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The effect of storage time after vitrification on pregnancy and neonatal outcomes among 24 698 patients following the first embryo transfer cycles

Jianghui Li†, Mingru Yin†, Bian Wang†, Jiaying Lin, Qiuju Chen, Ningling Wang, Qifeng Lyu, Yun Wang, Yanping Kuang*, and Qianqian Zhu*

Department of Assisted Reproduction, Shanghai Ninth people's hospital affiliated to JiaoTong University School of Medicine, Shanghai, China

*Correspondence address. Department of Assisted Reproduction, Shanghai Ninth People's hospital affiliated to JiaoTong University School of Medicine, Zhizaoju Road No. 639, Shanghai, China. E-mail: kuangyanp@126.com (Y.K.); qianqianzhu1988@126.com (Q.Z.)

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STUDY QUESTION: To evaluate the impact of storage time after vitrification on embryo viability, pregnancy outcomes and neonatal outcomes.

SUMMARY ANSWER: The prolonged storage time of vitrified embryos negatively affected pregnancy outcomes, including biochemical pregnancy rate, clinical pregnancy and live birth rate; but did not influence neonatal outcomes.

WHAT IS KNOWN ALREADY: Although vitrification has been the fundamental tool of ART treatments in recent years, few studies have explored the influence of storage period after vitrification on embryonic and clinical outcomes.

STUDY DESIGN, SIZE, DURATION: A retrospective study was performed among 24 698 patients with the first vitrified embryo transfer following a freeze-all strategy during the period from January 2011 to December 2017.

PARTICIPANTS/MATERIAL, SETTING, METHODS: A total of 24 698 patients met the inclusion criteria and were grouped according to the storage time (11 330 patients in Group 1 with storage time <3 months, 9614 patients in Group 2 with storage time between 3 and 6 months, 3188 patients in Group 3 with storage time between 6 and 12 months and 566 in Group 4 with storage time between 12 and 24 months). The pregnancy outcomes and neonatal outcomes were compared between different storage time groups. Multivariate logistic regression and linear regression were performed to evaluate the independent effect of storage time on clinical outcomes, adjusting for important confounders.

MAIN RESULTS AND THE ROLE OF CHANCE: After adjustment for potential confounding factors, the chance of biochemical pregnancy (Group 1 as reference; Group 2: adjusted odds ratio (aOR) = 0.92, 95% CI 0.87–0.97; Group 3: aOR = 0.83, 95% CI 0.76–0.90; Group 4: aOR = 0.68, 95% CI 0.56–0.81), clinical pregnancy (Group 2: aOR = 0.91, 95% CI 0.86–0.96; Group 3: aOR = 0.80, 95% CI 0.73–0.87; Group 4: aOR = 0.65, 95% CI 0.54–0.79) and live birth (Group 2: aOR = 0.89, 95% CI 0.85–0.95; Group 3: aOR = 0.83, 95% CI 0.76–0.91; Group 4: aOR = 0.59, 95% CI 0.48–0.72) significantly decreased with the increasing storage time, whereas the relationship between miscarriage, ectopic pregnancy and storage time did not reach statistical significance. In addition, there was no evidence of differences in adverse neonatal outcomes (preterm birth, low birthweight, high birthweight, macrosomia or birth defects) between groups.

LIMITATION, REASONS FOR CAUTION: Our study was limited by the retrospective design from a single center, the conclusion from our study needs to be verified in further studies.

†These authors contributed equally to this work.

WIDER IMPLICATIONS OF THE FINDINGS: This study provides new findings about the relationship between prolonged storage time of vitrified embryos and clinical outcomes and offers evidence for the safety of using long-stored embryos after vitrification.

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Key words: frozen embryo transfer / storage time / live birth rate / neonatal outcomes / vitrification

Introduction

Since the first report on successful clinical pregnancy following the transfer of a frozen embryo in 1983, the cryopreservation of embryos *in vitro* has increasingly been used in ART (Trounson and Mohr, 1983). The use of this technology improved the cumulative live birth rate because it allowed multiple embryo transfers with surplus frozen embryos from a single ovarian stimulation cycle, decreased the risk of ovarian hyperstimulation syndrome (OHSS) by delaying the embryo transfer and minimized the multiple pregnancy rate in patients without the need for transfer of multiple embryos simultaneously (Wirleitner *et al.*, 2013; Zhu *et al.*,2018). In addition, it can be used in patients who are not indicated for fresh embryo transfer because of uterine receptivity or preimplantation genetic diagnosis and in fertility preservation for patients with cancer or diminished ovarian reserve related to some gynecological diseases or aging (Liebermann, 2017).

With the rapid development of cryopreservation techniques, embryo vitrification has been the fundamental tool of ART treatments in recent years at the expense of the traditional slow-freezing method (Edgar and Gook, 2012). In contrast to slow freezing, vitrification is a kind of fast cryopreservation method that allows the solidification of the cell(s) and extracellular milieu into a glass-like state without ice crystal formation, which avoids damage to the cells (Rienzi *et al.*, 2017). The successful completion of this process requires a high concentration of cryoprotectants and ultra-rapid cooling-warming rates (Konc *et al.*, 2014). In routine practice, vitrification is performed by a short exposure (<1 min) of embryos to a high concentration of cryoprotectants, followed by loading on to a variety of microtools in a very small volume (0.1–2 µl) and immediate exposure to liquid nitrogen to achieve rapid cooling rates (Edgar and Gook, 2012). Rapid warming is achieved by rehydration in decreasing concentrations of cryoprotectant.

Embryo vitrification has proven to be a more effective alternative than slow freezing, not only because it is a simple, inexpensive and faster technique but also because it has higher survival rates and better clinical outcomes (Loutradi *et al.*, 2008; AbdelHafez *et al.*, 2010; Edgar and Gook, 2012). Consequently, the number and storage time of cryopreserved embryos has increased gradually. However, vitrification also raises concern over the potential toxic effects of the procedure related to exposure to higher concentrations of cryoprotectants and potential contamination by liquid nitrogen contact (Gosden, 2011). Whether vitrification affects embryo viability and subsequent implantation potential is still controversial (Testart *et al.*, 1987; Wirleitner *et al.*, 2013; Cobo *et al.*, 2015; Ueno *et al.*, 2018). The maximum length of embryo cryostorage that does not affect the embryonic or clinical outcomes is unknown. There are few studies exploring the influence of storage time on embryo survival and pregnancy outcomes, and they have had small sample sizes, only used the slow-freezing method, only involved cleavage-stage embryos or blastocysts, or did not consider embryo quality and neonatal outcomes (Quintans *et al.*, 2002; Revel *et al.*, 2004; Riggs *et al.*, 2010; Dowling-Lacey *et al.*, 2011; Wirleitner *et al.*, 2013; Liu *et al.*, 2014; Ueno *et al.*, 2018; Yuan *et al.*, 2019).

More than 60 000 frozen-thawed embryo transfer (FET) cycles were performed in our reproduction center since the initiation of our vitrification technique. In this study, we explored the impact of storage time on embryo viability, pregnancy outcomes and neonatal outcomes among 24 698 patients with the first vitrified embryo transfer cycles following a freeze-all strategy during the period from January 2011 to December 2017.

Materials and methods

Study design and participants

Based on the clinical application of vitrification technology since 2007, a freeze-all strategy was implemented in more than 85% of IVF cycles from 2011 to 2017 at the reproduction center of the Shanghai Ninth People's Hospital affiliated with Jiao Tong University School of Medicine (a large hospital-based tertiary care reproductive center in Shanghai, China). Specifically, the freeze-all strategy was performed in patients with high risk of developing OHSS, patients of advanced maternal age, patients with diminished ovarian reserve, patients with polycystic ovary syndrome or poor ovarian responders. In addition, patients with other infertility causes, such as tubal factor, endometriosis or male factor, may also be submitted to the freeze-all strategy based on each patient's preference or physician clinical expertise. This retrospective cohort study included 24 698 women undergoing their first FET cycles with autologous oocytes following the freeze-all strategy during the period from January 2011 to December 2017. Each woman was included only once in this study, and those having previous fresh or frozen embryo transfer were excluded. Women with no viable embryos available for transfer after vitrification were excluded. Women were excluded if they had mixed cleavage-stage embryo-blastocyst transfer or if they underwent PGD.

Procedures

Our previous studies have described in detail the ovulation induction, IVF/ICSI procedure, embryo culture, freezing, thawing and transfer (Kuang *et al.*, 2014a,b; Chen *et al.*, 2015; Du *et al.*, 2017). IVF or ICSI was performed depending on the semen quality. Normal fertilization was assessed 16–18 h after insemination/injection. Then the embryos were cultured with continuous single-culture medium (Irvine Scientific, USA) in a water-jacketed box incubator (Astec, Japan) at 37°C, under

5% CO_2 and 5% O_2 concentration throughout the entire developmental stage.

Embryos on Day 3 were graded according to Cummin's criteria (Cummins *et al.*, 1986; Reinblatt *et al.*, 2011). Grade I and II embryos were classified as good quality and cryopreserved by vitrification. The remaining embryos with poor quality were extendedly cultured and evaluated until the blastocyst stage. The Gardner classification system was used to evaluate the blastocyst quality (Gardner *et al.*, 2004). Only blastocysts better than grade 3CC were used for vitrification. Embryo grading was done by two trained embryologists and was verified by another senior embryologist with years of work experience.

The vitrification procedure was performed with a vitrification kit (Kitazato Company, Japan) according to the description by Kuwayama *et al.* (2005). Initially, the embryos were exposed to an equilibration solution containing the basic medium (mHTF medium, Irvine Scientific, USA)with 7.5% (v/v) ethylene glycol (EG), 7.5% dimethysulfoxide (DMSO) and 20% synthetic serum substitute (SSS) for 5–15 min at room temperature. After that, the embryos were transferred to a vitrification solution that consisted of mHTF medium with 15% EG, 15% DMSO, 0.5 mol/l sucrose and 20% SSS for 1 min. Finally, embryos were set on a Cryotop strip (Kitazato Company, Japan) in a small volume and were plunged into liquid nitrogen as soon as possible. The embryologists have been well trained to perform vitrification technically. The embryos were stored at a constant temperature of −196°C in the liquid phase of liquid nitrogen tank (Taylor Wharton HC35, Theodore, AL, USA), which has a liquid nitrogen capacity of 35 l. The level of liquid nitrogen is kept under constant surveillance manually by highly skilled embryologists to prevent suboptimal storage conditions, and the liquid nitrogen tank is refilled manually twice a week.

For the thawing process of vitrified embryos, the embryos unloaded from the carriers were immediately submerged into the thawing solution containing the basic medium with 1.0 mol/l sucrose and 20% SSS for 1 min at 37°C. Then, the embryos were transferred into the diluent thawing solution (basic medium with 0.5 mol/l sucrose and 20% SSS) for 3 min at room temperature. At the final step, the embryos were moved to the wash solution (basic medium with 20% SSS) twice for 5 min at room temperature. After that, the embryos were cultured in culture medium with 10% SSS at 37°C under the gas phase of 5% CO_2 and 5% O_2 in an incubator (Astec, Japan) until transfer. The same vitrification and thawing method was employed throughout the whole study period.

Endometrial preparation was performed as previously described (Du *et al.*, 2017). A natural cycle was used for patients with regular menstrual cycles, and a hormone therapy cycle or stimulation cycle was used for patients with irregular menstrual cycles. The allocation to blastocyst or cleavage-stage embryo transfer depended on the patient's age and the quality and number of embryos available. One or two embryos were transferred, and progesterone supplementation was provided until 8 weeks of gestation if pregnancy resulted. In order to accurately classify treatment outcomes, all patients were required to measure serum beta-HCG levels 14 days after embryo transfer in our center. The patients underwent a vaginal ultrasound examination approximately 35 days after embryo transfer if they had positive results in the serum beta-HCG test.

Patients were divided into four categories according to the storage time of transferred embryos: Group 1, storage time 0–3 months; Group 2, storage time 3–6 months; Group 3, storage time 6–12 months; and Group 4, storage time 12–24 months. The survival rate was defined as the number of survived embryos divided by the number of thawed embryos per cycle. The implantation rate was measured as the number of gestational sacs detected through vaginal ultrasound examination around 7 weeks of pregnancy divided by the number of embryos transferred, calculated for each patient.

The serum beta-HCG pregnancy test was performed on the 14th day after embryo transfer. Clinical pregnancy was defined as the observation of at least one gestational sac through vaginal ultrasound examination approximately 35 days after embryo transfer. Ectopic pregnancy was defined as at least one extrauterine cavity gestational sac. In this study, we classified heterotopic pregnancy in the group of ectopic pregnancies. The miscarriage rate was calculated as the number of clinical pregnancy losses divided by the number of clinical pregnancies. A live birth was defined as an infant born alive after 24 weeks of gestation who survived more than 28 days. Delivery of twins or higher-order-multiple births was counted as one live birth. Only singletons were included to evaluate the association of storage time with neonatal outcomes. The neonatal outcomes were gestational age, birth weight, preterm birth (PTM, <37 weeks' gestation), low birth weight (<2500g at birth), high birth weight (>4000g at birth) and macrosomia (birth weight >4500g at birth). Birth defects were defined according to the International Classification of Diseases, 10th Revision (ICD-10), and a detailed description can be found in our previously published paper (Zhu *et al.*, 2018).

Statistical analysis

The baseline characteristics and clinical outcomes are described as mean ± SD for continuous variables and as frequency with proportion for categorical variables. The differences between groups were tested using the ANOVA test for continuous variables and the Pearson's chi-square test for categorical variables. Multivariable logistic regression was performed to explore the effect of storage time on pregnancy outcome or neonatal outcome after controlling for potential confounders, including maternal age, maternal BMI, infertility type, parity, infertility causes, embryo quality, number of transferred embryos, stage of embryo development, endometrial preparation program and treatment years. Storage group was included as a categorical variable, and Group 1 was used as the reference. The results were reported as adjusted odds ratios (aORs) with 95% CIs. Multivariable linear regression was performed to investigate the association of storage time with gestational age or birth weight, adjusting for the same confounding factors as for the multivariable logistic regression. All statistical analyses were performed by using the two-sided 5% level of significance in the statistical package Stata, Version 12 (StataCorp, College Station, TX, USA).

Results

A total of 24 698 patients undergoing the first frozen embryo transfer met the inclusion criteria and were grouped according to the storage time (11 330 patients in Group 1 with storage time <3 months, 9614 patients in Group 2 with storage time between 3 and 6 months, 3188 patients in Group 3 with storage time between 6 and 12 months and 566 in Group 4 with storage time between 12 and 24 months). Maternal and treatment characteristics across storage time groups are

Table I Maternal and treatment characteristics of the frozen embryo transfer cycles, stratified by the storage time.

	Storage groups				P-value		
	1	2	3	4	P_1	P_2	P_3
Storage time (months)	(0–3)	(3–6)	(6–12)	(12–24)			
Storage time (days), mean ± SD	63.04 ± 13.75	128.26 ± 24.17	235.19 ± 46.99	468.21 ± 91.91			
Number of FET cycles	11 330	9614	3188	566			
Maternal age (years), mean ± SD	30.84 ± 4.31	31.68 ± 4.72	33.55 ± 5.25	35.59 ± 5.67	<0.001	<0.001	<0.001
Maternal BMI, mean ± SD	21.65 ± 2.94	21.72 ± 2.93	21.79 ± 3.01	21.86 ± 3.15	0.773	0.149	0.664
Type of infertility, *n* (%)					<0.001	<0.001	<0.001
Primary infertility	6781 (59.85)	4825 (50.19)	1491 (46.77)	260 (45.94)			
Second infertility	4549 (40.15)	4789 (49.81)	1697 (53.23)	306 (54.06)			
Parity, *n* (%)					<0.001	<0.001	<0.001
Nulliparous	10 307 (90.97)	8507 (88.49)	2755 (86.42)	486 (85.87)			
Pluriparous	1023 (9.03)	1107 (11.51)	433 (13.58)	80 (14.13)			
Infertility causes, *n* (%)							
Tubal infertility	7749 (68.39)	7128 (74.14)	2318 (72.71)	429 (75.80)	<0.001	<0.001	<0.001
PCOS	1138 (10.04)	921 (9.58)	232 (7.28)	31 (5.48)	0.261	<0.001	<0.001
DOR	433 (3.82)	436 (4.54)	294 (9.22)	75 (13.25)	0.010	<0.001	<0.001
Endometriosis	859 (7.58)	902 (9.38)	461 (14.46)	87 (15.37)	<0.001	<0.001	<0.001
Uterine factor	887 (7.83)	1072 (11.15)	454 (14.24)	90 (15.90)	<0.001	<0.001	<0.001
Unexplained infertility	403 (3.56)	244 (2.54)	65 (2.04)	13 (2.30)	<0.001	<0.001	0.111
Male factor	4197 (37.04)	3241 (33.71)	1057 (33.16)	175 (30.92)	<0.001	<0.001	0.003
Fertilization method, *n* (%)					<0.001	<0.001	<0.001
IVF	6883 (60.75)	6419 (66.77)	2140 (67.13)	403 (71.20)			
ICSI	2805 (24.76)	2127 (22.12)	798 (25.03)	135 (23.85)			
IVF+ICSI	1642 (14.49)	1068 (11.11)	250 (7.84)	28 (4.95)			
Number of embryos transferred, *n* (%)					0.663	<0.001	<0.001
1	1408 (12.43)	1214 (12.63)	656 (20.58)	177 (31.27)			
2	9922 (87.57)	8400 (87.37)	2532 (79.42)	389 (68.73)			
Embryo quality at transfer, *n* (%)					0.611	<0.001	<0.001
Good-quality embryo	10 954 (96.68)	9307 (96.81)	3038 (95.29)	521 (92.05)			
Poor-quality embryo	376 (3.32)	307 (3.19)	150 (4.71)	45 (7.95)			
Development stage of embryos transferred, *n* (%)					0.403	0.069	0.032
Cleavage stage	10 521 (92.86)	8956 (93.16)	2930 (91.91)	512 (90.46)			
Blastocyst	809 (7.14)	658 (6.84)	258 (8.09)	54 (9.54)			
Endometrial preparation program, *n* (%)					<0.001	<0.001	<0.001
Natural cycle	3525 (31.11)	2331 (24.25)	692 (21.71)	138 (24.38)			
Stimulated cycle	5864 (51.76)	3921 (40.78)	1152 (36.14)	173 (30.57)			
Hormonal replacement cycle	1941 (17.13)	3362 (34.97)	1344 (42.16)	255 (45.05)			
Year of treatment, *n* (%)					<0.001	<0.001	<0.001
2011–2013	4114 (36.31)	2111 (21.96)	576 (18.07)	79 (13.96)			
2014–2015	3195 (28.20)	4038 (42.00)	1203 (37.74)	198 (34.98)			
2016–2017	4021 (35.49)	3465 (36.04)	1409 (44.20)	289 (51.06)			
Number of oocytes retrieved, mean ± SD	11.82 ± 7.75	10.69 ± 7.35	8.40 ± 7.09	6.38 ± 6.39	<0.001	<0.001	<0.001
Number of embryos cryopreserved, mean ± SD	4.83 ± 3.07	4.47 ± 2.86	3.55 ± 2.67	2.84 ± 2.40	<0.001	<0.001	<0.001
Number of embryos warmed, *n* (mean/cycle)	21 388 (1.89)	18 111 (1.88)	5743 (1.80)	965 (1.70)			
Number of embryos transferred, *n* (mean/cycle)	21 252 (1.88)	18 014 (1.87)	5720 (1.79)	955 (1.69)			

BMI, body mass index; FET, frozen-thawed embryo transfer; PCOS, polycystic ovary syndrome; DOR, declined ovarian reserve.
P_1: Group 2 vs. Group 1, P_2: Group 3 vs. Group 1, P_3: Group 4 vs. Group 1.

shown in Table I. Maternal age at embryo transfer increased with longer storage time ($P < 0.001$). No difference was seen in maternal BMI across all storage time groups. The proportion of primary infertility and nulliparity decreased with increasing storage time. The main infertility causes were tubal infertility, followed by male infertility, and IVF was the major fertilization method across four groups. The proportion of patients with declined ovarian reserve (DOR), endometriosis or uterine factor was gradually rising with the extended storage time. More than 90% of patients transferred good-quality embryos and transferred embryos at the cleavage stage in all four groups. The proportion of good-quality embryos transferred was lower in Groups 3 and 4 compared with Group 1. More patients underwent blastocyst transfer in Group 4 than in Group 1. The mean number of oocytes retrieved and the mean number of embryos cryopreserved before the first FET cycle was fewer in Groups 2, 3 and 4 compared with Group 1.

Figure 1 presents the pregnancy outcomes per FET cycle for the four storage time groups. There was no significant difference in the survival rate between groups. The rates of implantation, positive HCG, clinical pregnancy, multiple pregnancies, live birth and multiple live births declined with prolonged storage time. Specifically, the implantation rate ranged from 39.84% for storage times <3 months to 25.88% for storage times between 12 and 24 months. The clinical pregnancy rate significantly decreased with increasing storage time from 55.60% in Group 1 (embryo storage <3 months) to 25.80% in Group 4 (storage period between 12 and 24 months). The live birth rate fell from 47.16% in Group 1 to 25.80% in Group 4. The miscarriage rate and ectopic pregnancy rate increased with longer storage time; however, statistical significance was only found for the miscarriage rate.

The results of multivariable logistic analysis of pregnancy outcomes are shown in Table II. After adjustment for potential confounding factors, the chance of positive HCG, clinical pregnancy, multiple pregnancies, live birth and multiple live births was still significantly reduced with increasing storage time, whereas the relationship between miscarriage, ectopic pregnancy and storage time did not reach statistical significance.

Among all singletons born after FET, the neonatal outcomes across the four storage groups were analyzed (Table III). The sex ratio was similar between groups. No differences were observed across groups in terms of gestational age and birth weight. There was no evidence of differences in adverse neonatal outcomes between the groups in terms of preterm birth, low birth weight, high birth weight, macrosomia and birth defects. Results from the multivariable logistic regression exploring the relationship of adverse neonatal outcomes with storage are shown in Fig. 2. The risk of adverse neonatal outcomes, including preterm, low birth weight, high birth weight and birth defects, did not change significantly with the length of storage time, adjusting for a number of confounding factors. The multivariable linear regression also showed that the gestation age and birth weight were not significantly related to storage time (Table IV).

Considering the increasing proportion of older patients and patients with diminished ovarian reserve in Groups 3 and 4, we repeated the analysis in the subset of patients with age <36 years and the diagnosis of tubal infertility only. The results were consistent with the analysis among the whole population.

Discussion

Principal findings

Given the gradual increase in the number and time of cryopreserved embryos with vitrification, it is important to understand the influence of extended storage time on clinical outcomes. Our study demonstrated the safety of using long-stored embryos on neonatal health. As one of the larger retrospective cohort studies, our study suggests that although the storage time of vitrified embryos negatively affected pregnancy outcomes, including implantation rate, positive HCG rate, clinical pregnancy and live birth rate, neonatal outcomes were not influenced by storage time.

Results and research implications

Concerns have arisen over the safety of prolonged storage time of vitrified embryos worldwide following the wide application of vitrification. Some animal studies have been conducted to investigate the impact of extended storage time on embryos after vitrification. The results from Mozdarani and Moradi (2007) indicated that the viability of mouse embryos decreased and the chromosome abnormalities increased with

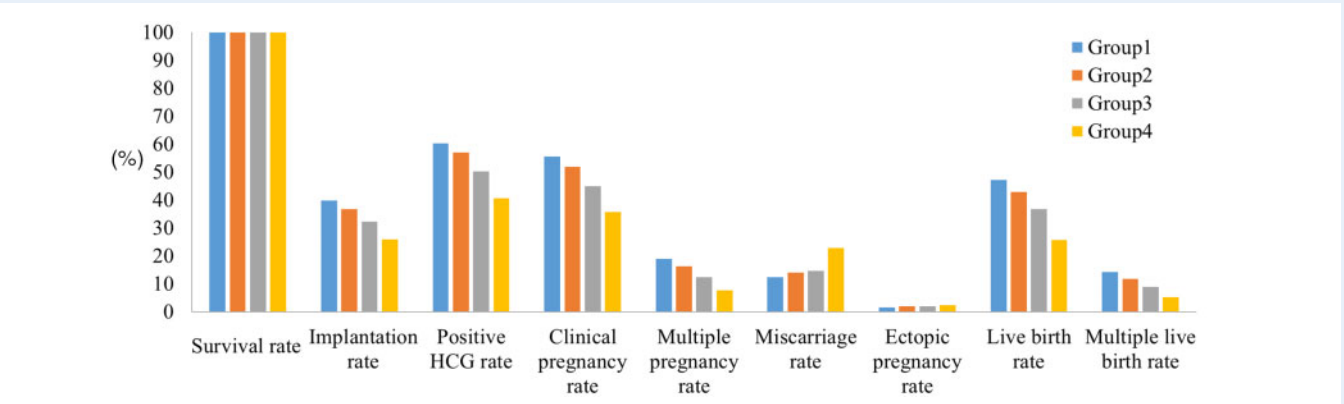

Figure 1. Pregnancy outcomes following frozen embryo transfer with different storage times.

Table II Odds ratios of pregnancy outcomes with different storage times of vitrified embryos.

	Group 2	P_1-value	Group 3	P_2-value	Group 4	P_3-value
Positive HCG rate						
Unadjusted OR(95%CI)	0.87 (0.83, 0.92)	<0.001	0.67 (0.62, 0.72)	<0.001	0.45 (0.38, 0.54)	<0.001
Adjusted OR(95%CI)[a]	0.92 (0.87, 0.97)	0.004	0.83 (0.76, 0.90)	<0.001	0.68 (0.56, 0.81)	<0.001
Clinical pregnancy rate						
Unadjusted OR(95%CI)	0.86 (0.82, 0.91)	<0.001	0.65 (0.60, 0.71)	<0.001	0.44 (0.37, 0.53)	<0.001
Adjusted OR(95%CI)[a]	0.91 (0.86, 0.96)	0.001	0.80 (0.73, 0.87)	<0.001	0.65 (0.54, 0.79)	<0.001
Multiple pregnancy rate						
Unadjusted OR(95%CI)	0.84 (0.78, 0.90)	<0.001	0.61 (0.55, 0.69)	<0.001	0.36 (0.27, 0.49)	<0.001
Adjusted OR(95%CI)[a]	0.89 (0.83, 0.96)	0.004	0.82 (0.72, 0.92)	0.001	0.63 (0.46, 0.87)	0.005
Miscarriage rate						
Unadjusted OR(95%CI)	0.89 (0.82, 0.96)	0.005	0.75 (0.66, 0.85)	<0.001	0.54 (0.39, 0.76)	<0.001
Adjusted OR(95%CI)[a]	1.05 (0.94, 1.18)	0.412	0.96 (0.80, 1.14)	0.622	1.32 (0.92, 1.89)	0.127
Ectopic pregnancy rate						
Unadjusted OR(95%CI)	1.26 (0.95, 1.66)	0.108	1.30 (0.86, 1.96)	0.212	1.54 (0.62, 3.83)	0.350
Adjusted OR(95%CI)[a]	1.14 (0.86, 1.52)	0.361	1.18 (0.77, 1.82)	0.440	1.59 (0.63, 4.03)	0.330
Live birth rate						
Unadjusted OR(95%CI)	0.84 (0.79, 0.89)	<0.001	0.65 (0.60, 0.71)	<0.001	0.39 (0.32, 0.47)	<0.001
Adjusted OR(95%CI)[a]	0.89 (0.85, 0.95)	<0.001	0.83 (0.76, 0.91)	<0.001	0.59 (0.48, 0.72)	<0.001
Multiple live birth rate						
Unadjusted OR(95%CI)	0.81 (0.74, 0.87)	<0.001	0.58 (0.51, 0.66)	<0.001	0.33 (0.23, 0.48)	<0.001
Adjusted OR(95%CI)[a]	0.87 (0.80, 0.94)	0.001	0.79 (0.68, 0.91)	0.001	0.60 (0.41, 0.88)	0.008

[a]Adjusted for maternal age, maternal BMI, infertility type, parity, infertility causes, embryo quality, number of transferred embryos, stage of embryo development, endometrial preparation program, and treatment years.
OR, odds ratio.
P_1: Group 2 vs. Group 1, P_2: Group 3 vs. Group 1, P_3: Group 4 vs. Group 1.

Table III Neonatal outcomes of singletons born after frozen embryo transfer, stratified by the storage times of vitrified embryos.

	Group 1 (n = 3377)	Group 2 (n = 2705)	P_1-value	Group 3 (n = 814)	P_2-value	Group 4 (n = 110)	P_3-value
Newborn gender, *n* (%)			0.606		0.504		0.968
Female	1603 (47.47)	1302 (48.13)		397 (48.77)		52 (47.27)	
Male	1774 (52.53)	1403 (51.87)		417 (51.23)		58 (52.73)	
Gestational age, mean ± SD	38.54 ± 1.52	38.53 ± 1.71	0.922	38.43 ± 1.56	0.059	38.38 ± 1.78	0.289
Preterm (<37weeks), *n* (%)	231(6.84)	186(6.88)	0.956	49(6.02)	0.400	11(10.00)	0.199
Birth weight, mean ± SD	3342.22 ± 479.54	3348.47 ± 513.97	0.625	3339.96 ± 488.33	0.904	3295.73 ± 513.82	0.318
Low birth weight (<2500g), *n* (%)	120 (3.55)	120 (4.44)	0.079	30 (3.69)	0.856	4 (3.64)	0.963
High birth weight (>4000g), *n* (%)	206 (6.10)	184 (6.80)	0.267	51 (6.27)	0.860	6 (5.45)	0.780
Macrosomia (>4500g), *n* (%)	30 (0.89)	23 (0.85)	0.874	3 (0.37)	0.183	1 (0.91)	0.982
Birth defects, *n* (%)	36 (1.07)	29 (1.07)	0.982	8 (0.98)	0.834	1 (0.91)	0.874

P_1: Group 2 vs. Group 1, P_2: Group 3 vs. Group 1, P_3: Group 4 vs. Group 1.

increasing storage duration. However, other animal studies reported that cryostorage duration of vitrified embryos had no significant effect on embryo survival rate, pregnancy rate or live birth rate (Eum *et al.*, 2009; Sanchez-Osorio *et al.*, 2010; Lavara *et al.*, 2011). Considering the significant differences in anatomy and physiology between animals and humans, the results derived from animal experiments could not be accurately applied to humans.

Few cases have been reported about successful deliveries of healthy babies from human-thawed embryos after cryostorage for an extended period of time. López-Teijón *et al.* (2006) reported the

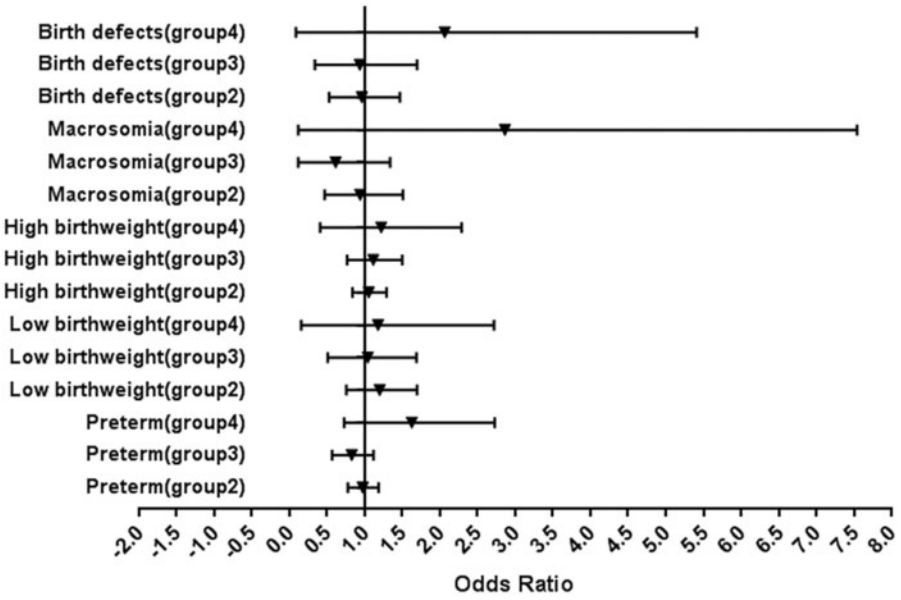

Figure 2. **Odds ratios of adverse neonatal outcomes among singletons with different storage time as vitrified embryos.**

Table IV **Results of multiple regression analysis of gestational age and birth weight among singletons following frozen embryo transfer with different storage times.**

	Group 2			Group 3			Group 4		
	B[a]	Standard error	P-value	B[a]	Standard error	P-value	B[a]	Standard error	P-value
Gestational age	−0.03	0.04	0.501	−0.12	0.06	0.073	−0.14	0.16	0.385
Birth weight	8.55	10.68	0.424	21.41	16.22	0.187	−20.95	39.60	0.597

[a]Adjusted for maternal age, maternal BMI, infertility type, parity, infertility causes, number of transferred embryos, stage of embryo development, embryo quality, endometrial preparation program, treatment years and neonatal gender.

successful birth of a healthy baby from donated pronuclear embryos in storage for 13 years. Twenty years was the longest storage period of human cryopreserved embryos resulting in successful live births of healthy babies, which was from the report by Dowling-Lacey *et al.* (2011). However, subjects from case reports might not be representative of the entire population of vitrified embryo transfers, so population studies are needed.

Human population studies evaluating the effect of cryopreservation storage time on ART outcomes have been reported, but the results were contradictory. The earlier study by Testart *et al.* (1987) found a decrease in the survival rate of human embryos and pregnancy rate after several months of storage. In contrast, a retrospective study performed from 1986 to 2007 by Riggs concluded that survival, implantation potential and pregnancy outcomes were not influenced by cryostorage duration for patients using autologous cryopreserved oocytes and donor oocytes (Riggs *et al.*, 2010). Similarly, Liu *et al.* (2014) and Yuan *et al.* (2019) also reported that storage time did not affect survival and pregnancy outcomes. The freezing method used in all the above studies was slow freezing. To our knowledge, there were only two studies exploring the influence of storage period after vitrification on embryonic and clinical outcomes (Wirleitner *et al.*, 2013; Ueno *et al.*, 2018). A more recent study performed by Ueno *et al.* revealed that cryostorage duration of vitrified blastocysts had no significant relationship with pregnancy and neonatal outcomes. However, in this study, the inclusion was limited to patients aged 35–39 years with a diagnosis of tubal factor infertility and having previous failed transfer cycles. The inappropriate inclusion criteria could lead to selection bias, which precluded drawing solid conclusion and extrapolating to the general population (Ueno *et al.*, 2018). Wirleitner *et al.* (2013) performed a retrospective study to investigate the effect of storage duration of vitrified blastocysts on survival rate, implantation rate and neonatal outcomes among 603 embryo transfer cycles. The results from their study showed that prolonged storage time did not have a significantly negative consequence on either embryo or offspring. Although Wirleitner pointed out this was the first study to evaluate the effect of extended storage time of vitrified blastocysts on embryonic and clinical outcomes, they also admitted the limitation of the small sample size and proposed that larger-scale studies on this subject

were needed. Our study was performed to analyze the impact of storage time on both vitrified blastocysts and cleavage-stage embryos in 24 698 frozen transfer cycles, and findings from this large study could enrich the current research in this field.

The mechanisms underlying the relationship between prolonged storage time and negative pregnancy outcomes are unclear. We think there are a few possible mechanisms. First, free radicals and toxic effects related to cryoprotectants could lead to an increase in DNA fragmentation (Kopeika *et al.*, 2015). Second, vitrification can solidify the cells and extracellular milieu into a glass-like state and reduce thermomechanical stress that can lead to fracturing. Although the diffusional mobility of large molecules under the glass transition temperature (Tg) is restricted, small local movements of molecules may still be mobile near or below Tg, which is related to cryobiology (Wowk, 2010). Molecular mobility below Tg can cause relaxation, which is associated with degradation risk of biological materials stored in the glassy state. In a recent study, Walters *et al.* reported time-dependent deterioration in seeds stored at cryogenic temperatures over a period of years, and also explained this was driven by the small movements of adjacent molecules (Walters *et al.*, 2004; Walters, 2007).

Previous studies reported increasing birth weight after vitrified embryo transfer and speculated that this increase was related to modifications of the epigenome of embryos (Wikland *et al.*, 2010; Liu *et al.*, 2013; Litzky *et al.*, 2018; Ginström Ernstad *et al.*, 2019). For example, Liu *et al.* (2013) reported a significantly higher median birth weight after vitrified cleaved embryo transfer than after slow freezing or fresh cleaved embryo transfer. Wikland *et al.* (2010) also found that the birth weight was higher after vitrified blastocyst transfer compared with after fresh blastocyst transfer. However, our study showed that the longer storage time increased the miscarriage rate but did not influence the neonatal outcomes, which was consistent with a previous study (Aflatoonian *et al.*, 2010). In that study, the spontaneous abortion rate was reported to be significantly higher after FET (14.5%) than after fresh embryo transfer (ET, 9%), but the neonatal outcomes, including prematurity, birth weight, mortality and birth defects, were comparable between FET and fresh ET. In terms of neonatal health, our study also provides evidence for the safety of long-term storage embryos.

In the present study, the proportion of patients with DOR, endometriosis or uterine factor was gradually rising with the extended storage time, and correspondingly with the decrease in the proportion of embryos transferred with good quality. For these patients, the number of oocytes retrieved and the number of embryos cryopreserved per ovarian stimulation cycle was few and the embryo quality was not good, so they had to take more time to obtain adequate quantity and quality of cryopreserved embryos before the embryo transfer. In order to exactly illustrate the relationship of storage duration with pregnancy and neonatal outcomes, we performed the following analysis. On the one hand, we adjusted infertile causes, embryo quality and other confounder factors in the multivariable regression to explore the effect of storage time on clinical outcomes. On the other hand, we reanalyzed the data among patients who were no older than 35 years and were infertile only because of tubal factors, and found the results for pregnancy and neonatal outcomes were consistent with previous analysis among the whole patient population.

Although being limited to the maximum cryostorage duration of 24 months, the present result can reflect the trend that the clinical pregnancy and live birth rate decreased with the extended storage duration, which potentially revolutionized clinical and laboratory practice of assisted reproduction. The result suggests that clinicians should consider the effect of storage duration before making the decision on the day of embryo transfer. Studies are needed to explore the potential causes of this decrease and improve the vitrification methodology. With the increased demand for fertility preservation in recent years, the safety of long-term cryopreservation on vitrified embryos has been an important issue for the preservation of fertility. Our result indicated that long-term storage of vitrified embryos led to decreased live birth rate but did not affect the neonatal outcome. This finding provides data to help make decision on embryo cryopreservation based on the risk and benefit analyses for specific group of patients who wish to delay childbirth because of medical or personal reasons. In addition, we offer evidence for the safety of long-stored vitrified embryos on neonatal health by performing a retrospective study with a large sample size. Prospective studies with long-term follow-up of the offspring born after vitrified embryo transfer evaluating physiological and psychological health are needed to ensure the long-term safety of vitrification with regard to storage time.

Strengths and limitations

There were some strengths to our study. Over the course of the study, the clinical and laboratory practices did not substantially change, which should minimize the possible confounders associated with pregnancy and neonatal outcome. The known factors related to the clinical outcomes, including maternal age, maternal BMI, infertility type, parity, infertility causes, number of transferred embryos, stage of embryo development, embryo quality, endometrial preparation program and treatment years, were all included in the multivariate logistic regression as independent variables.

Our study also has several limitations. First, this was a retrospective study. However, we took some measures, including setting strict inclusion criteria and restricting the subjects to the first FET cycle, to make the research more rigorous. Second, the proportion of older patients or poor-prognosis patients (patients with diminished ovarian reserve) increased with extended storage duration. We attempted to correct for age, embryo quality and infertility causes with the sub-analysis of patients <36 years of age and the diagnosis of tubal infertility only, which presumably were the good prognosis patients, and the results were consistent with the whole cohort. Third, we did not undertake a long-term follow-up of the offspring, so information about offspring growth and development in the long term was not available.

Conclusions

In summary, our study revealed that although the prolonged storage time of vitrified embryos negatively affected pregnancy and live birth outcomes, it did not have a significant influence on neonatal outcomes. This study provides new findings about the relationship between prolonged storage time of vitrified embryos and clinical outcomes and offers evidence for the safety of using long-stored embryos after vitrification.

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Authors' roles

Y.P.K. and Q.Q.Z. supervised the entire study, including the procedures, conception, design and completion. L.J.H., M.R.Y., B.W., J.Y.L., Q.J.C., N.L.W., Q.F.L. and Y.W. were responsible for the collection of data. Q.Q.Z. and J.H.L. contributed the data analysis and drafted the article. Q.Q.Z. participated in the interpretation of the study data and in revisions to the article.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

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