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The Effect of Embryo Vitrification Duration on Frozen-Thawed Transfer Outcomes: The Key Moderating Role of Age—A Retrospective Cohort Study of 13,054 Cycles

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Abstract

Purpose

To investigate how embryo vitrification duration affects frozen-thawed transfer outcomes, focusing on the interaction between age (at transfer and at freezing) and storage time.

Methods

This retrospective cohort study included 13,054 first vitrified embryo transfers (2015–2025). Cycles were divided by cryopreservation duration: ≤0.5, 0.5-2, 2–5, and > 5 years. Primary outcomes were clinical pregnancy, implantation, and live birth rates; secondary outcomes included biochemical pregnancy, miscarriage, ectopic, and multiple pregnancy. Multivariate logistic regression adjusted for confounders, followed by age-stratified analyses and interaction tests. Subgroup analyses used age at transplantation (ROC cutoff 36 years) and at freezing (cutoff 34.5 years).

Results

Compared to ≤ 0.5 years, the > 5 years group showed lower clinical pregnancy (aOR = 0.757, P = 0.036), implantation (aOR = 0.604, P = 0.001), and live birth rates (aOR = 0.881, P = 0.003). For 2–5 years, only live birth rate decreased (aOR = 0.875, P = 0.049). Age significantly interacted with freezing duration (P = 0.001). Women aged ≤ 34.5 years at freezing also exhibited a dose-response decline in live birth rates with longer storage.

Conclusions

Long-term cryopreservation (>5 years) has an adverse effect on the outcomes of frozen-thawed embryo transfer, but this effect is observed only in younger women. In older women, the deterioration of the uterine environment may mask the additional risks associated with prolonged cryopreservation. Clinicians should make individualized decisions regarding the use of long-term cryopreserved embryos by considering both the patient's age at the time of transfer and the age of the embryos at the time of cryopreservation.

I. Introduction

In recent years, assisted reproductive technology (ART) has advanced rapidly. In particular, the maturation and application of embryo vitrification have become indispensable cornerstones of modern reproductive medicine. With the implementation of national policies encouraging childbirth, an increasing number of couples who have undergone in vitro fertilization and embryo transfer (IVF-ET) are choosing to thaw and transfer previously frozen embryos to achieve pregnancy. Over time, reproductive centers have accumulated large numbers cryopreserved embryos. The duration of cryopreservation varies widely, ranging from several months to several years or even more than a decade or two. Previously, slow-freezing (programmed freezing) was the standard method, but vitrification was first applied in China in 2003 and was fully implemented in 2012[1, 2]. During fresh-cycle embryo transfer, embryos that divide too rapidly or too slowly struggle to synchronize with endometrial development; therefore, they are not considered the preferred option for embryo transfer[3]. In contrast, frozen embryo transfer (FET) offers patients who have experienced a failed fresh cycle embryo transfer or whose transfer had to be canceled due to ovarian hyperstimulation syndrome (OHSS) the possibility of undergoing another embryo transfer and achieving clinical pregnancy. Furthermore, the risk of OHSS is significantly reduced in patients undergoing FET [4], and it minimizes the rate of multiple pregnancies without the need to transfer multiple embryos simultaneously[5].

However, the high concentrations of cryoprotectants used in vitrification, as well as the open system that exposes embryos directly to liquid nitrogen during storage, may damage to embryos undergoing long-term cryopreservation or induce osmotically[6]. From a theoretical perspective, embryos cryopreserved in liquid nitrogen (-196°C) experience near-complete inhibition of intracellular enzyme activity, with all cellular metabolic processes coming to a standstill. This effectively halts biological processes over time, maintaining the cells' initial viable state and thereby achieving long-term stable cryopreservation[7]. However, the potential effects of cryopreservation on embryos remain to be fully investigated, and the maximum duration of long-term cryopreservation that does not adversely affect embryos or clinical outcomes remains unknown. Kopeika et al.[8] point out that although cryopreservation technology can maintain the original morphological characteristics of cells, it may still pose potential risks to the structural integrity and functional stability of the genomes of germ cells and embryos, thereby triggering chromosomal aberrations. This phenomenon may be associated with direct or indirect interference with genetic stability caused by physicochemical changes during the freeze-thaw process, manifesting as abnormal changes in DNA, nucleoproteins, and mitochondrial DNA, accompanied by issues such as chromosomal structural or numerical abnormalities and imbalances in DNA damage repair mechanisms, ultimately adversely affecting the normal mitotic process of cells after thawing. During clinical cryopreservation, high concentrations of the cryoprotectant dimethyl sulfoxide (DMSO) exert direct cytotoxic effects; even low-dose DMSO (≤10%) can affect gene transcription and epigenetic regulatory mechanisms, thereby inducing persistent epigenetic alterations[9]. Meanwhile, the stress response triggered by vitrification, including osmotic shock, changes in temperature and pH, and cryoprotectant toxicity, may lead to epigenetic and transcriptomic changes in oocytes and embryos[10]. Although there are reported cases worldwide of successful pregnancies and the birth of healthy newborns after embryos with extremely prolonged cryopreservation durations were transferred, these remain isolated instances. Therefore, a retrospective analysis of the outcomes of thawed and transferred vitrified embryos is necessary.

In existing studies, the impact of cryopreservation duration on pregnancy outcomes remains controversial; some studies have demonstrated that the duration of vitrification cryopreservation does not affect clinical pregnancy outcomes[11]. The 2018 "Chinese Expert Consensus on the Storage Duration of

Frozen Embryos" states that embryo cryopreservation has no significant effect on FET pregnancy rates or the incidence of birth defects in offspring; embryo cryopreservation (for up to 6 years) does not affect embryo survival rates, implantation rates, pregnancy rates, live birth rates, or the incidence of birth defects in offspring following thawing. Other studies have shown no significant differences in pregnancy outcomes, including biochemical pregnancy, implantation, clinical pregnancy, ongoing pregnancy, and live birth rates, between groups at different storage time points. No differences were found among storage duration groups regarding preterm birth, birth length, or low birth weight[12, 13]. A 2011 study by Lavara et al.[14]demonstrated that vitrified embryos can be stored in liquid nitrogen for at least 15 years while maintaining good pregnancy, conception, and live birth rates.

Conversely, other findings indicate that for vitrified embryos, longer storage durations lead to varying degrees of decline in biochemical pregnancy, clinical pregnancy, and live birth rates[15]. Li et al. [15]conducted a retrospective analysis of 24,698 patients and found that clinical pregnancy and live birth rates decreased significantly with increasing storage duration, while the relationship between miscarriage, ectopic pregnancy, and storage duration did not reach statistical significance. There were no significant differences in neonatal adverse outcomes (preterm birth, low birth weight, high birth weight, macrosomia, or congenital anomalies) between the groups. Zhan et al.[6] conducted an analysis involving 36,665 frozen-thawed embryo transfer cycles and reported that when the duration of blastocyst vitrification storage exceeded five years, both the implantation rate (IR) and live birth rate (LBR) following blastocyst transfer were reduced. In contrast, prolonged cryopreservation of cleavage-stage embryos did not exert a significant effect on either IR or LBR. For singleton live births, an increase in the duration of cryopreservation was associated with a decline in the incidence of small-for-gestational-age (SGA) infants, alongside a rise in the rate of large-for-gestational-age (LGA) infants. A meta-analysis[16] showed that, compared with fresh cleavage-stage embryo transfer, singleton pregnancies following fresh blastocyst transfer were associated with a higher risk of LGA and very preterm births. Singleton pregnancies following frozen blastocyst transfer are associated with higher risks, but the risks of preterm birth and perinatal mortality are reduced. In sensitivity analyses, the trend toward increased risks of macrosomia and preterm birth following frozen blastocyst transfer persisted, and the analysis also showed a significant increase in the risk of preterm birth following fresh blastocyst transfer. Therefore, when examining the association between embryo cryopreservation duration and implantation outcomes, separate consideration of cleavage-stage embryos and blastocysts is required. However, the vast majority of studies on human embryo vitrification have been conducted with cryopreservation durations of less than 2 or 5 years, and the effects on reproductive outcomes have been inconsistent[12, 17, 18].

Against this backdrop, this study aimed to investigate the relationship between vitrification duration and clinical outcomes basing on a large-scale retrospective cohort analysis of patients receiving frozen-thawed embryo transfer.

II. Materials and Methods

1. Study Design and Participants

This study conducted a retrospective analysis at the Reproductive and Genetic Center of Shandong University of Traditional Chinese Medicine Affiliated Hospital (hereinafter referred to as "the Center") of women who underwent vitrified embryo thaw and transfer at the Center between January 2015 and January 2025. If a woman underwent multiple transfer cycles, only the first cycle was included in the analysis. Inclusion criteria: patients who underwent in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) at this hospital due to tubal infertility, ovulatory dysfunction, or male factor infertility; patients in whom both partners had normal chromosomes; and patients whose embryos were all cryopreserved via vitrification. Exclusion criteria: patients with no embryos suitable for transfer after vitrification; patients with chromosomal abnormalities in one or both partners; patients with anatomical abnormalities of the female reproductive organs, including unicornuate, bicornuate, and septate uterus, intrauterine adhesions, and endometriosis; patients with a history of recurrent miscarriage or repeated implantation failure; and patients with severe concomitant medical or surgical conditions. A total of 13,054 cycles were included and divided into four groups based on the duration of cryopreservation: Frozen Group 1 (≤0.5 years), Frozen Group 2 (0.5–2 years), Frozen Group 3 (2–5 years), and Frozen Group 4 (>5 years).

2. ART Protocol

Based on each patient's clinical characteristics (age, BMI, number of antral follicles, basal hormone levels, and anti-Müllerian hormone levels) to administer recombinant follicle-stimulating hormone (FSH) or human menopausal gonadotropin (hMG) at a dose of 150–450 IU/day, and selects the appropriate protocol from gonadotropin-releasing hormone agonist (GnRH-a) long protocol, GnRH-a short protocol, or gonadotropin-releasing hormone antagonist (GnRH-ant). Oocyte retrieval was performed 34–36 h after the trigger. Depending on oocyte maturity and semen processing time, insemination is conducted within 4–6 h after oocyte culture. After fertilization was verified, the embryos were transferred to a cleavage-stage culture medium for further development. Morphological evaluation and transfer of cleavage-stage embryos typically occur on the second or third day after oocyte retrieval.

Currently, our center primarily evaluates embryo morphology based on cell count (C), fragmentation (F), and symmetry of the cleavage cells (S). Generally, D3 embryos scoring less than 6 points are considered non-viable, while those scoring 6 points or more are viable, and those scoring 7 points or more are high-quality embryos. Usable embryos are either transferred or frozen based on the patient's condition or cultured further to the blastocyst stage for subsequent transfer or freezing, and non-usable embryos are discarded. Blastocysts are primarily scored based on the size of the blastocyst cavity, inner cell mass, and condition of the trophoblast cells, and are denoted by a number followed by two uppercase letters (e.g., 4AA, 3AB). In principle, if a D5/D6 embryo has not developed into a blastocyst, embryos with an inner cell mass or trophoblast cell score of "C" on D5/D6 are not recommended for transfer. If an embryo had not developed to the blastocyst stage by D5, it was cultured until D6 for re-evaluation.

All ART procedures were conducted in accordance with standard hospital operating procedures.

3. Embryo Cryopreservation and Thawing Techniques

Cryopreservation: At room temperature, prepare the cryopreservation solutions: equilibrium solution (ES) and vitrification solution (VS). Shake well before use. Using a 100-μL pipette tip, dispense 50 μL of ES and three droplets of VS, each approximately 20 μL. Seal the bottle after use and store it in the designated refrigerator compartment. Prepare the VS in advance and allow it to return to room temperature. Turn off the lights; temporarily place the embryos to be cryopreserved in the incubator on the left and place the basket into the liquid nitrogen tank. Apply ES for 5 minutes, followed by three drops of VS for 20 seconds each. Start timing once the embryos are placed into the solution. Continuously pipette the embryos in the VS, then mount them onto the loading rod, aspirating as little liquid as possible. Quickly immerse the loading rod into liquid nitrogen, secure the cap while submerged, ensuring the tip of the loading rod never leaves the liquid nitrogen. Rapidly insert the loading rod into the holder sleeve and place it in the designated position within the liquid nitrogen tank.

Thawing: Remove the thawing solutions from the refrigerator. Shake well before use. Using a 1-mL pipette, add 0.5 mL of S4, 0.5 mL of S3, 0.5 mL of S2, and 1 mL of S1 into the inner circle of a 3037 dish. Then warm S1 to 37°C in an incubator. Remove the cap from the loading rod while it is submerged in liquid nitrogen, and quickly place the rod into S1 for 1 minute with gentle agitation. Keep observing the embryo under the microscope throughout this process until it becomes visible. Subsequently, immerse the rod in S2, S3, and S4 for 3 minutes each. Then record a video. Transfer the embryo to a four-well dish, holding the pipette high to avoid the tip touching the walls of the dish. Finally, transfer the embryo to a transfer dish, place it in the incubator, and await transfer.

During the study, the same freezing and thawing procedures, embryo cryopreservation methods, technical equipment, and storage vials were consistently employed.

4. FET Protocol

Endometrial preparation was selected based on the patient's menstrual cycle and ovulation status. A natural cycle was used for women with regular cycles and normal ovulation, utilizing the patient's own ovulatory cycle and endogenous hormones for endometrial preparation. An artificial cycle was primarily applied to patients with irregular cycles, ovulatory dysfunction, or no dominant follicle growth (diameter >10 mm) on transvaginal ultrasound in previous cycles. These patients returned to the clinic on days 2–4 of menstruation. After confirming hormonal eligibility and the absence of follicles >10 mm in both ovaries, oral Complex Packing Estradiol Tablets/Estradiol and Dydrogesterone Tablets (Femoston) or Estradiol Valerate Tablets (Progynova) was initiated. Transvaginal ultrasound was repeated after 7 days, and the dose was adjusted according to endometrial thickness. When treatment duration reached 12–14 days, with serum estradiol (E_2) ≥100 ng/mL and endometrial thickness ≥8 mm, endometrial transformation was induced. Embryo transfer was performed under ultrasound guidance, with timing selected according to the embryonic stage: cleavage-stage embryos were transferred on day 3 after ovulation or progesterone administration, while blastocysts were transferred on day 5 after ovulation or day 6 after progesterone. All patients received progesterone supplementation after transfer to support luteal function.

5. Clinical Outcomes

Primary outcomes included clinical pregnancy rate (CPR), cycle implantation rate (CIR), and live birth rate (LBR). Secondary outcomes included multiple pregnancy rate (MPR), biochemical pregnancy rate (BPR), miscarriage rate (MR), and ectopic pregnancy rate (EPR). Clinical pregnancy was defined as positive urinary hCG and serum β-hCG 14 days post-transfer, confirmed by intrauterine gestational sac on ultrasound at 4 weeks. CPR = number of clinical pregnancy cycles / total frozen-thawed transfer cycles. Implantation was defined as detection of a gestational sac by transvaginal ultrasound. CIR = number of implantation cycles / total transfer cycles. Live birth was delivery of a live infant after 28 weeks' gestation. LBR = number of live birth cycles / total transfer cycles. Multiple pregnancy was presence of >1 intrauterine fetus. MPR = number of multiple pregnancy cycles / clinical pregnancy cycles. Biochemical pregnancy was elevated serum hCG at 14 days post-transfer without a gestational sac. BPR = number of biochemical pregnancy cycles / total transfer cycles. Miscarriage was loss of fetal heart activity within 28 weeks after clinical confirmation. MR = number of miscarriage cycles / clinical pregnancy cycles. Ectopic pregnancy was diagnosed by ultrasound or laparoscopy showing at least one ectopic gestational sac. EPR = number of ectopic pregnancy cycles / clinical pregnancy cycles.

6. Statistical Analysis

Data were analyzed using SPSS 25.0. Normally distributed continuous variables were expressed as mean ± standard deviation and compared using ANOVA. Categorical variables were expressed as counts and percentages and compared using the chi-square test or Fisher's exact test. P-value <0.05 was considered statistically significant. After adjusting for confounding factors (maternal age at FET, BMI, infertility type and cause, number of transferred embryos, embryo development status, embryo quality, and endometrial preparation), multivariate logistic regression examined the effect of storage duration on pregnancy outcomes. Cryopreservation duration was treated as a categorical variable with Group 1 as reference, and adjusted odds ratios (aOR) with 95% confidence intervals (CI) were calculated. Multivariate logistic analyses were also performed after stratification by embryo developmental stage and transfer strategy, with adjustment for the same confounders.

To analyze the interaction between age and freezing duration on live birth rates, we first described freezing duration and age groups using frequencies and percentages. Age groups were divided into three categories using 35 and 38 years as cutoffs per 2024 ESHRE guidelines[19]: <35, 35–37, and ≥38 years. We then performed multivariable logistic regression analyses stratified by age at transfer and age at freezing, with live birth rate as the dependent variable and freezing duration as the independent variable. Confounding factors (BMI, number of transferred embryos, embryo quality, developmental stage) were adjusted to calculate aORs and 95% CIs. In the full-sample model, a product term of age group and freezing duration was included; the Wald test assessed interaction significance (P<0.05). A ROC curve for live birth was plotted. Based on the maximum Youden index, the optimal cutoff for age at transfer was 35.5 years, rounded to 36 years (≤35 vs. ≥36), for stratified analysis.

A critical confounding factor remains: the discrepancy between “age at freezing” and “age at transfer.” In clinical practice, patients often preserve embryos at a young age (e.g., egg freezing, embryo cryopreservation, or delayed transfer) but undergo transfer many years later. Age at freezing determines embryo quality (chromosomal aneuploidy rate and egg quality), while age at transfer determines the uterine environment, endometrial receptivity, and endocrine status. For example, a woman who froze embryos at age 30 (good embryo quality) and transferred them at age 42 (aged uterus) might fail to achieve a live birth. Is this due to 12 years of freezing damage or the aged uterus? To analyze this, we applied a ROC curve to “age at freezing” and identified an optimal inflection point of 34.5 years. Participants were then divided using this cutoff: ≤34.5 years (AgeFreeze_Group1) and >34.5 years (AgeFreeze_Group2). Multivariate logistic regression was subsequently performed on the two groups.

III. Results

This study included 13,054 cycles. Patients were divided into four groups according to cryopreservation duration: FrozenGroup 1 (n=8,622, ≤0.5 years), FrozenGroup 2 (n=3,240, 0.5–2 years), FrozenGroup 3 (n=939, 2–5 years), and FrozenGroup 4 (n=253, >5 years).

Table 1 presents the baseline and clinical characteristics of patients in each group. The mean age at frozen embryo transfer (FET) was 33.35±5.28 years, mean BMI was 23.75±3.88 kg/m², and mean embryo cryopreservation duration was 0.74±1.88 years (maximum 12.3 years). With increasing storage duration, maternal age at FET increased ($P<0.05$), and the proportions of secondary infertility and female infertility rose significantly. The proportion of cleavage-stage embryo transfers increased, whereas that of blastocyst transfers decreased ($P<0.05$). Additionally, significant differences were observed among groups in the number of embryos transferred, embryo quality, and endometrial preparation protocols ($P<0.05$). Clinical outcomes, including clinical pregnancy rate, implantation rate, biochemical pregnancy rate, and live birth rate, declined with longer cryopreservation duration, with significant differences across the four groups ($P<0.05$). However, no significant differences were found in miscarriage rate, ectopic pregnancy rate, or multiple pregnancy rate ($P>0.05$).

Table 1: Baseline and Clinical Characteristics of Study Participants by Freezing Duration Group						
	All cycles	**FrozenGroup1**	**FrozenGroup2**	**FrozenGroup3**	**FrozenGroup4**	**P**
Number of frozen-thawed transfer cycles,n(%)	13054	8622	3240	939	253	
Female age at FET (years)	33.35±5.28	33±5.35	33.92±5.21	33.83±4.77	36.22±4.17	0.05
Female BMI (kg/m^2)	23.75±3.88	23.77±3.89	23.73±3.91	23.64±3.77	23.59±3.74	0.7
Embryo cryopreservation duration (years)	0.74±1.88	0.23±0.12	0.94±0.38	3.22±0.80	6.45±1.42	0.05
Type of infertility,n (%)						0.05
Primary	5295(40.56%)	3989(46.26%)	1193(34.88%)	131(13.95%)	18(7.11%)	
Secondary	7759(59.44%)	4635(53.76%)	2047(59.85%)	808(86.05%)	234(92.89%)	
Main causes of infertility, n (%)						0.05
Female factor	10447(80.03%)	6556(76.04%)	2910(85.09%)	761(81.04%)	220(86.96%)	
Male factor	340(2.60%)	254(2.95%)	52(1.52%)	24(2.56%)	10(3.95%)	
Male and female factors	2267(17.37%)	1812(21.02%)	458(13.39%)	154(16.40%)	23(9.09%)	
Number of embryos transferred, n (%)						0.05
1	4142(31.73%)	2757(31.98%)	1081(33.36%)	250(26.62%)	54(21.34%)	
2	8579(65.72%)	5750(66.69%)	2013(62.13%)	629(66.97%)	187(73.91%)	
2	333(2.55%)	115(1.33%)	146(4.51%)	60(6.41%)	12(4.75%)	
Development stage of embryo trans ferred, n (%)						0.05
Cleavage embryo	8623(66.06%)	5735(66.52%)	2063(63.67%)	628(66.88%)	197(77.87%)	
Blastocyst	3261(24.98%)	2163(25.09%)	864(26.67%)	207(22.04%)	27(10.67%)	
Sequential transplantation	1170(8.96%)	724(8.39%)	313(9.66%)	104(11.08%)	29(11.46%)	
Quality of transferred embryos, n (%)						0.05
Good-quality	6562(50.27%)	4489(52.06%)	1450(44.75%)	471(50.16%)	152(60.08%)	
Non-Good-quality	6492(49.73%)	4133(47.93%)	1790(55.25%)	468(49.84%)	101(39.92%)	
FET Endometrial Preparation Protocol, n (%)						0.05
Artificial cycle	8548(65.48%)	5692(66.02%)	2137(65.96%)	567(60.38%)	152(60.08%)	
Natural cycle	4506(34.52%)	2930(33.98%)	1103(34.04%)	372(39.61%)	101(39.92%)	
Clinical pregnancy rate (%)	6465/13054(49.53%)	4321/8622(50.12%)	1571/3240(48.49%)	463/939(49.31%)	110/253(43.48%)	0.026
Cycle implantation rate (%)	6306/13054(48.31%)	4221/8622(48.96%)	1536/3240(47.41%)	422/939(44.94%)	107/253(42.29%)	0.005
Live birth rate (%)	5225/13054(40.03%)	3537/8622(41.02%)	1240/3240(38.27%)	355/939(37.81%)	93/253(36.76%)	0.035
Biochemical pregnancy rate (%)	295/13054(2.26%)	199/8622(2.31%)	82/3240(2.53%)	5/939(0.53%)	9/253(3.56%)	0.01
Miscarriage rate (%)	1098/6465(16.98%)	691/4321(15.99%)	304/1571(19.35%)	85/463(18.36%)	18/110(16.36%)	0.378
Incidence of ectopic pregnancy rate (%)	138/6465(2.13%)	101/4321(2.34%)	27/1571(1.72%)	9/463(1.94%)	1/110(0.91%)	0.283
Multiple pregnancy rate (%)	1342/6465(20.76%)	988/4321(22.87%)	283/1571(18.01%)	57/463(12.31%)	14/110(12.73%)	0.076

Compared to storage ≤0.5 years, the >5 years group had significantly lower clinical pregnancy (aOR=0.757, P=0.036), cycle implantation (aOR=0.604, P=0.001), and live birth rates (aOR=0.881, P=0.003). No significant differences in these primary outcomes were observed for the 2–5 years group. Longer storage (2–5 years and >5 years) did not significantly affect secondary outcomes (biochemical pregnancy, miscarriage, ectopic pregnancy, multiple pregnancy) (Table 2).

Table 2: Multivariate Logistic Regression: Storage Time and Pregnancy Outcomes		
	P	**aOR (95% CI)**
Clinical pregnancy rate		
FrozenGroup1	ref.	
FrozenGroup2	0.027	0.912(0.840-0.990)
FrozenGroup3	0.196	0.913(0.796-1.048)
FrozenGroup4	0.036	0.757(0.583-0.983)
Cycle implantation rate		
FrozenGroup1	ref.	
FrozenGroup2	0.026	0.911(0.839-0.989)
FrozenGroup3	0.434	0.947(0.825-1.086)
FrozenGroup4	0.001	0.604(0.460-0.794)
Live birth rate		
FrozenGroup1	ref.	
FrozenGroup2	0.120	0.814(0.628-1.055)
FrozenGroup3	0.049	0.875(0.762-0.995)
FrozenGroup4	0.003	0.881(0.811-0.956)
Miscarriage rate		
FrozenGroup1	ref.	
FrozenGroup2	0.015	1.190(1.034-1.370)
FrozenGroup3	0.200	0.841(0.645-1.096)
FrozenGroup4	0.150	0.670(0.389-1.155)
Incidence of ectopic pregnancy rate		
FrozenGroup1	ref.	
FrozenGroup2	0.180	0.763(0.497-1.140)
FrozenGroup3	0.569	0.820(0.413-1.627)
FrozenGroup4	0.277	0.335(0.047-2.409)
Multiple pregnancy rate		
FrozenGroup1	ref.	
FrozenGroup2	0.172	1.091(0.963-1.236)
FrozenGroup3	0.000	0.517(0.392-0.682)
FrozenGroup4	0.006	0.467(0.271-0.803)

Table 3 presents stratified analyses by embryo development stage and quality, the results showed: Cleavage-stage embryos (n=8,623): Using storage ≤0.5 years as reference, storage >5 years was associated with significantly lower implantation rate (aOR=0.861, 95%CI 0.777–0.955, P=0.004) and live birth rate (aOR=0.861, 95%CI 0.777–0.955, P=0.014). No significant differences were observed for storage durations of 0.5–2 years or 2–5 years; Blastocysts (n=3,261): For storage of 2–5 years, clinical pregnancy rate (aOR=0.889, 95%CI 0.750–1.054) and implantation rate (aOR=0.819, 95%CI 0.373–1.798) showed decreasing trends without statistical significance; live birth rate was also non-significant. For storage >5 years, live birth rate was significantly decreased, while clinical pregnancy and implantation rates showed numerical declines but were not significant, possibly due to small sample size; Sequential transfer group (n=1,170): Compared with the reference (≤0.5 years), none of the storage durations (0.5–2, 2–5, >5 years) showed significant differences in clinical pregnancy, implantation, or live birth rates, indicating relatively stable outcomes across storage durations; High-quality embryos: Storage >5 years was associated with significantly lower clinical pregnancy rate (P=0.042), implantation rate (P=0.004), and live birth rate (P=0.014) compared with the reference, with all aORs <1. No significant differences were found for 0.5–2 or 2–5 years; Non-high-quality embryos: For storage >5 years, implantation rate was

significantly reduced (P=0.009, aOR=0.534). Clinical pregnancy and live birth rates also showed decreasing trends but did not reach statistical significance (P>0.05). Other storage durations showed no significant differences.

Table 3: Stratified Logistic Regression: Embryo Development Stage and Quality

	Clinical pregnancy rate	P	aOR (95% CI)	Cycle implantation rate	P	aOR (95% CI)	Live birth rate	P	aOR (CI)
Cleavage embryo(n=8623)	4177/8623(48.44%)			5497/16298(33.73%)			3410/8623 (39.55%)		
FrozenGroup1	2823/5735(49.22%)	ref.		3701/10802(34.26%)	ref.		2332/5375 (43.39%)	ref.	ref.
FrozenGroup2	964/2063(46.73%)	0.032	0.893(0.806-0.990)	1264/3889(32.50%)	0.040	0.897(0.809-0.995)	772/2063 (37.42%)	0.071	0.760 1.024
FrozenGroup3	310/628(49.36%)	0.969	1.003(0.848-1.187)	417/1229(33.93%)	0.630	1.042(0.881-1.233)	238/628 (37.89%)	0.175	0.889 1.054
FrozenGroup4	81/197(41.11%)	0.070	0.759(0.562-1.023)	115/378(30.42%)	0.004	0.631(0.462-0.862)	68/197 (34.52%)	0.014	0.861 0.955
Blastocyst(n=3261)	1676/3261(51.39%)			1762/3645(48.34%)			1327/3261 (40.71%)		
FrozenGroup1	1123/2163(51.92%)	ref.		1168/2352(49.66%)	ref.		902/2163 (41.70%)	ref.	ref.
FrozenGroup2	448/864(51.85%)	0.283	0.917(0.783-1.074)	487/1020(47.16%)	0.186	0.899(0.767-1.053)	348/864 (40.28%)	0.619	0.819 1.798
FrozenGroup3	92/207(44.44%)	0.001	0.610(0.452-0.823)	97/240(40.42%)	0.002	0.626(0.465-0.845)	67/207 (32.37%)	0.371	0.930 1.091
FrozenGroup4	13/27(48.15%)	0.359	0.693(0.316-1.519)	10/28(35.71%)	0.051	0.423(0.178-1.005)	10/27 (37.04%)	0.011	0.677 0.915
Sequential transplantation(n=1170)	611/1170(52.81%)			792/2353(33.66%)			486/1170 (41.54%)		
FrozenGroup1	375/724(50.79%)	ref.		486/1446(33.61%)	ref.		302/724 (41.71%)	ref.	ref.
FrozenGroup2	159/313(50.80%)	0.771	0.961(0.737-1.254)	204/633(32.22%)	0.845	0.974(0.746-1.271)	120/313 (38.34%)	0.297	0.866 1.134
FrozenGroup3	61/104(58.65%)	0.466	1.165(0.772-1.760)	80/217(36.87%)	0.331	1.227(0.812-1.852)	50/104 (48.08%)	0.224	1.291 1.950
FrozenGroup4	16/29(55.17%)	0.953	1.023(0.485-2.158)	22/57(38.59%)	0.588	0.810(0.311-1.739)	14/29 (48.28%)	0.487	1.302 2.737
Good-quality (n=6562)	3688/6562(56.20%)			4829/12025(40.16%)			3014/6562 (45.93%)		
FrozenGroup1	2590/4489(57.70%)	ref.	ref.	3319/8133(40.81%)	ref.	ref.	2150/4489 (47.89%)	ref.	ref.
FrozenGroup2	766/1450(52.83%)	0.153	0.917(0.815-1.033)	1061/2691(39.41%)	0.177	0.922(0.819-1.038)	595/1450 (41.03%)	0.154	0.788 1.094
FrozenGroup3	258/471(54.78%)	0.618	0.953(0.787-1.152)	347/915(37.92%)	0.768	0.972(0.803-1.176)	205/471 (43.52%)	0.066	0.835 1.012
FrozenGroup4	74/152(48.68%)	0.042	0.721(0.518-0.983)	102/284(35.92%)	0.004	0.607(0.432-0.853)	64/152 (42.11%)	0.014	0.749 0.844
Non-Good-quality (n=6492)	2777/6492(42.78%)			3222/10273(31.36%)			2209/6492 (34.03%)		

FrozenGroup1	1731/4133(41.88%)	ref.	ref.	2036/6467(31.48%)	ref.	ref.	1386/4133 (33.53%)	ref.	ref.
FrozenGroup2	805/1790(44.97%)	0.565	0.967(0.861-1.085)	894/2851(31.36%)	0.474	0.958(0.853-1.076)	645/1790 (36.03%)	0.103	1.101 1.236
FrozenGroup3	205/468(43.80%)	0.242	0.886(0.724-1.085)	247/771(32.04%)	0.518	0,936(0.764-1.145)	150/468 (32.05%)	0.559	0.941 1.154
FrozenGroup4	36/101(35.64%)	0.168	0.738(0.480-1.136)	45/179(25.14%)	0.009	0.534(0.334-0.854)	28/101 (27.73%)	0.194	0.747 1.160

Figure 1 shows the distribution of the Frozen Group (thawed embryo group) and Age Group by live birth outcome.

Table 4 showed: for AgeGroup1, live birth rates in Frozen Groups 2–4 were lower than in Group 1; for AgeGroups2 and 3, no significant differences among groups; and all three groups indicated embryo quality and total transferred embryos were independent factors for live birth.

Table 4: Multivariate Logistic Regression: Storage Duration and Pregnancy Outcomes, by Age Group

		P	aOR(95%CI)
AgeGroup1	FrozenGroup1	ref.	ref.
	FrozenGroup2	0.029	0.888(0.799-0.988)
	FrozenGroup3	0.003	0.762(0.637-0.911)
	FrozenGroup4	0.004	0.508(0.320-0.805)
	BMI	0.398	1.005(0.994-1.016)
	Quality of transferred embryos	<0.001	1.573(1.435-1.724)
	Number of embryos transferred	0.001	1.172(1.064-1.292)
	Development stage of embryo trans	0.143	1.054(0.982-1.132)
AgeGroup2	FrozenGroup1	ref.	ref.
	FrozenGroup2	0.673	1.045(0.853-1.280)
	FrozenGroup3	0.149	1.260(0.921-1.724)
	FrozenGroup4	0.723	1.084(0.694-1.693)
	BMI	0.841	1.002(0.980-1.025)
	Quality of transferred embryos	0.000	1.717(1.433-2.057)
	Number of embryos transferred	0.017	1.245(1.040-1.490)
	Development stage of embryo trans	0.005	1.221(1.061-1.404)
AgeGroup3	FrozenGroup1	ref.	ref.
	FrozenGroup2	0.927	1.009(0.833-1.223)
	FrozenGroup3	0.744	0.944(0.668-1.333)
	FrozenGroup4	0.175	1.390(0.864-2.237)
	BMI	0.253	1.015(0.990-1.041)
	Quality of transferred embryos	0.000	1.471(1.237-1.749)
	Number of embryos transferred	0.002	1.308(1.102-1.552)
	Development stage of embryo trans	0.011	1.187(1.040-1.355)

Note: Confounding factors such as BMI, number of embryos transferred, embryo quality, and embryo development stage have been controlled for.

The full model's interaction analysis (Table 5) showed a significant Age Group × Frozen Group interaction (OR = 1.112, 95% CI 1.045–1.184, p = 0.001), and it indicated that age moderated the relationship. Specifically, the OR > 1 implied that the negative effect of frozen group on live birth was attenuated in older age groups (OR approached 1) but more pronounced in younger age groups.

Table 5: Interaction Tests for the Full Model		
	P	aOR(95%CI)
BMI	0.206	1.006(0.997-1.015)
Quality of transferred embryos	<0.001	1.573(1.461-1.694)
Number of embryos transferred	<0.001	1.213(1.124-1.309)
Development stage of embryo trans	<0.001	1.110(1.049-1.175)
AgeGroup	<0.001	0.601(0.543-0.667)
FrozenGroup	<0.001	0.792(0.707-0.888)
AgeGroup by FrozenGroup	0.001	1.112(1.045-1.184)

Based on the ROC curve and coordinate table generated by SPSS, we reclassified the data into younger (≤35) and older (≥36) groups according to the statistically optimal cutoff, then performed multivariate logistic regression on the two groups again.

The results are presented in Table 6. In the younger group, freezing time significantly affected live birth rate (overall P = 0.003). Compared with freezing time ≤0.5 years, no significant change was observed for 0.5–2 years (OR = 0.91, 95% CI: 0.83–1.01, P = 0.077), but live birth rates were significantly lower for 2–5 years (OR = 0.80, 95% CI: 0.67–0.95, P = 0.009) and >5 years (OR = 0.61, 95% CI: 0.42–0.90, P = 0.013), indicating that in younger women, freezing time exceeding 2 years led to a reduced live birth rate. In the older group, freezing time had no significant effect on live birth rate (overall P = 0.448). The OR values for all freezing time subgroups were not statistically significant (all P > 0.05), suggesting that freezing time is not a major factor affecting live birth rate in older women.

Table 6: Multivariate Logistic Regression: Freezing Time and Live Birth Rate, by Age		
Variable	Younger group (≤35 years old)	Older group (≥36 years old)
FrozenGroup1	ref.	ref.
FrozenGroup2	0.913(0.825-1.010)	0.994(0.851-1.160)
FrozenGroup3	0.799(0.674-0.947)	1.126(0.874-1.450)
FrozenGroup4	0.614(0.417-0.903)	1.283(0.894-1.840)
P for trend	0.003	0.448
BMI	1.003(0.992-1.013)	1.014(0.995-1.034)
Quality of transferred embryos	0.637(0.584-0.695)	0.632(0.550-0.725)
Number of embryos transferred	1.181(1.077-1.294)	1.284(1.121-1.470)
Development stage of embryo trans	1.066(0.997-1.141)	1.236(1.113-1.373)

To test whether age modifies the effect of freezing time on live birth rate, we included age group (Age_ROC), freezing time group (FrozenGroup), and their interaction term in a multivariate logistic regression analysis in the full sample (Table 7). After adjusting for confounders, a significant interaction was found between age and freezing time (overall interaction P=0.008). Specifically, compared with freezing ≤0.5 years, the effect of age on live birth rate differed significantly for freezing durations of 2–5 years (interaction OR=1.42, 95%CI: 1.05–1.92, P=0.024) and >5 years (OR=2.09, 95%CI: 1.23–3.53, P=0.006), indicating that the impact of freezing time on live birth rate varies by age. These findings support the rationale for stratified analysis.

Table 7: Analysis of the interaction between age and freezing time on live birth rates		
Variable	P	aOR(95% CI)
Main effect		
Age group (younger vs. older)	<0.001	0.532(0.481-0.588)
FrozenGroup	0.002	
FrozenGroup2	0.064	0.909(0.822-1.006)
FrozenGroup3	0.008	0.794(0.670-0.941)
FrozenGroup4	0.013	0.613(0.416-0.902)
AgeRoc by FrozenGroup	0.008	
AgeRoc1 by FrozenGroup2	0.347	1.092(0.909-1.313)
AgeRoc1 by FrozenGroup3	0.024	1.420(1.048-1.923)
AgeRoc1 by FrozenGroup4	0.006	2.085(1.230-3.534)

Note: Adjustments were made for BMI, total number of transferred embryos, embryo quality, and embryo development; P-values for interaction terms are based on the Wald test.

The results in Table 8 showed that among women aged ≤34.5 years at embryo formation (better oocyte quality), longer cryopreservation significantly decreased live birth rate, while no effect was seen in older women (>34.5 years). In the younger group, 0.5–2 years reduced live birth rate by ~11.5%, 2–5 years by 23.9%, and >5 years by 33.4%, showing a clear dose-response relationship.

Table 8 : Multivariate Logistic Regression: Storage Duration and Pregnancy Outcomes, by Frozen Embryo Age Group			
		P	aOR(95%CI)
AgeFreeze_Group1	FrozenGroup1	ref.	ref.
	FrozenGroup2	0.019	0.885(0.800-0.980)
	FrozenGroup3	0.001	0.761(0.650-0.890)
	FrozenGroup4	0.004	0.666(0.505-0.877)
	BMI	0.380	1.005(0.994-1.015)
	Quality of transferred embryos	<0.001	1.560(1.428-1.705)
	Number of embryos transferred	<0.001	1.195(1.089-1.311)
	Development stage of embryo trans	0.038	1.075(1.004-1.151)
AgeFreeze_Group2	FrozenGroup1	ref.	ref.
	FrozenGroup2	0.280	0.920(0.791-1.070)
	FrozenGroup3	0.466	0.884(0.635-1.231)
	FrozenGroup4	0.408	0.692(0.290-1.656)
	BMI	0.483	1.007(0.988-1.025)
	Quality of transferred embryos	<0.001	1.609(1.407-1.841)
	Number of embryos transferred	0.002	1.231(1.078-1.841)
	Development stage of embryo trans	<0.001	1.211(1.092-1.342)

IV. Discussion

This study, based on a large cohort of 13,054 frozen-thawed embryo transfer cycles, systematically analyzed the effect of vitrified embryo storage duration on clinical outcomes and specifically explored the interaction between age and storage time. The main findings were: storage exceeding 5 years significantly reduced clinical pregnancy, cycle implantation, and live birth rates; cleavage-stage and high-quality embryos were more sensitive to long-term storage, while blastocysts and sequential transfer showed relatively stable outcomes; age significantly moderated the effect on live birth rate: in women aged ≤35 years at transfer, storage >2 years was associated with a decreased live birth rate, but no independent effect was seen in those aged ≥36 years; in women aged ≤34.5 years at cryopreservation, longer storage also led to a dose-dependent decline in live birth rate, whereas no such effect occurred in older women at cryopreservation. These results suggested that the impact of cryopreservation duration on pregnancy outcomes did not exist independently but interacted complexly with female age (both at transfer and at cryopreservation).

The core innovation of this study was analyzing age's moderating effect on freezing duration and live birth rate. In the younger group (≤35 years at transfer), freezing durations of 2–5 years and >5 years significantly reduced live birth rate (trend test P=0.003); in the older group (≥36 years), ORs were not significant (overall P=0.448). An interaction test confirmed effect modification (interaction P=0.001, OR=1.112). To explain this, we hypothesized that age acted as an "effect-masking factor." In older women, the uterine environment, endometrial receptivity, endocrine status, and vascular function had degenerative changes. The negative impact of these age-related host factors on pregnancy outcomes far outweighed additional risks from freezing duration. Thus, at age ≥36 years at transfer, uterine aging was the primary factor limiting live birth, and freezing duration no longer exerted an additive effect. In contrast, younger women had superior uterine conditions, making embryo quality key; thus, the detrimental effects of freezing duration were fully manifested.

This study also separately analyzed the role of age at freezing, and the results were highly consistent: in women aged ≤34.5 years at freezing, prolonged freezing significantly reduced live birth rate (OR decreased from 0.885 to 0.666); whereas in those aged >34.5 years, freezing duration had no additional effect. This further reinforced the logic that maternal age at embryo formation determined baseline embryo quality; embryos from younger oocytes were more sensitive to freezing damage, while those from older women lacked sufficient quality reserves, which made freezing duration no longer the primary factor.

This study had limitations: As a retrospective study, unmeasured biases (e.g., embryo karyotype, endometrial receptivity) might still exist despite confounder adjustment. The long-term freezing group (>5 years) had a small sample size, limiting statistical power. No long-term newborn follow-up was conducted, so the impact on offspring safety requires further study. Future prospective, multicenter studies with PGT and epigenetic analysis are needed to investigate molecular mechanisms and guide individualized transfer strategies. Based on these results, we recommended: For young women (≤35 years) or embryos frozen young, implantation within 2 years is ideal. For embryos frozen >5 years, patients should be informed of potential risks, but such embryos may still be used. Older women should focus on embryo quality over freezing duration.

Conclusion

In conclusion, prolonged vitrification storage (> 5 years) significantly reduced clinical pregnancy, implantation, and live birth rates, while age critically moderated these effects. In younger women (≤ 35 years at transfer or ≤ 34.5 years at freezing), longer storage duration was associated with a dose-dependent decline in live birth rate. However, no independent effect of storage time was observed in older women. These findings highlight the importance of individualized embryo transfer strategies.

Declarations

Consent to participate

All participants in this study signed the relevant informed consent forms while undergoing ART treatment. This study was approved by the Ethics Review Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (Approval No.: (2026)□□□(082)□-KY). All procedures performed were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments.

Consent for publication

All authors have reviewed and approved the final draft, are responsible for the content of the submission, and have resolved any disputes regarding authorship prior to submission.

Ethical approval and its number

This study was reviewed and approved by the Institutional Review Board (IRB) of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. Approval number: (2026)□□□(082)□-KY

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Author Contribution

JYX was responsible for data collection, data analysis, and manuscript preparation; ZY and CHZ contributed to data analysis and manuscript revision; and ZN contributed to the conceptualization and research design of the study.All authors read andapproved the final manuscript.

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Data Availability

Because of the retrospective nature of this study and the use of anonymized patient data from the affiliated hospital's database, the data that support the findings of this study are available from the corresponding author upon reasonable request. No new data were generated or analyzed beyond those used for the clinical outcomes assessment described.

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Figures

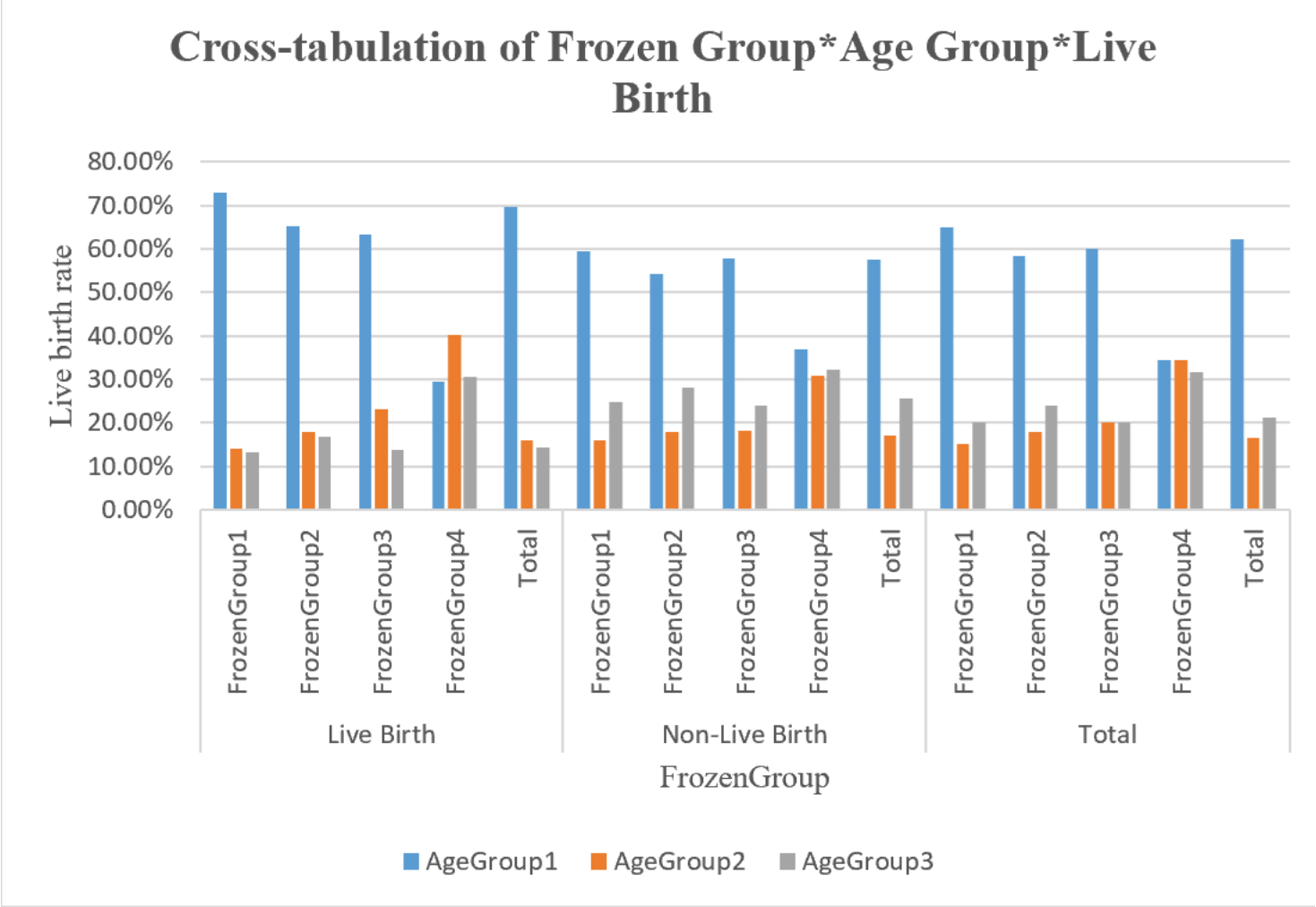


Figure 1

Distribution of the Frozen Group and Age Group by Live Birth Outcome