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Effect of embryo cryopreservation duration on pregnancy-related complications and birthweight after frozen-thawed embryo transfer: a retrospective cohort study

Jing-Jing Xu[1,2,†], Lei Chen[1,2,†], Cheng Li[1,2], Chen-Chi Duan[1,2], He-Feng Huang[1,2,3] and Yan-Ting Wu[1,2,3]

[1]International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, 200030, China; [2]Shanghai Key Laboratory of Embryo Original Diseases, Shanghai, 200030, China and [3]Obstetrics and Gynecology Hospital, Fudan University, Shanghai, 200011, China

Abstract

Frozen embryo transfer (FET) has been adopted by growing number of reproductive medicine centers due to the improved outcome compared with fresh embryo transfer. However, few studies have focused on the impact of embryo cryopreservation duration on pregnancy-related complications and neonatal birthweight. Thus, a retrospective cohort study including all FET cycles with livebirth deliveries in a university affiliated hospital from May 2010 to September 2017 was conducted. These deliveries were grouped by the cryopreservation duration of the transferred embryo (≤3 months, 4–6 months, 7–12 months, and >12 months). The associations between embryo cryopreservation duration and pregnancy-related complications were evaluated among the groups using multinomial logistic regression. Neonatal birthweight was compared according to the stratification of singletons and multiples using multinomial and multilevel logistic regression, respectively. Among all 12,158 FET cycles, a total of 3864 livebirth deliveries comprising 2995 singletons and 1739 multiples were included. Compared with those within 3 months, women undergoing FET after a cryopreservation time longer than 3 months did not show any increased risk of gestational diabetes mellitus, gestational hypertension, preeclampsia, meconium staining of the amniotic fluid, or preterm birth. Furthermore, the risk of lower birthweight, macrosomia, small-for-gestational-age, or large-for-gestational-age for either singletons or multiples was not affected by long-term cryopreservation. In summary, embryo cryopreservation duration does not have negative effects on pregnancy-related complications or birthweight after FET.

Introduction

Since it was first reported in 1984, frozen-thawed embryo transfer (FET) has been increasingly used in assisted reproductive technology (ART).[1] As an essential part of ART, embryo cryopreservation has been used to store surplus embryos after oocyte retrieval and in vitro fertilization (IVF). With the development of cryobiology and refinement of cryopreservation over the last few decades, vitrification has been adopted as a preferred method by most reproductive medicine centers.[2,3] However, due to the use of cryoprotectants in vitrification, concerns regarding the safety of transferring vitrified embryos have been raised,[4–6] particularly in terms of pregnancy and perinatal outcomes.

The effect of cryopreservation on the human embryo and health of the offspring is still a matter of debate. Many studies have demonstrated that the incidences of macrosomia and large-for-gestational-age (LGA) babies after FET are significantly higher than those following fresh embryo transfer or spontaneous conception.[7,8] Notably, there is no report in the literature regarding the adverse effects of FET in terms of birth defects. Thus, embryo cryopreservation offers many infertile patients the opportunity to undergo FET under ideal conditions, such as after an appropriate endometrial lining is prepared, and to avoid severe ovarian hyperstimulation syndrome (OHSS).[9,10] With the recent abolition of the one-child policy in China, some infertile patients with pregnancy intentions have resorted to transferring surplus embryos that had been cryopreserved during their previous IVF cycles. Although there are several previous reports of cases undergoing FET of embryos that had been cryopreserved for up to 16 years,[11–13] there has been no systematic study on long-term cryopreservation duration and perinatal outcomes.

Several recent reports have indicated that embryo cryopreservation duration has no effect on the survival rate of the embryo after thawing and neonatal birthweight in singletons.[14–17] Nevertheless, no study has focused on the effect of embryo cryopreservation duration on

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Address for correspondence:
Yan-Ting Wu and He-Feng Huang, International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, 910, Hengshan Rd., Shanghai, 200030, China; Obstetrics and Gynecology Hospital, Fudan University, 419, Fangxie Rd., Shanghai, 200011, China.
Emails: yanting_wu@163.com; huanghefg@sjtu.edu.cn

†Jing-Jing Xu and Lei Chen contributed equally to this work.

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maternal safety in terms of pregnancy-related complications. To fill this knowledge gap, this retrospective cohort study was designed to explore whether embryo cryopreservation duration has adverse effects on maternal health and neonatal outcomes after FET and to provide more data on the safety of the vitrification technique.

Method

Study design and participants

This retrospective cohort study included all infertile women undergoing FET and had live births in the International Peace Maternity and Child Health Hospital (IPMCH) from May 2010 to September 2017. Women who received donated oocytes or sperm, or women who underwent preimplantation genetic testing (PGT) were excluded. Mixed transfers with two embryos retrieved from different oocyte retrieval cycles were also excluded as they were cryopreserved at different times. Eligible FET cycles which resulted in live birth were eventually recruited in the analysis, and categorized into four groups according to the duration of embryo cryopreservation (Group I: ≤3 months; Group II: 4–6 months; Group III: 7–12 months; and Group IV: >12 months). Written informed consent about follow-up until delivery was routinely obtained from all women when they initiated their ART cycles in IPMCH. Ethical approval was obtained from the Institutional Review Board of the International Peace Maternity and Child Health Hospital (GKLW 2016-21).

ART procedures

The ART procedures, including ovarian stimulation, oocyte retrieval, and insemination, by either conventional IVF or intracytoplasmic sperm injection (ICSI), were conducted according to our standard protocols. Fertilization assessment was carried out 16–20 h after insemination. Embryo cryopreservation was performed by vitrification due to one of the following indications: 1) there was a maternal condition that was unsuitable for fresh embryo transfer, such as a high estrogen level, OHSS, or a desynchronized endometrium; 2) embryos had been harvested in a previous, unsuccessful IVF cycle, or there were surplus embryos after a fresh embryo transfer; or 3) the infertile couple had chosen to delay the transfer for personal reasons. FET was performed following endometrial preparation by natural monitoring, an ovarian stimulation cycle, or hormone replacement therapy (HRT).

Embryo vitrification and warming procedures

All embryos were vitrified and warmed with the open device. Before November 9, 2015, the embryo vitrification and thawing kit of JieYing Laboratory Inc (Longueuil, Quebec, Canada) was applied,[18,19] and after that, the Cryotop® of Kitazato BioPharma Co. Ltd (Fuji, Japan) was used.[20] The operation procedures were in accordance with the manufacturer's instructions. When vitrification: 1) Transfer embryos to Equilibration Solution (ES) for 5 min (JieYing Kit)/7–8 min (Cryotop® Kit) at room temperature; 2) Transfer embryos to Vitrification Solution (VS) with minimal volume of ES and equilibrate for 1 mi (JieYing Kit)/30–60 s (Cryotop® Kit); 3) Quickly place the embryos on the JY straw/Cryotop straw with minimal volume of VS (each straw contains 1–2 embryos); 4) Plunge the straw into sterile liquid nitrogen and fit the straw cap; 5) Transfer the straw to storage dewar (MVE XC47/11-6SQ, Chart Industries, GA, USA). When thawing: 1) JieYing Kit: warm Thawing Solution (TS) 1, 2, 3, and 4 to room temperature; Cryotop® Kit: warm TS to 37°C, Diluent Solution (DS) and Washing Solution (WS) to room temperature; 2) Remove the straw cap from the straw in liquid nitrogen; 3) Quickly immerse the straw into TS1 (JieYing Kit)/TS (Cryotop® Kit) and gently wash for 1 min; 4) Transfer the embryos to TS2 (JieYing Kit)/DS (Cryotop® Kit) for 3 min; 5) Transfer the embryos to TS3 (JieYing Kit) for 5 min/WS (Cryotop® Kit) for 3 min; 6) Transfer the embryos to TS4 (JieYing Kit) for 5 min/another WS (Cryotop® Kit) for 3 min; 7) Transfer and incubate the embryos to culture medium at a 37°C incubator to complete recovery. The liquid nitrogen dewars were only opened when the embryos need to be taken out, and closed immediately after taking out. Sterile liquid nitrogen was refilled regularly every week. Only embryologists who have achieved a recovery rate of more than 98% on discarded embryos were allowed to take up the job. The quality control assessment was carried out every year, and if the embryologist failed, he/she would be retrained.

Data collection and variable definition

Sociodemographic characteristics (including maternal age at oocyte retrieval and embryo transfer, residence, educational attainment, occupation, smoking status during pregnancy), reproductive history (including parity, number of previous abortions, previous ectopic pregnancy, primary infertility, cause of infertility [tubal infertility, anovulation, endometriosis, male-factor infertility, unexplained infertility, or combined cause], and duration of infertility [1–2, 3–4 or ≥5 years]) were extracted from the ART files, which were recorded at the first visit. The maternal height and weight were measured, and her body mass index (BMI) was calculated.

The patient's clinical data regarding the ART procedure, including oocyte retrieval and embryo transfer, were collected from the patient's hospital records as previously described.[21] ART procedures were conducted per routine protocols, and patient information during the ART procedure, including controlled ovarian hyperstimulation (COH) protocol (gonadotropin-releasing hormone [GnRH] agonist protocol, GnRH antagonist protocol, microflare protocol or other protocol), type of insemination (IVF or ICSI), number of oocytes retrieved (≤10, 11–20 or >20), type of endometrial preparation (natural cycle, HRT cycle, or ovarian stimulation cycle), day of embryo transfer (day 3, day 4, or day 5), and number of embryos transferred (1 or 2), was documented. Endometrial thickness before embryo transfer was measured by highly trained sonographers via transvaginal ultrasound (Acuson X300, Siemens, Germany).

The follow-up interview on the pregnancy-related complications and neonatal outcomes were preformed after their deliveries. Data on pregnancy-related complications (including gestational age, gestational hypertensive disorder, gestational diabetes mellitus (GDM), intrahepatic cholestasis of pregnancy (ICP), meconium staining of the amniotic fluid, preterm birth, and mode of delivery) and neonatal outcomes (including birthweight and sex of the neonates) were extracted from hospital records provided by participants. Small-for-gestational-age (SGA) or LGA was defined according to a global reference for birthweight for a given gestational age and sex.[22]

Statistical analysis

Continuous variables with a normal distribution are represented as the means ± standard deviations, and differences among groups were tested by one-way analysis of variance. Categorical variables

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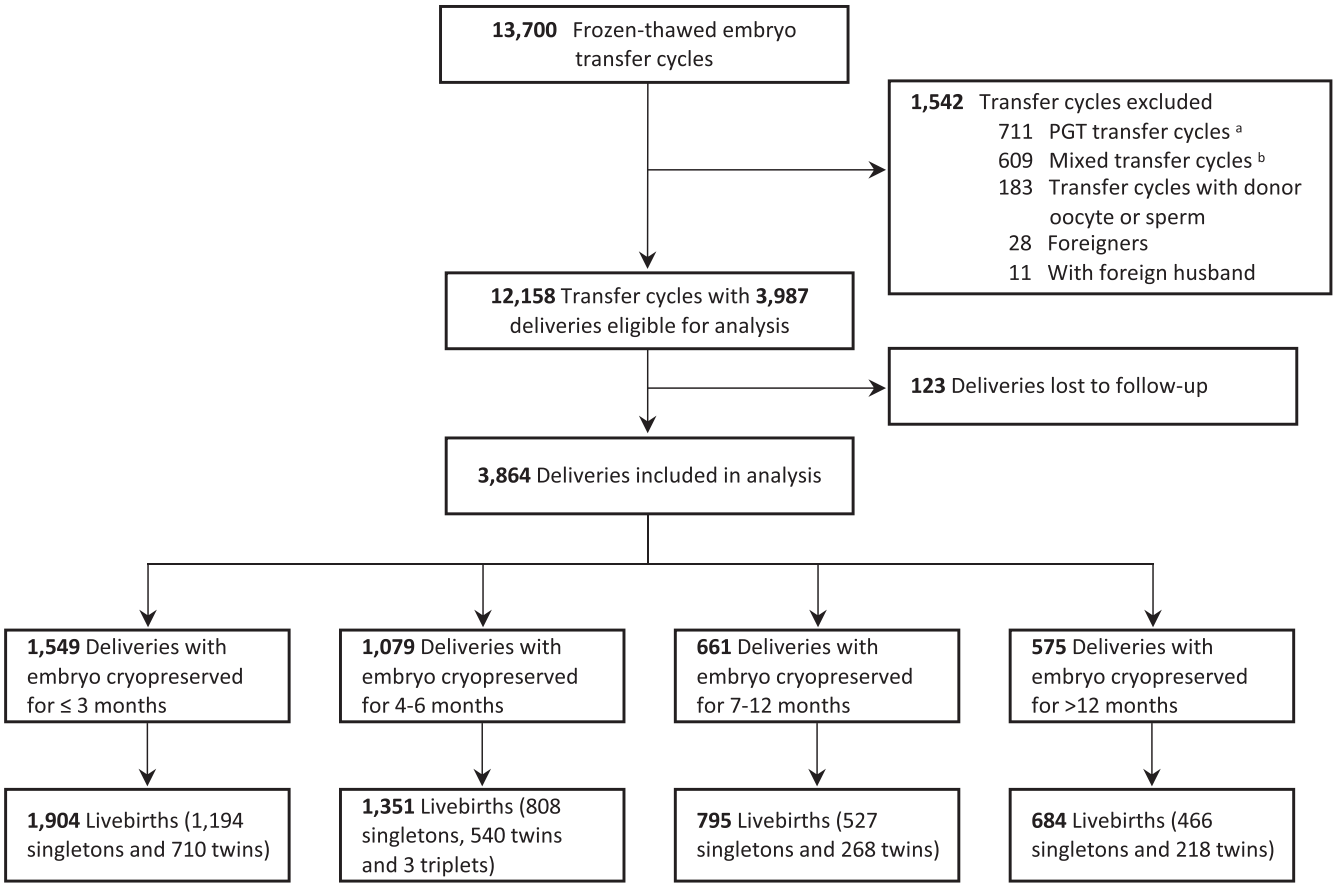

Fig. 1. Patient inclusion flow chart. a. PGT, preimplantation genetic testing. b. Mixed transfer cycle was defined as the transfer of two embryos from different oocyte retrieval cycles.

are represented as frequencies with proportions, and differences in trends were detected by the Cochran–Mantel–Haenszel χ^2 test.

To investigate the associations between the embryo cryopreservation duration and pregnancy-related complications, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated and adjusted for potential confounding factors for each outcome using multinomial logistic regression. Neonatal outcomes were stratified according to the delivery of a singleton or multiples. To analyze the neonatal outcomes of singletons, multinomial logistic regression analyses were performed to adjust ORs for potential confounding factors. When we analyzed the neonatal outcomes of multiples, ORs and 95% CIs were obtained using multilevel logistic regression, and the analysis was adjusted for the same confounding factors as those used for the singletons, according to Carlin et al.[23]

SAS software version 9.3 (SAS Institute, Inc., Cary, NC) was used to perform all statistical analyses. All p values were calculated using two-sided tests. Differences were considered statistically significant at a p value of less than 0.05.

Results

The flow chart of the study is shown in Fig. 1. A total of 3987 women with live birth deliveries from 12,158 FET cycles were included in the analysis. Among them, 123 women who could not provide delivery medical records were defined as lost to follow-up. With the increasing of embryo cryopreservation duration among groups, the number of participants was decreasing, and the longest cryopreservation duration was 6.7 years. The frequency distribution graph with number of live birth deliveries from FET per year is shown in Fig. 2.

The distributions of the maternal sociodemographic characteristics and reproductive history data among groups are shown in Table 1. Although maternal age at embryo transfer was comparable among groups ($p_{trend} = 0.318$), women who underwent transfers of embryos with longer cryopreservation times were younger at oocyte retrieval ($p_{trend} = 0.007$). Additionally, all groups showed comparable proportions in terms of residence, educational attainment, occupation, and smoking status during pregnancy. The proportions of women who experienced previous abortions ($p_{trend} < 0.001$) were much lower in the groups of women who underwent transfers of embryos with longer cryopreservation times. The proportions of parous women were higher in the 7–12-month and over-12-month cryopreservation groups than in the ≤3-month and 4–6-month cryopreservation groups ($p_{trend} < 0.001$). No significant differences were found among groups in terms of previous ectopic pregnancy, duration of infertility, primary infertility, or cause of infertility.

The results of the differences in the procedures for oocyte retrieval and frozen-thawed embryo transfer in each group are provided in Table 2. The distributions of COH protocol, type of insemination, number of oocytes retrieved, day of embryo transfer and number of embryos transferred were not different between any groups. Endometrial thickness was comparable among groups before embryo transfer. However, the endometrial preparation

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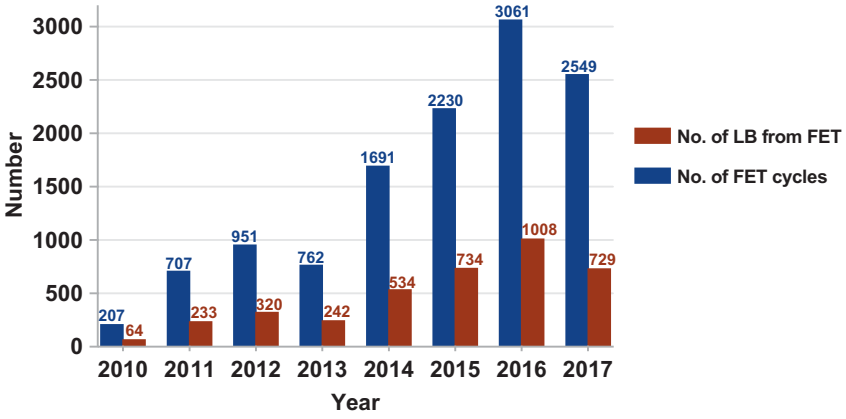

Fig. 2. Frequency distribution graph with number of live birth deliveries from frozen-thawed embryo transfer (FET) per year. Blue bars represent the number of FET cycles in this year. Red bars represent the number of live birth deliveries from the FET in this year.

protocol was found to be significantly different among the groups. Women who underwent FET within 3 months of cryopreservation were much more likely to undergo a natural cycle protocol and less likely to undergo a HRT cycle (58.62% in the natural cycle and 30.54% in the HRT cycle, $p_{trend} < 0.001$).

Table 3 shows the associations between embryo cryopreservation duration and pregnancy-related complications after adjusting for confounding factors, including age at oocyte retrieval, age at embryo transfer, parity, number of previous abortions, type of fertilization, type of endometrial preparation, and number of fetus. Compared to cryopreservation for up to 3 months, long-term cryopreservation duration did not increase the risk of any pregnancy-related complication, including GDM, gestational hypertension, preeclampsia, ICP, meconium staining of the amniotic fluid, preterm birth, and cesarean section deliveries. The associations between embryo cryopreservation duration and neonatal outcomes are presented in Table 4. FETs after different embryo cryopreservation durations had similar proportions in terms of neonatal sex for both singletons and multiples. No significant trends in the association between birthweight and increased embryo cryopreservation duration were found for singletons (Group I: 3323.76 ± 522.00, Group II: 3334.63 ± 525.53, Group III: 3300.23 ± 512.75, and Group IV: 3292.02 ± 565.46, $p_{trend} = 0.447$) or multiples (Group I: 2503.91 ± 441.64, Group II: 2499.27 ± 438.26, Group III: 2532.34 ± 450.39, and Group IV: 2495.78 ± 501.33, $p_{trend} = 0.761$). The rates of low birthweight and macrosomia were also comparable among groups in singletons, and no association was found between the risk of LBW or macrosomia and embryo cryopreservation duration. A similar null effect was also observed in multiples with respect to LBW, and no case of macrosomia was found among multiples in the four groups. Additionally, there was no evidence of an association between SGA or LGA and embryo cryopreservation duration among either singletons or multiples.

Considering the possible impact of the embryo vitrification/thawing kits replacement, a stratified analysis according to the date of embryo frozen and thawed was conducted. And it was shown that regardless of whether embryos were frozen before or after November 9, 2015, different cryopreservation duration has no effect on pregnancy complications and neonatal outcomes (Supplementary Table S1-S2). The same results were also found in the stratified analysis of embryo thawed date (Supplementary Table S3-S4).

Discussion

In this retrospective cohort study, we found no association between embryo cryopreservation duration before FET and pregnancy-related complications, including GDM, gestational hypertension, preeclampsia, meconium staining of the amniotic fluid, and preterm birth. In addition, embryo cryopreservation duration seemed to have no adverse effect on abnormal birthweight, including LBW, SGA, macrosomia, and LGA. The findings of our study suggest that it is safe to cryopreserve human embryos for a longer time period and long-term cryopreservation will not result in adverse effects on maternal health or neonatal birthweight.

Since FET was first introduced, the safety of the procedures has been a concern. Although FET has been regarded as having a higher live birth rate than fresh embryo transfer and a comparable rate of birth defects,[24,25] there are still problems resulting from embryo cryopreservation technology. Our previous study indicated that blastomere loss after embryo thawing could lead to a decreased pregnancy rate after embryo transfer.[21] In addition to blastomere loss, other factors, namely, embryo vitrification, open vitrification systems, and vitrification duration, have raised concern regarding their impacts on pregnancy outcomes and neonatal safety.[15,26,27] Although the long duration of cryopreservation makes it difficult to study the impact of duration on the safety of vitrification, some studies have indicated comparable pregnancy rates for FETs after short- and long-term cryopreservation and reported that birthweight in singletons is not influenced by vitrification duration.[14–17] Our findings were consistent with these documented findings. In addition, our study was the first to compare the relationship between embryo cryopreservation duration and birthweight in multiples, which also reached the same conclusion as singletons.

Besides, these studies failed to evaluate the impacts of cryopreservation duration on adverse maternal health conditions during pregnancy.[14] Pregnancy-related complications, especially GDM and gestational hypertensive disorder, have been regarded as risk factors for chronic noninfectious diseases of the offspring in adulthood,[28,29] which cannot be detected in the short-term postpartum follow-up in these studies. Thus, it is critical to assess maternal health during pregnancy after transferring long-term cryopreserved embryos. From our findings, transferring embryos cryopreserved within or more than 12 months did not have any effect on

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Table 1. Maternal characteristics of all FET groups with different embryo cryopreservation durations

	Group I: ≤3 months ($n = 1549$)	Group II: 4–6 months ($n = 1079$)	Group III: 7–12 months ($n = 661$)	Group IV: >12 months ($n = 575$)	
	n (%)	n (%)	n (%)	n (%)	*p*
Maternal sociodemographic characteristics					
Age at oocyte retrieval, mean ± SD, years	30.87 ± 3.61	31.05 ± 3.70	30.48 ± 3.87	30.15 ± 3.92	0.007
Age at embryo transfer, mean ± SD, years	30.86 ± 3.61	31.05 ± 3.70	31.16 ± 3.85	31.95 ± 3.91	0.318
Pregestational BMI, mean ± SD, kg/m^2	22.05 ± 2.89	22.15 ± 3.07	22.01 ± 3.04	22.09 ± 3.18	0.777
Residence					
Residents	1013 (65.40)	713 (66.08)	434 (65.66)	392 (68.17)	0.314
Immigrants	536 (34.60)	366 (33.92)	227 (34.34)	183 (31.83)	
Educational attainment					
Primary school or lower	16 (1.03)	15 (1.39)	9 (1.36)	6 (1.04)	0.379
Middle school	197 (12.72)	150 (13.90)	70 (10.59)	56 (9.74)	
High school	246 (15.88)	182 (16.87)	100 (15.13)	117 (20.35)	
College or above	1090 (70.37)	732 (67.84)	482 (72.92)	396 (68.87)	
Occupation					
Employed	1066 (68.82)	748 (60.32)	454 (68.68)	416 (72.35)	0.387
Self-employed	343 (22.14)	213 (19.74)	139 (21.03)	111 (19.30)	
Unemployed	140 (9.04)	118 (10.94)	68 (10.29)	48 (8.35)	
Smoking during pregnancy					
No	1530 (98.77)	1069 (99.07)	658 (99.55)	569 (98.96)	0.320
Yes	19 (1.23)	10 (0.93)	3 (0.45)	6 (1.04)	
Reproductive history					
Parity					
No	1456 (94.00)	1002 (92.86)	596 (90.17)	519 (90.26)	<0.001
Yes	93 (6.00)	77 (7.14)	65 (9.83)	56 (9.74)	
Number of previous abortions					
0	1002 (64.69)	713 (66.08)	473 (71.56)	431 (74.96)	<0.001
1–2	500 (32.28)	325 (30.12)	165 (24.96)	141 (24.52)	
≥3	47 (3.03)	41 (3.80)	23 (3.48)	3 (0.52)	
Previous ectopic pregnancy					
No	1342 (86.64)	935 (86.65)	562 (85.02)	522 (90.78)	0.109
Yes	207 (13.36)	144 (13.35)	99 (14.98)	53 (9.22)	
Duration of infertility					
1–2	575 (37.12)	428 (39.67)	243 (36.76)	225 (39.13)	0.473
3–4	510 (32.92)	334 (30.95)	228 (34.49)	185 (32.17)	
≥5	464 (29.95)	317 (29.38)	190 (28.74)	165 (28.70)	
Primary infertility					
No	893 (57.65)	620 (57.46)	374 (56.58)	349 (60.70)	0.408
Yes	656 (42.35)	459 (42.54)	287 (43.42)	226 (39.30)	
Cause of infertility					
Tubal infertility	719 (46.42)	456 (42.26)	286 (43.27)	271 (47.13)	0.430
Anovulatory	101 (6.52)	75 (6.95)	28 (4.24)	34 (5.91)	
Endometriosis	40 (2.58)	26 (2.41)	23 (3.48)	14 (2.43)	

(*Continued*)

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Table 1. (*Continued*)

	Group I: ≤3 months (*n* = 1549)	Group II: 4–6 months (*n* = 1079)	Group III: 7–12 months (*n* = 661)	Group IV: >12 months (*n* = 575)	
	n (%)	n (%)	n (%)	n (%)	*p*
Male-factor infertility	81 (5.23)	80 (7.41)	57 (8.62)	65 (11.30)	
Unexplained infertility	228 (14.72)	178 (16.50)	117 (17.70)	64 (11.13)	
Combined[a]	380 (24.53)	264 (24.47)	150 (22.69)	127 (22.09)	

FET, frozen-thawed embryo transfer; BMI, body mass index; SD, standard deviations.
[a]Combined is defined as two or more infertility causes mentioned above.

Table 2. ART procedures in all FET groups with different embryo cryopreservation

	Group I: ≤3 months (*n* = 1549)	Group II: 4–6 months (*n* = 1079)	Group III: 7–12 months (*n* = 661)	Group V: >12 months (*n* = 575)	
	n (%)	n (%)	n (%)	n (%)	*p*
COH protocol					
GnRH-agonist regimen	644 (41.58)	458 (42.45)	242 (36.61)	236 (41.04)	0.183
GnRH-antagonist regimen	806 (52.03)	570 (52.83)	370 (55.98)	297 (51.65)	
Microflare protocol	84 (5.42)	37 (3.43)	40 (6.05)	35 (6.09)	
Others	15 (0.97)	14 (1.30)	9 (1.36)	7 (1.22)	
Type of insemination					
IVF	1112 (71.79)	750 (69.51)	469 (70.95)	402 (69.91)	0.425
ICSI	437 (28.21)	329 (30.49)	192 (29.05)	173 (30.09)	
Number of oocytes retrieved					
≤10	517 (33.38)	377 (34.94)	241 (36.46)	200 (34.78)	0.511
11–20	730 (47.13)	495 (45.88)	300 (45.39)	270 (46.96)	
>20	302 (19.50)	207 (19.18)	120 (18.15)	105 (18.26)	
Endometrial preparation protocol					
Natural cycle	908 (58.62)	478 (44.30)	283 (42.81)	251 (43.65)	<0.001
OS cycle	168 (10.85)	108 (10.01)	45 (6.82)	39 (6.78)	
HRT cycle	473 (30.54)	493 (45.69)	333 (50.38)	285 (49.57)	
Day of embryo transfer					
Day 3	1151 (74.31)	785 (72.75)	480 (72.62)	428 (74.43)	0.492
Day 4	234 (15.11)	186 (17.24)	119 (18.00)	91 (15.83)	
Day 5	164 (10.59)	108 (10.01)	62 (9.38)	56 (9.74)	
Number of embryos transferred					
1	153 (9.88)	121 (11.21)	77 (11.65)	67 (11.65)	0.154
2	1396 (90.12)	958 (88.79)	584 (88.35)	508 (88.35)	
Endometrial thickness, mean ± SD, mm	9.56 ± 1.50	9.51 ± 1.45	9.49 ± 1.39	9.57 ± 1.43	0.593

ART, assisted reproductive technology; FET, frozen-thawed embryo transfer; COH, controlled ovarian stimulation; GnRH, Gonadotropin-releasing hormone; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; OS, ovarian stimulation; HRT, hormonal replacement therapy.

the risk of GDM, gestational hypertensive disorder, ICP, or other pregnancy-related complications.

Some studies have found that the embryo cryopreservation does not increase the incidence of chromosomal abnormalities[30,31] and DNA damage,[32] while increasing evidence suggests that cryopreservation may be associated with deviations from the physiological epigenetic marks, such as DNA methylation,[33] histone modifications,[34] and noncoding RNA.[35] Although owing to the ethical issues, most of these studies were on animals. A multi-omics study found that FET seemed to introduce more disturbance into infant epigenomes than fresh embryo transfer did, and the epigenetic alterations highly enriched in the processes related to nervous

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Table 3. Complications of pregnancies following the transfer of frozen-thawed embryos with different cryopreservation durations

	Group I: ≤3 months	Group II: 4–6 months	Group III: 7–12 months	Group V: >12 months			
	(*n* = 1549)	(*n* = 1079)	(*n* = 661)	(*n* = 575)	aOR (95%CI)[a]	aOR (95%CI)[a]	aOR (95%CI)[a]
	n (%)	n (%)	n (%)	n (%)	II vs. I	III vs. I	IV vs. I
Gestational diabetes mellitus							
No	1383 (89.28)	967 (89.62)	602 (91.07)	529 (92.00)	Reference	Reference	Reference
Yes	166 (10.72)	112 (10.38)	59 (8.93)	46 (8.00)	0.96 (0.74–1.24)	0.85 (0.60–1.22)	0.75 (0.43–1.32)
Hypertensive disorder							
No	1266 (81.73)	894 (82.85)	550 (83.21)	474 (82.43)	Reference	Reference	Reference
Gestational hypertension	111 (7.17)	77 (7.14)	46 (6.96)	42 (7.30)	0.94 (0.69–1.28)	0.97 (0.65–1.46)	1.11 (0.61–2.04)
Preeclampsia	172 (11.10)	108 (10.01)	65 (9.83)	59 (10.26)	0.85 (0.65–1.10)	0.81 (0.58–1.14)	0.76 (0.45–1.27)
Intrahepatic cholestasis of pregnancy							
No	1479 (95.48)	1029 (95.37)	626 (94.70)	549 (95.48)	Reference	Reference	Reference
Yes	70 (4.52)	50 (4.63)	35 (5.30)	26 (4.52)	1.04 (0.71–1.51)	1.15 (0.72–1.84)	0.84 (0.40–1.78)
Meconium staining of the amniotic fluid							
No	1302 (84.05)	907 (84.06)	562 (85.02)	483 (84.00)	Reference	Reference	Reference
Yes	247 (15.95)	172 (15.94)	99 (14.98)	92 (16.00)	1.03 (0.83–1.27)	0.89 (0.67–1.18)	0.84 (0.55–1.29)
Preterm birth							
No	1217 (78.57)	827 (76.65)	525 (79.43)	460 (80.00)	Reference	Reference	Reference
Preterm	332 (21.43)	252 (23.35)	136. (20.57)	115 (20.00)	1.05 (0.85–1.29)	0.98 (0.77–1.26)	0.98 (0.75–1.28)
Mode of delivery							
Vaginal	508 (32.80)	353 (32.72)	220 (33.28)	205 (35.65)	Reference	Reference	Reference
Cesarean section	1041 (67.20)	726 (67.28)	441 (66.72)	370 (64.35)	0.95 (0.80–1.14)	0.95 (0.75–1.20)	0.76 (0.54–1.08)

aOR, adjusted odds ratio; CI, confidence interval.
[a]aOR was adjusted for age at oocyte retrieval, age at embryo transfer, parity, number of previous abortions, type of insemination, type of endometrial preparation, and number of fetus.

system, cardiovascular system, and glycolipid metabolism.[36] These epigenetic alterations remind us that the cryopreservation may have long-term effects on the offspring of FET. Hiura *et al.* have observed that ART offspring has increased incidences of normally rare imprinting disorders such as Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), Prader-Willi syndrome (PWS), and Silver-Russell syndrome (SRS).[37,38] Due to the long follow-up period and difficulty in obtaining human biological samples, the long-term effects of embryo cryopreservation on FET offspring still need more research to confirm.

Although embryo cryopreservation and FET have been widely applied, to the best of our knowledge, this is the first broad study to evaluate whether vitrified embryos that are cryopreserved for a longer time are associated with adverse maternal health or neonatal outcomes. Our results indicated that cryopreservation duration did not have a negative impact on these outcomes. However, it should be noted that the longest cryopreservation duration in our study was up to 6.7 years, and the mean duration of cryopreservation in Group IV was approximately 2.7 years. Therefore, it is apparently safe to transfer embryos that have been cryopreserved for approximately 3 years. Additionally, the transfer of these embryos could reduce the economic burden and physical pain of these women by allowing them to avoid undergoing a new ovarian stimulation cycle.

Due to the patients' concern on the adverse effects of extremely long-term embryo cryopreservation on both mothers and babies, they refused to have these embryos transferred; thus, the study population with extremely long-term cryopreservation duration is lacking in this study. This is one of the limitations of our study. Yuan *et al.* reported an analysis of long-term embryo cryopreservation (≥12 years) in 20 patients; 4 successfully conceived. Among them, one patient developed GDM, while one developed GDM and had a preterm delivery.[11] It is worth noting that the sample size of their study was quite small, that the embryos were cryopreserved by means of slow-freezing methods, and that the patients were at an advanced age when the embryos were transferred (38–51 years old).[11] On the other hand, we must also be aware of the influence of iatrogenic damage during long-term cryopreservation, such as human errors of freezing, preservation, the daily use of liquid nitrogen tanks and even the equipment failures rather than the increasing storage time itself.[39] Due to concerns from both patients and clinical doctors, more robust evidence on the safety of transferring long-term cryopreserved embryos is urgent and necessary.

During the 7 years in this study, embryo vitrification and thawing kits have been replaced. In order to study the impact of kit replacement, we added a stratified analysis, and the result showed that it was comparable with the overall result. Parmegiani *et al.* conducted a randomized controlled trial to study the efficacy

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Table 4. Outcomes of neonates born after FET with different embryo cryopreservation durations

	Group I: ≤3 months	Group II: 4–6 months	Group III: 7–12 months	Group V: >12 months	aOR (95%CI)[a]	aOR (95%CI)[a]	aOR (95%CI)[a]
	n (%)	n (%)	n (%)	n (%)	II vs. I	III vs. I	IV vs. I
Singletons	*n* = 1194	*n* = 808	*n* = 527	*n* = 466			
Sex							
Male	577 (48.32)	421 (52.10)	266 (50.47)	242 (51.93)	Reference	Reference	Reference
Female	617 (51.68)	387 (47.90)	261 (49.53)	224 (48.07)	0.96 (0.72–1.03)	1.03 (0.82–1.30)	1.24 (0.87–1.76)
Birthweight, mean ± SD	3323.76 ± 522.00	3334.63 ± 525.53	3300.23 ± 512.75	3292.02 ± 565.46	*P* = 0.447		
<2500 g	79 (6.62)	58 (7.18)	35 (6.64)	31 (6.65)	1.04 (0.73–1.48)	1.03 (0.65–1.64)	1.22 (0.61–2.47)
2500–4000 g	1022 (85.59)	693 (85.77)	457 (86.72)	399 (85.62)	Reference	Reference	Reference
>4000 g	93 (7.79)	57 (7.05)	35 (6.64)	36 (7.73)	0.90 (0.64–1.27)	0.95 (0.60–1.50)	1.35 (0.70–2.61)
Birthweight for gestational age							
SGA	99 (8.29)	56 (6.93)	34 (6.45)	31 (6.65)	0.84 (0.60–1.19)	0.72 (0.45–1.13)	0.81 (0.52–1.24)
AGA	812 (68.01)	557 (68.94)	394 (74.76)	334 (71.67)	Reference	Reference	Reference
LGA	283 (23.70)	195 (24.13)	99 (18.79)	101 (21.67)	1.01 (0.82–1.25)	0.92 (0.68–1.22)	0.91 (0.70–1.19)
Multiples	*n* = 710	*n* = 543	*n* = 268	*n* = 218			
Sex							
Male	367 (51.69)	281 (51.75)	131 (48.88)	105 (48.17)	Reference	Reference	Reference
Female	343 (48.31)	262 (48.25)	137 (51.12)	113 (51.83)	1.01 (0.81–1.27)	1.18 (0.85–1.64)	1.29 (0.78–1.64)
Birthweight, mean ± SD	2503.91 ± 441.64	2499.27 ± 438.26	2532.34 ± 450.39	2495.78 ± 501.33	*P* = 0.761		
<2500 g	347 (48.87)	260 (47.88)	123 (45.90)	115 (52.75)	0.91 (0.72–1.14)	0.95 (0.68–1.32)	1.42 (0.86–2.36)
2500–4000 g	363 (51.13)	283 (52.12)	145 (54.10)	103 (47.25)	Reference	Reference	Reference
>4000 g	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	NA	NA	NA
Birthweight for gestational age							
SGA	135 (19.01)	99 (18.23)	59 (22.01)	35 (16.06)	0.91 (0.68–1.22)	1.36 (0.89–2.09)	1.11 (0.56–2.18)
AGA	537 (75.63)	423 (77.90)	197 (73.51)	173 (79.36)	Reference	Reference	Reference
LGA	38 (5.35)	21 (3.87)	12 (4.48)	10 (4.59)	0.74 (0.43–1.29)	1.69 (0.75–3.80)	3.54 (0.96–12.97)

FET, frozen-thawed embryo transfer; aOR, adjusted odds ratio; CI, confidence interval; NA, not accessible; AGA, appropriate for gestational age; SGA, small for gestational age; LGA, large for gestational age; SD, standard deviations.
[a]aOR was adjusted for age at oocyte retrieval, age at embryo transfer, parity, number of previous abortions, type of insemination, and type of endometrial preparation.

and efficiency of a universal warming protocol on vitrified embryos with two different embryo vitrification/thawing kits, including Cryotop® Kit (Kitazato Japan) and Sage Kit (Origio, Denmark), and indicated that the survival rates and implantation rates among the combination of kits of different manufacturers were comparable, and a thawing kit of a given manufacturer could be used to warm embryos vitrified with another kit.[20] Similarly, in our study, although the embryo vitrification/thawing kits was replaced, it did not affect the results.

In summary, this retrospective cohort study proves the safety of transferring long-term cryopreserved embryos in terms of pregnancy-related complications. Further studies with long-term follow-up are still required to assess the possible effects of long-term cryopreservation on child growth and development.

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Conflicts of Interest. The authors declare no conflict of interest.

Ethical Standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines and with the Helsinki Declaration of 1975, as revised in 2008, and has been approved by the Institutional Review Board of the International Peace Maternity and Child Health Hospital (GKLW 2016-21). Written informed consent was obtained from all participants before inclusion.

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