes. When the storage time exceeded five years, IR and LBR gradually decreased with the duration of embryo preservation. The IR and LBR with 2–5 years storage time were similar to those stored for <0.5 years, while the IR and LBR with a 1–2 years of storage time were lower than those stored for <0.5 years.

Discussion
Principal findings
This is a comprehensive assessment of the relationship between vitrified embryo cryopreservation time and clinical outcomes, exploring whether long-term embryo preservation poses additional risks to pregnancy and neonatal outcomes after FET. Our study found that when the vitrification cryopreservation time of blastocysts was longer than 5 years, the IR and LBR of blastocyst transfer decrease, while long-term cryopreservation of cleavage-stage embryos had no effect on IR and LBR. In the neonatal outcomes of singleton live births, as the cryopreservation time increased, the rates of SGA decreased while LGA rates increased.

Results and research implications
To obtain the first fetus, most individuals undergo embryo transfer within 2 years of oocyte retrieval. With the full implementation of the two-child policy in China, many individuals with early frozen embryos flocked to hospitals, hoping to achieve their desire to give birth again through FET. The first child of these individuals may be 2 years old or older, indicating that the remaining embryos were frozen for at least 2 years, with some frozen for 5 years or longer. Previous studies have shown that prolonged cryopreservation times not only increase chromosomal aberrations in embryos (Mozdarani and Moradi, 2007; Yan *et al.*, 2011), but also allow embryos to undergo irreversible damage caused by ionizing radiation and cosmic rays (Chen *et al.*, 2000). At low temperatures, enzymes cannot repair this damage, which may lead to DNA breakage or other harmful forms of DNA damage in the embryos. In addition, racemization of amino acids accumulated during long-term cryopreservation and DNA detoxification can

Table 2. Multivariate logistic regression analysis of the impact of storage time on pregnancy outcomes.

	P-value	aOR (95% CI)
Implantation rate		
0–2 years	ref.	
2–5 years	0.225	0.96 (0.89–1.03)
>5 years	0.020	0.82 (0.69–0.97)
Live birth rate		
0–2 years	ref.	
2–5 years	0.052	0.93 (0.87, 1.00)
>5 years	0.002	0.76 (0.64, 0.91)
Biochemical pregnancy rate		
0–2 years	ref.	
2–5 years	0.353	1.09 (0.91, 1.29)
>5 years	0.527	1.14 (0.75, 1.73)
Multiple pregnancy rate		
0–2 years	ref.	
2–5 years	0.786	1.02 (0.89, 1.17)
>5 years	0.465	1.14 (0.81, 1.59)
Ectopic pregnancy rate		
0–2 years	ref.	
2–5 years	0.07	1.12 (0.99, 1.27)
>5 years	0.314	1.17 (0.86, 1.61)
Miscarriage rate		
0–2 years	ref.	
2–5 years	0.324	1.07 (0.94, 1.21)
>5 years	0.089	1.31 (0.96, 1.8)

Table 3. Pregnancy outcomes and adjusted odds ratio after stratification analyses based on development stage of vitrified embryos transfer.

	Implantation rate	P-value	aOR (95% CI)	Live birth rate	P-value	aOR (95% CI)
Cleavage embryo (n = 10 630)	4899/17 567 (27.89%)			3065/10 630 (28.83%)		
0–2 years	4262/15 663 (27.21%)	ref.		2681/9543 (28.09%)	ref.	
2–5 years	558/1654 (33.74%)	0.433	1.06 (0.92–1.22)	334/948 (35.23%)	0.395	0.94 (0.81–1.09)
>5 years	79/250 (31.60%)	0.102	0.74 (0.52–1.06)	50/139 (35.97%)	0.414	0.86 (0.60–1.24)
Extended culture of cleavage embryos (n = 2388)	1252/3871 (32.3%)			802/2388 (33.58%)		
0–2 years	814/2757 (29.52%)	ref.		493/1591 (30.99%)	ref.	
2–5 years	349/877 (39.79%)	0.538	0.93 (0.74–1.17)	245/617 (39.71%)	0.483	1.08 (0.87–1.35)
>5 years	89/237 (37.55%)	0.650	0.92 (0.65–1.31)	64/180 (35.56%)	0.843	1.04 (0.73–1.48)
Blastocyst (n = 23 414)	14 215/32 977 (43.11%)			9784/23 414 (41.79%)		
0–2 years	12 365/28 251 (43.77%)	ref.		8531/20 252 (42.12%)	ref.	
2–5 years	1677/4089 (41.01%)	0.088	0.93 (0.85–1.01)	1146/2848 (40.24%)	0.156	0.94 (0.86–1.02)
>5 years	173/464 (37.28%)	0.033	0.78 (0.62–0.98)	107/314 (34.08%)	0.002	0.68 (0.53–0.87)

Table 4. Pregnancy outcomes and adjusted odds ratio based on the number and quality of vitrified blastocyst transplantation.

	Implantation rate	P-value	aOR (95% CI)	Live birth rate	P-value	aOR (95% CI)
Number of transplanted blastocysts						
Single blastocyst transferred (n = 14 024)	6989/14 024 (49.84%)			5590/14 024 (39.86%)		
0–2 years	6157/12 253 (50.25%)	ref.		4929/12 253 (40.23%)	ref.	
2–5 years	755/1607 (46.98%)	0.620	1.03 (0.92–1.16)	606/1607 (37.71%)	0.108	0.91 (0.80–1.02)
>5 years	77/164 (46.95%)	0.997	0.99 (0.76–1.38)	55/164 (33.54%)	0.047	0.71 (0.50–0.99)
Double blastocyst transferred (n = 9390)	7226/18 780 (38.48%)			4194/9390 (44.66%)		
0–2 years	6208/15 998 (38.80%)	ref.		3602/7999 (45.03%)	ref.	
2–5 years	922/2482 (37.15%)	0.098	0.90 (0.79–1.02)	540/1241 (43.51%)	0.223	0.92 (0.81–1.05)
>5 years	96/300 (32.00%)	0.008	0.64 (0.46–0.89)	52/150 (34.67%)	0.004	0.60 (0.42–0.85)
Quality of transplanted blastocysts						
Non-good-quality (n = 4511)	1751/6327 (27.68%)			1130/4511 (25.05%)		
0–2 years	1516/5438 (27.88%)	ref.		971/3858 (25.17%)	ref.	
2–5 years	213/825 (25.82%)	0.153	0.86 (0.70–1.06)	144/604 (23.84%)	0.135	0.85 (0.68–1.05)
>5 years	22/64 (34.38%)	0.238	1.44 (0.79–2.63)	15/49 (30.61%)	0.562	1.20 (0.64–2.25)
At least one good-quality (n = 18 903)	12 464/26 477 (47.07%)			8654/18 903 (45.78%)		
0–2 years	10 849/22 813 (47.56%)	ref.		7560/16 394 (46.11%)	ref.	
2–5 years	1464/3264 (44.85%)	0.434	0.96 (0.88–1.06)	1002/2244 (44.65%)	0.704	1.02 (0.91–1.15)
>5 years	151/400 (37.75%)	0.020	0.74 (0.58–0.95)	92/265 (34.72%)	0.013	0.70 (0.53–0.93)

Table 5. Neonatal outcomes of singleton live births after vitrified embryo transfer cycles.

						Group 2 vs Group 1		Group 3 vs Group 1	
Variable	All cycles	Group 1 0–2 years	Group 2 2–5 years	Group 3 >5 years	P-value	aOR(95% CI)	P-value	aOR(95% CI)	P-value
Single birth live babies, n (%)	10 759	9272	1318	169					
Gestational age (weeks)	38.29 ± 1.82	38.33 ± 1.82	38.07 ± 1.7	37.53 ± 2.23	<0.001				
Preterm birth, n (%)	920 (8.55%)	788 (8.50%)	114 (8.65%)	18 (10.65%)	0.606	0.93 (0.75–1.16)	0.529	1.15 (0.69–1.92)	0.601
Birth weight (g)	3214.13 ± 513.80	3218.06 ± 512.74	3219.51 ± 479.04	3199.02 ± 574.36	0.861				
LGA, n (%)	589 (5.47%)	484 (5.22%)	89 (6.75%)	16 (9.47%)	0.005	1.38 (1.09–1.75)	0.009	2.00 (1.17–3.43)	0.012
AGA, n (%)	9595 (89.18%)	8269 (89.18%)	1175 (89.15%)	151 (89.35%)	0.997	0.97 (0.80–1.19)	0.800	0.98 (0.59–1.61)	0.928
SGA, n (%)	575 (5.34%)	519 (5.60%)	54 (4.10%)	2 (1.18%)	0.004	0.69 (0.52–0.92)	0.013	0.20 (0.05–0.80)	0.023
Newborn gender, n (%)					0.908				
Male	5834 (54.22%)	5033 (54.28%)	708 (53.72%)	93 (55.03%)		1.03 (0.91–1.17)	0.653	1.16 (0.84–1.60)	0.354
Female	4925 (45.78%)	4239 (45.72%)	610 (46.28%)	76 (44.97%)					
Congenital defects, n (%)	70 (0.65%)	65 (0.60%)	5 (0.38%)	0					

LGA, large for gestational age; AGA, appropriate for gestational age; SGA, small for gestational age.

lead to cell damage (Kennedy *et al.*, 1994). Therefore, to provide patients with safe and satisfactory FET results, it is necessary to understand the effect of embryo freezing times exceeding 2 years or even 5 years on pregnancy and newborn outcomes.

Almost all studies on human embryo vitrification have been conducted within a cryopreservation period of less than 2 years (Li *et al.*, 2020; Zhang *et al.*, 2021; Mao *et al.*, 2022) or 5 years (Li *et al.*, 2017; Ueno *et al.*, 2018), and the impact on reproductive outcomes is not consistent. In addition, clinical pregnancy and LBRs are influenced by double embryo transfer, and therefore IRs can better reflect the potential of embryos. Therefore, it was necessary to conduct a comprehensive analysis of long-term embryo freezing, including implantation and LBRs, as well as known factors that affect clinical outcomes, such as embryo stage, number, and quality, in order to better solve problems in clinical practice. Our study included 36 665 freeze-thaw cycles, aiming to evaluate the effect of vitrification preservation time on cleavage-stage embryos and blastocysts, in order to provide clinical evidence.

We studied embryos that had been cryopreserved for more than 5 years, and found that when the embryo cryopreservation time

exceeded 5 years, IR and LBR decreased significantly. However, IR and LBR did not decrease in the 2–5 years storage group, which is different from the results of previous studies showing that clinical outcomes decreased with the extension of embryo cryopreservation time (Li *et al.*, 2020; Hu *et al.*, 2022; Mao *et al.*, 2022). This may be related to the short duration of embryo cryopreservation (mostly within 2 years) in previous studies, which included patients who underwent their first vitrified embryo transfer following a freeze-all strategy or fresh transfer. If these patients failed to undergo their first transfer or to conceive within 1 year, it indicates that other medical factors may be present that are more likely to reduce clinical outcomes than extended cryopreservation (1–2 years). This is consistent with our line chart, where the IR and LBR of embryos frozen for 1–2 years decreased compared to those of embryos frozen within 1 year. The IR and LBR of embryos frozen for 2–5 years increased to levels similar to those frozen for 0.5 years. Therefore, a survey questionnaire can be considered to confirm the reasons for delayed transfer in patients.

We found that with prolonged cryopreservation times, the proportion of patients undergoing their first transfer significantly
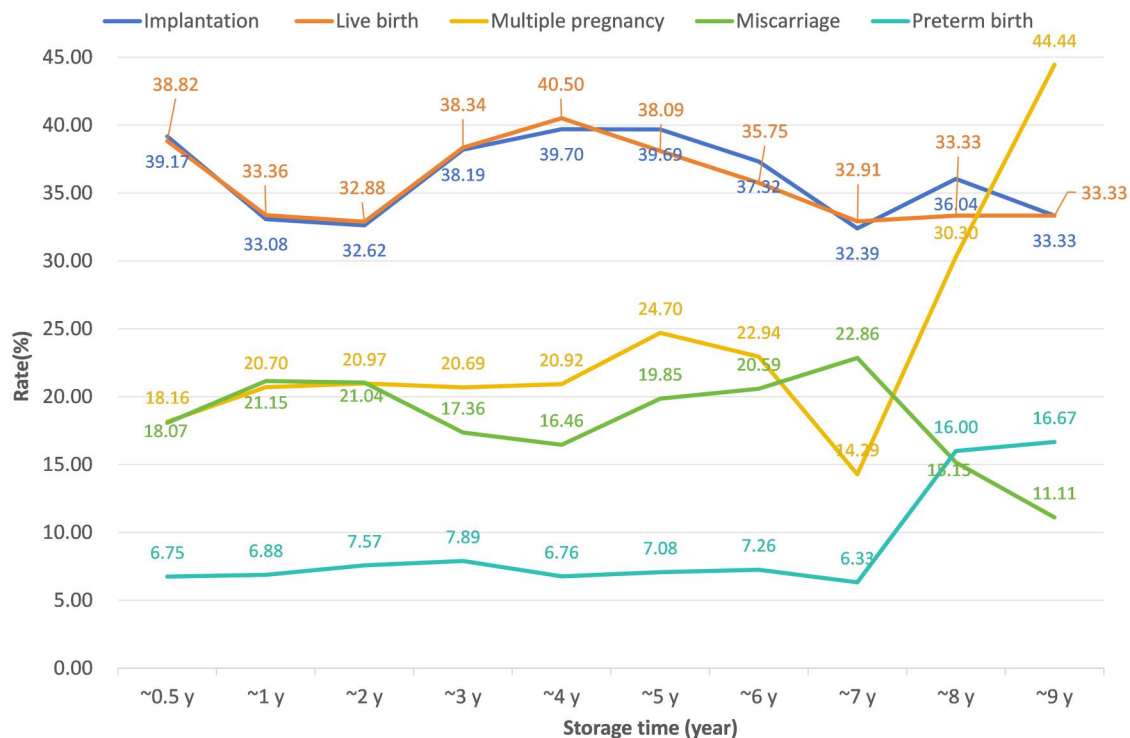
Figure 1. Line chart showing various clinical outcome indicators changing with embryo storage time. The horizontal axis represents the storage time of frozen-thawed embryos in FET cycles, while the vertical axis indicates the percentage of clinical indicators corresponding to each time interval, including the implantation and live birth rates, as well as the multiple pregnancy, miscarriage, and preterm birth rates.

decreased (69.57%, 26.54%, and 24.49%, respectively) (Table 1). This difference may be due to the different proportion of patients who had already given birth among the three groups. Only 0.87% of patients in the <2 years storage group had already given birth through a previous transfer, while 39.27% and 56.87% of patients in the 2–5 and >5 years storage groups had already given birth and wished to conceive again (Table 1). Therefore, successful transfer and the need for a second child led to long-term freezing and subsequent thawing of the embryos. We adjusted for multiple confounding factors, including the number of previous embryos transferred, and still found that long-term freezing for >5 years storage time would reduce IR and LBR.

Embryonic stage has been documented to affect the LBR (Zhang *et al.*, 2021; Glujovsky *et al.*, 2022). Considering the possibility of confounding factors affecting the results, hierarchical logistic regression analyses were performed. Previous studies had shown that there was no significant difference in FET results after long-term cryopreservation of cleavage stage embryos, but they were all small-scale studies (Li *et al.*, 2017, 2023) or only analyzed the preservation time within 2 years (Zhang *et al.*, 2021). Our research indicates that there is no significant difference in IR and LBR for cleavage stage embryo transfer after long-term cryopreservation. This may be related to changes in clinical practice. Prior to 2018, embryologists selected multiple high-quality day-3 embryos for cryopreservation in order to transfer two times, and the surplus embryos from the same batch were frozen after blastocyst culture. After 2018, embryologists only guaranteed a maximum of one opportunity for day-3 embryo transfer or freezing, and the surplus embryos or all embryos were cultured into blastocysts and then frozen. When thawing, blastocysts were preferred, so this clinical change may have led to the existence of potential residual confounding factors. In addition, some patients undergo extended culture of their cleavage embryos

after thawing to further screen the embryos, resulting in changes in clinical outcomes.

After stratifying the embryonic stages, we found a significant negative association between cryopreservation of blastocyst-stage embryos for more than 5 years and pregnancy outcomes, especially for good-quality blastocysts. The conclusions of most studies on the cryopreservation time of blastocysts have been inconsistent. Some studies have shown that long-term cryopreservation does not affect clinical outcomes, but the sample size was very small (Li *et al.*, 2023), closed carriers were used, and confounding factors were not adjusted for Wirleitner *et al.* (2013), or a large time span of 2–7 years was used as the long-term cryopreservation group (Ueno *et al.*, 2018; Ma *et al.*, 2023). On the contrary, other studies have suggested that long-term cryopreservation of vitrified blastocysts might have a negative impact on pregnancy outcomes, but there were still some biases, such as small sample size (Zheng *et al.*, 2023) and preservation times within 2 years (Zhang *et al.*, 2021). The theoretical risk of vitrification is the use of very high concentrations of cryoprotectants, which may lead to osmotic damage and biochemical cytotoxicity (Nagy *et al.*, 2020). Secondly, during long-term cryopreservation, the viability of embryos may be affected by temperature fluctuations and radiation. Improper maintenance of the cryo-tanks, failure to maintain sufficient liquid nitrogen levels, repeated opening of the cryo-tanks, or movement of the samples, may cause the embryo may experience transient warming during these processes, which may affect the developmental potential of the embryos. Although the incidence rate is low, these risks may increase with the extension of frozen storage time. In addition, studies have shown that vitrification freezing can reduce mouse embryo viability and increase chromosomal aberrations, depending on the duration of cryopreservation (Mozdarani and Moradi, 2007; Yan *et al.*, 2011). Another study suggests that repeated fluctuations in temperature to −80°C can lead to a

significant decrease in the number, activity, and metabolic characteristics of placental pluripotent stromal cells after thawing, as well as an increase in apoptosis (Pogozhykh *et al.*, 2017).

It is worth noting that we observed a significant increase in the rate of multiple pregnancies due to the transfer of more than one embryo in patients who were at least 5 years older than when their embryos were frozen. This may be related to the decrease in LBRs. Therefore, we performed a stratification on the number of transferred embryos to further analyze the impact of long-term cryopreservation on LBRs. We found that the LBR was still significantly decreased in patients who were transferred with a single blastocyst that had been cryopreserved for over 5 years. In addition, compared to embryos frozen for 5 years (line chart in Fig. 1), the LBR of embryos frozen for 6–7 years was also decreased, even without an increase in the rate of multiple pregnancies. This might exclude the potentially confounding probability of the multiple pregnancy *per se* on the LBR. Although patients with an embryo storage period of 8–9 years showed a high multiple pregnancy rate, premature birth rate, and low LBR, the number of patients in these categories was relatively small and therefore further research is needed. These results may indicate the potential risks of long-term cryopreservation of the blastocysts.

Consistent with previous research findings, our study showed that, compared to a preservation time of less than 2 years, a preservation time of 2–5 years had no effect on the pregnancy outcomes of cleavage embryos (Li *et al.*, 2017), nor did it affect the pregnancy outcomes of blastocysts (Ma *et al.*, 2023). This also indicates that vitrification cryopreservation of embryos or blastocysts for less than 5 years is relatively safe. The decline in fecundity is attributed to a lower oocyte yield and, most importantly, higher oocyte aneuploidy rates in older women (Franasiak *et al.*, 2014). There is no doubt that long-term preservation can reduce the negative impact of increased age at oocyte retrieval on pregnancy outcomes and improve cumulative outcomes (Loreti *et al.*, 2024). Therefore, when providing medical consultation to patients, it is necessary to inform them that cryopreservation of embryos for more than 5 years is more effective compared to an increase in oocyte retrieval age, but it is still recommended to thaw and transfer embryos as soon as possible within 5 years to achieve a live birth, rather than relying on medical technology to delay childbirth.

At present, there is limited data on the impact of cryopreservation time on perinatal outcomes, mainly focusing on average gestational age and weight, and it is believed that it will not affect neonatal outcomes (Li *et al.*, 2017; Ma *et al.*, 2023). Similar to these studies, our results showed no significant differences in the preterm birth rates, birth weight, or newborn sex ratio among the groups. However, it is noteworthy that the 2–5 years storage group and the >5 years storage group had an increased incidence of LGA and decreased incidence of SGA compared with the 0–2 years storage group, which was used as the control group. This is consistent with previous studies (Cui *et al.*, 2021; He *et al.*, 2023). The underlying mechanisms of these changes are still largely unclear and speculated to be related to modifications in the embryonic epigenome (Ishihara *et al.*, 2014), which requires further research. Interestingly, we also found that the gestational age of newborns in the 2–5 years storage group and the >5 years storage group decreased with the duration of embryo cryopreservation compared with the 0–2 years storage group, but the average gestational age still exceeded 37 weeks (Ueno *et al.*, 2018).

This study has several advantages. Firstly, due to the large sample size, it reports results with a storage time of >5 years, providing more evidence for the safety of long-term storage. Secondly, this study included as many known factors as possible related to pregnancy outcomes to ensure a more objective analysis. In addition, the stage, number, and quality of embryos transferred have been shown to be associated with FET outcomes (Zhu *et al.*, 2020). This study conducted a comprehensive stratified analysis based on these factors to further explore the impact of cryopreservation time on reproductive outcomes in different subgroups.

However, there are also some limitations. Firstly, owing to the retrospective nature and acquisition of data from a single fertility center, inevitable selection bias persists. Secondly, although the sample size of the cryopreserved embryo group over a longer time span is larger compared to previous studies, it remains relatively small, which affects further stratified analysis, such as combining the number and quality of blastocysts. Thirdly, although the freezing and thawing procedures in the laboratory have not changed, personnel turnover may have an impact on the outcome. Lastly, we did not conduct a long-term follow-up of children born after FET treatment, limiting our ability to provide more convincing evidence regarding potential effects on offspring development.

Conclusion

In conclusion, the duration of vitrification preservation did not affect the IR and LBR of cleavage stage embryos, however, good-quality blastocyst vitrification preservation for >5 years was associated with a lower IR and LBR. It is important to note that this study still has some limitations that warrant cautious interpretation.

Data availability

The data will be available on reasonable request to the corresponding author.

Acknowledgements

Authors' roles

S.Z. and C.L. analyzed the data and drafted the manuscript; Q.L. selected the data and revised the manuscript; J.G., C.W., and Y.L. selected the data and provided critical feedback on the content; J.L. revised the manuscript and participated in the interpretation of results and discussion; H.D. and H.L. designed the study and revised the manuscript. All authors approved the final version of the manuscript for publication.

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Conflict of interest

None declared.

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