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Noneffectiveness of cryostorage duration on clinical and neonatal outcomes after single vitrified-warmed blastocyst transfers

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Abstract

Background In the current era of assisted reproductive technology, the strategy of single vitrified-warmed blastocyst transfer (SVBT) is acknowledged for positively impacting clinical outcomes and preventing the risk of multiple conceptions. Previous studies have indicated that blastocyst grade and the day of blastocyst positively correlate with outcomes. Moreover, storage duration has no significant effect on survival rates, clinical outcomes, and neonatal outcomes. However, some researchers express controversial opinions on cryostorage duration, and their findings suggest a negative impact on clinical outcomes. These results remain subject to controversy, and limited studies exist regarding the outcomes after SVBT. Therefore, our study aims to investigate the impact of the day of blastocyst, blastocyst grade, and blastocyst cryostorage duration on clinical and neonatal outcomes following SVBT in patients who underwent clomiphene-citrate-based minimal stimulation.

Material and methods In this study, retrospective cohort study data collected from June 2015 to April 2023 included 2107 patients with first SVBT cycles who underwent a clomiphene-based minimal ovarian stimulation protocol or a drug-free natural protocol at the Ojinmed IVF Center. Patients were categorized into four groups based on blastocyst cryostorage duration: group 1 (< 2 months, $n = 882$), group 2 (3–6 months, $n = 794$), group 3 (7–12 months, $n = 187$), group 4 (13–24 months, $n = 126$), and group 5 (25–81 months, $n = 118$). The patient's clinical and neonatal outcomes were compared with cryostorage duration after the propensity score matched.

Result Multivariable logistic regression analysis revealed that prolonged cryostorage duration insignificantly correlated with clinical outcomes. Additionally, neonatal outcomes are not correlated with cryostorage duration.

Conclusions The patient must consider several parameters when selecting embryos for transfer, including the duration of cryostorage. Our study results show that for the first single vitrified-warmed blastocyst transfer of patients who underwent clomiphene citrate-based minimal stimulation, cryostorage duration does not affect outcomes.

Keywords Cryostorage duration, Neonatal outcomes, Clinical outcomes, Vitrification, Cryopreservation

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Background

In 1983, the first transfer of cryopreserved human embryos at the fourth and eighth cell stages occurred after 4 months [1]. Currently, with the refinement of cryopreservation technology, clinical outcomes are better in frozen embryo transfer (FET) cycles than in fresh embryo cycles. Single vitrified-thawed blastocyst transfer (SVBT) has gained recognition for its ability to improve clinical outcomes and prevent multiple conceptions [2–4]. A noticeable trend in assisted reproductive technology (ART) is the increasing prevalence of FET cycles, with more than 50% of cases reported in some European countries (Armenia, Czech Republic, the Netherlands), constituting an overall proportion of 36.3%, worldwide [5]. Moreover, higher delivery rates are recorded after FET cycles than after fresh embryo transfer cycles. In Japan, the number of FET cycles has continuously increased since 2007, in 2019 at 51.7% freezing all cycles of total oocyte retrieval [6]. FET cycles in ART offer several advantages, such as endometrial preparation, flexibility in scheduling, improved pregnancy outcomes due to the selecting embryo, and preimplantation genetic testing [7].

The freezing embryos undergo vitrification to prevent the formation of ice crystals, which is presumed to cause less damage. However, the high concentration of cryoprotectants employed in vitrification could be cytotoxic, potentially leading to osmotic shock. Compared studies of vitrification and slow freezing may induce additional damage to embryos that may not be readily visible under microscopic examination but could adversely affect embryo viability [8, 9]. Vitrification is a rapid frozen technique that improves the survival rate of embryos, clinical pregnancy rate, and live birth rate [10, 11]. However, the potential toxicity of high concentrations of dimethyl sulfoxide (DMSO) during vitrification, and whether it affects the quality of blastocysts, remains unknown. Additionally, the impact of long-term storage of embryos in liquid nitrogen on blastocysts is uncertain. Previous studies have suggested that storage duration has no significant effect on survival rates, clinical outcomes, and neonatal outcomes. Despite some researchers holding controversial opinions on cryostorage duration, their findings indicate a negative impact on clinical outcomes. Long-term cryostorage of blastocysts in liquid nitrogen has shown adverse effects on clinical results. Furthermore, prolonged storage durations may lead to increased birth weight [12].

In current ART practices, FET cycles have continuously increased. However, the clinical and neonatal outcomes associated with blastocyst cryostorage duration remain not fully understood. Our study aims to investigate patients who underwent selectively single SVBT using minimal ovarian stimulation protocols. We aim to investigate the impact of cryostorage duration on clinical and neonatal outcomes, emphasizing the importance of considering this factor in the selection of blastocysts for transfer.

Materials and methods

Participants and study design

In this study, retrospective cohort study data collected from June 2015 to April 2023 included 2107 patients' first SVBT cycles from those who underwent a clomiphene-based minimal ovarian stimulation protocol or a drug-free natural protocol at the Ojinmed IVF Center, Mongolia. The blastocyst cryostorage duration was divided into four groups: group 1 (< 2 months, $n=882$), group 2 (3–6 months, $n=794$), group 3 (6–12 months, $n=187$), group 4 (12–24 months, $n=126$), and group 5 (24–81 months, $n=118$). Patients were matched using propensity score matching. After PSM, group 1 (< 2 months, $n=502$), group 2 (3–6 months, $n=443$), group 3 (6–12 months, $n=112$), group 4 (12–24 months, $n=84$), and group 5 (24–81 months, $n=91$). Patients who underwent a preimplantation genetic testing (PGT-A) cycle, using donor oocytes and sperm collected by PESA/TESE (percutaneous epididymal sperm aspiration and testicular sperm extraction), were excluded from this study. The study design and inclusion of participants followed the scheme illustrated in Fig. 1. All procedures were performed in accordance with the ethical standards of the ethics committee of our hospital (approval number: EA2023080502).

Stimulation, oocyte retrieval, fertilization procedures, and embryo culture

All patients underwent treatment with clomiphene citrate (CC; Fuji Pharma, Tokyo, Japan) based on a minimal ovarian stimulation protocol or natural cycle. This minimal stimulation protocol or natural cycle was developed by Kato O. and others [13, 14]. In minimal stimulation protocol, clomiphene citrate (50 mg/day) was administered from the third day of the menstrual cycle until final oocyte maturation, and, if necessary, the total dose of follicle-stimulating hormone (GONAL-f, Merck, Darmstadt, Germany) was 300–600 IU at menstrual cycle day 8 to day 12. In a natural cycle, it was drug-free. Oocyte retrieval was conducted without anesthesia when the main follicle was larger than 18 mm and used a 21- or 22-G needle (Kitazato OPU Needle, Kitazato, Tokyo, Japan). Follicular flushing was performed using an HTF solution (m-HTF with HEPES; Kitazato, Shizuoka, Japan) with 1% heparin during oocyte retrieval. Oocyte until insemination after maturation checks place in dish with HTF with 1% serum protein substitute (SPS, Kitazato,

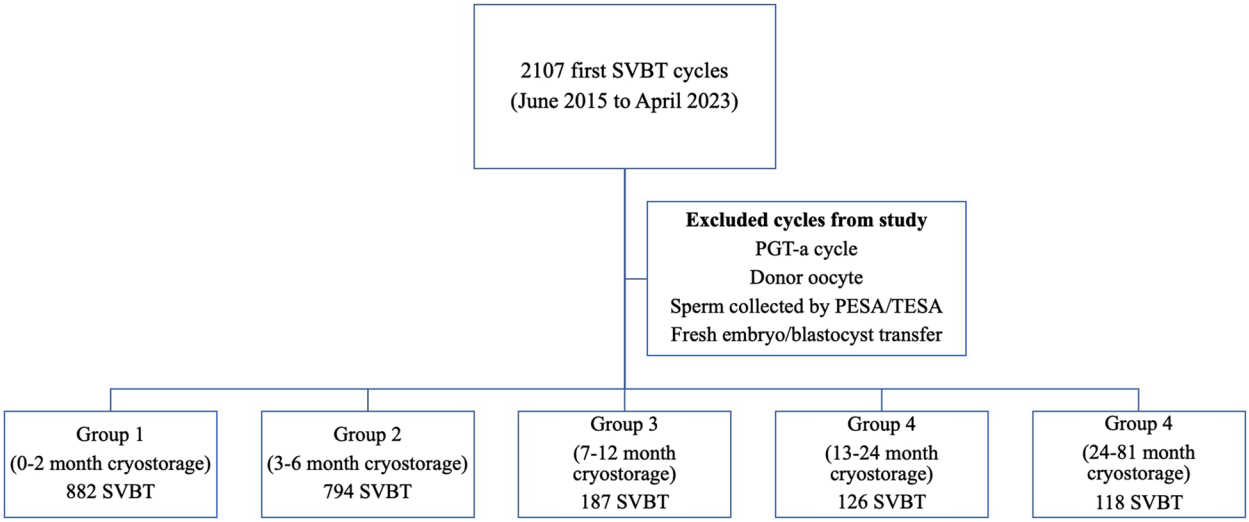

Fig. 1 Flowchart of study design. Process of data selection for study. Note: SVBT, single vitrified-warmed blastocyst transfer; PGT, preimplantation genetic test; *PESA/TESE*, percutaneous epididymal sperm aspiration/testicular epididymal sperm extraction

Shizuoka, Japan). The sperm source used only ejaculated sperm, and the samples were washed with the density gradient centrifuge method. HTF supplemented with an SPS was used as the sperm was kept until insemination. Conventional insemination was carried out approximately 3 h after, and ICSI (ICSI, TPC Micropipettes, CooperSurgical, Denmark) was carried out 4–5 h after retrieval of the mature oocyte.

Embryo culture, blastocyst monitoring, and cryopreservation

At 16–20 h after insemination, normal fertilization was confirmed by observing two pronuclei, and the zygotes were cultured individually in a drop of 30 µl of one-step medium (ONESTEP Medium, Naka IVF Medium, Naka Medical, Tokyo, Japan). All embryos were cultured to the blastocyst stage at 37 °C in 5% O_2, 6% CO_2, and 89% N_2 in water-jacketed incubators (APC-50DR, Penguin AQ series, Astec, Fukuoka, Japan) or dry incubators (EC-6S dry incubator, Astec, Fukuoka, Japan). On days 5 to 6, hatching blastocysts and blastocysts that reached an inner diameter > 160 µm were vitrified immediately according to the Cryotop method (Kitazato, Japan). Blastocyst inner diameter was measured using inverted microscopy (Eclipse TE-2000U, Nikon, Tokyo, Japan; IX71 Inverted Microscope, Olympus, Tokyo, Japan) and the corresponding imaging software (Octax EyeWare, Vitrolife, Sweden). If the blastocyst's inner diameter did not fulfill the desired > 160 µm, it continued to be cultured. We followed the standard protocols for vitrification and thawing, and blastocysts were equilibrated in an equilibrium solution consisting of 7.5% (volume/volume) ethylene glycol (EG) and 7.5% (volume/volume) DMSO for 15 min. Then the blastocysts were transferred to a vitrification solution consisting of 15% (volume/volume) EG, 15% (volume/volume) DMSO, and 0.5-M sucrose for 1.5 min. Next, the blastocyst was placed on the Cryotop and immediately plunged into liquid nitrogen. Labeled Cryotops were stored in a liquid nitrogen tank (XC 34/18, MVE Biological Solution, USA), and tanks were refilled with liquid nitrogen every day. For thawing, the Cryotop was placed in a thawing solution of 1.0-M sucrose at 37 °C for 1 min. The blastocyst was then removed from the thawing solution and transferred to a diluted solution of 0.5-M sucrose at room temperature for 6 min. Then blastocysts were transferred to the washing solution without sucrose for 3 min. Finally, the blastocysts were transferred to the washing solution for 1 min [15]. After the thawing process, the blastocyst was cultured in a one-step median until transfer at 37 °C in 5% O_2, 6% CO_2, and 89% N_2 in the incubator.

Post-thaw embryo culture, embryo transfer procedure, and outcome measures

During the study period, we generated single vitrified-warmed blastocysts that were transferred 4.5 to 5 days after ovulation during preparation hormone replacement or natural cycles [16]. Previously thawed blastocysts were selected based on blastocyst morphological parameters obtained by Gardner and Schoolcraft [17]. After thawing, the blastocysts were cultured for 3 to 4 h, after which blastocoel re-expansion was confirmed. Degenerating blastocysts were discarded. The blastocyst transfer procedure was conducted with guidance of vaginal

ultrasonography. A single blastocyst in minimal medium (m-HTF with HEPES in 10% SPS) was placed in the upper part of the uterine cavity using a designed soft silicone inner catheter (Kitazato ET Catheter, Kitazato, Japan) [18]. The clinical outcomes were the clinical pregnancy rate (with a confirmed gestational sac at 6–7 weeks of pregnancy), implantation rate (determined by heart beat in ultrasound), and live birth rate (live birth at 22 weeks of pregnancy) per embryo transfer procedure. The miscarriage rate was defined as the number of patients with a miscarriage among patients who attained clinical pregnancy. The neonatal outcomes included gestational age, birth weight, birth length, low birth weight (< 2500 g at birth), and macrosomia (birth weight > 4500 g at birth). The sex ratio at birth was calculated as the proportion of males among all live births.

Statistical analysis

All the data analyses were performed using SPSS 15.0 statistical software (IBM SPSS Statistics, IBM, New York, USA). To adjust for confounding factors related to achieving clinical outcomes, propensity score matching was performed. The PSM allowed each patient who underwent a cryostorage duration. The variables in the PSM include patient age, BMI, infertility diagnosis, oocyte number, baseline hormones, and fertility methods. The continuous data are presented as the mean and standard deviation (SD). Nominal variables were analyzed by Fisher's exact test, and two categorical variables were analyzed by the chi-square test for trend, as appropriate. Differences between groups for continuous variables were assessed using ANOVA. $p < 0.05$ was considered to indicate statistical significance. A multivariable logistic regression analysis was adjusted for age, number of cycles, and cause of infertility diagnosis, body mass index, and type of fertilization. The data are reported as adjusted odds ratios (aORs) and 95% confidence intervals (95% CIs).

Results

This retrospective cohort study analyzed 2107 patients' first single vitrified-warmed blastocyst transfer (SVBT) cycles, who underwent clomiphene-based minimal stimulation. Participants were grouped based on cryostorage duration (882 SVBT cycles in group 1 with < 2 months, 794 SVBT cycles in group 2 with 3–6 months, 187 SVBT cycles in group 3 with 7–12 months, 126 SVBT cycles in group 4 with 13–24 months, and 118 SVBT cycles in group 5 with 25–81 months) as shown in Table 1. After propensity score matching (PSM), there was no statistically significant difference in patient characteristics between the groups (Table 2). The mean age of the

Table 1 Baseline characteristics of the participants with by unmatched

	0–2 months Group 1	3–6 months Group 2	7–12 months Group 3	13–24 months Group 4	25–81 months Group 5	*P*-value
Maternal age at oocyte pickup (years ± SD)	34.96 ± 5.4	36.04 ± 5.5	35.73 ± 5.32	35.52 ± 6.02	33.92 ± 5.08	0.001
Maternal age at blastocyst transfer (years ± SD)	34.87 ± 5.3	35.99 ± 5.4	36.01 ± 5.22	35.19 ± 5.89	36.44 ± 5.01	0.001
SVBT cycles (n)	882	794	187	126	118	
Baseline hormones (mean ± SD)						
Estradiol pg/mL	41.4 ± 21.4	41.34 ± 23.03	41.42 ± 25.13	33.86 ± 20.7	44.5 ± 27.8	0.07
Thyroid-stimulating hormone uIU/mL	2.48 ± 4.63	2.04 ± 1.66	1.89 ± 0.96	2.32 ± 1.45	3.26 ± 3.94	0.29
Prolactin ng/mL	16.3 ± 11.2	15.53 ± 9.5	14.62 ± 6.4	15.33 ± 7.7	13.52 ± 6.1	0.55
Follicle-stimulating hormone mIU/mL	10.01 ± 5.7	10.25 ± 5.8	10.26 ± 4.72	10.51 ± 7.15	10.43 ± 5.54	0.29
Anti-Mullerian hormone ng/mL	1.79 ± 1.4	1.67 ± 1.4	1.75 ± 1.19	1.08 ± 0.78	1.25 ± 1.34	0.15
BMI (kg/m^2)	24.1 ± 4.4	23.9 ± 4.2	24.2 ± 4.1	24.01 ± 3.9	23.8 ± 4.9	0.19
Causes of infertility n, (%)						
Female factor	489 (55.44)	451 (56.80)	105 (56.15)	68 (53.97)	65 (55.08)	0.25
Male factor	224 (25.40)	174 (21.92)	41 (21.93)	27 (21.43)	28 (23.73)	
Mix factor	89 (10.09)	90 (11.34)	22 (11.76)	14 (11.11)	13 (11.02)	
Unexplained	80 (9.07)	79 (9.94)	19 (10.16)	17 (13.49)	12 (10.17)	
Fertilization n, (%)						
cIVF	483 (54.76)	390 (46.12)	102 (54.55)	64 (53.17)	56 (47.46)	0.29
ICSI	399 (45.24)	404 (50.88)	85 (45.45)	59 (46.82)	62 (52.54)	

Data are shown as the mean ± SD and proportion (%)

$P < 0.05$ statistically significant

BMI Body mass index, *cIVF* Conventional IVF, *ICSI* Intracytoplasmic sperm injection, *SVBT* Single vitrified-warmed blastocyst transfer

Table 2 Baseline characteristics of the participants by propensity score matched

	0–2 months Group 1	3–6 months Group 2	7–12 months Group 3	13–24 months Group 4	25–81 months Group 5	*P*-value
Maternal age at oocyte pickup (years ± SD)	34.55 ± 3.68	34.42 ± 3.98	33.9 ± 3.77	33.77 ± 4.3	33.73 ± 3.41	0.11
Maternal age at blastocyst transfer (years ± SD)	34.73 ± 3.66	34.6 ± 4.05	34.56 ± 3.81	34.85 ± 4.01	35.81 ± 3.53	0.45
SVBT cycles (n)	502	443	112	84	91	
Baseline hormones (mean ± SD)						
Estradiol pg/mL	42.44 ± 21.56	39.87 ± 20.85	39.32 ± 24.14	32.82 ± 21.14	41.47 ± 30.01	0.84
Thyroid-stimulating hormone uIU/mL	2.78 ± 6.23	1.99 ± 1.58	1.76 ± 1.03	2.47 ± 1.55	3.18 ± 3.66	0.64
Prolactin ng/mL	16.61 ± 10.45	16.12 ± 8.76	14.17 ± 5.96	14.77 ± 6.38	14.52 ± 5.83	0.51
Follicle-stimulating hormone mIU/mL	9.64 ± 5.16	10.23 ± 5.16	10.31 ± 5.16	10.30 ± 5.16	10.14 ± 5.16	0.61
Anti-Mullerian hormone ng/mL	1.68 ± 1.33	2.01 ± 1.66	1.89 ± 1.34	1.31 ± 0.89	1.64 ± 0.95	0.61
BMI (kg/m^2)	23.9 ± 3.9	24.5 ± 3.2	24.5 ± 4.5	23.8 ± 4.2	23.8 ± 4.7	0.25
Causes of infertility n, (%)						
Female factor	248 (49.40)	225 (50.79)	56 (50.00)	43 (51.19)	46 (50.55)	0.31
Male factor	153 (30.48)	121 (21.31)	31 (27.68)	20 (23.81)	24 (26.37)	
Mix factor	56 (11.16	50 (11.29)	14 (12.50)	9 (10.71)	12 (13.19)	
Unexplained	45 (8.96)	47 (10.61)	11 (9.82)	12 (14.29)	9 (9.89)	
Fertilization n, (%)						
cIVF	260 (51.79)	230 (51.91)	60 (53.57)	44 (52.38)	50 (54.94)	0.45
ICSI	242 (48.21)	213 (48.09)	52 (46.43)	40 (47.62)	41 (45.06)	

Data are shown as the mean ± SD and proportion (%)

$P < 0.05$ statistically significant

BMI Body mass index, *cIVF* Conventional IVF, *ICSI* Intracytoplasmic sperm injection, *SVBT* Single vitrified-warmed blastocyst transfer

patients did not significantly differ among the five groups. Additionally, patient BMI (body mass index), baseline hormones (estrogen, thyroid-stimulating hormone, prolactin, follicle-stimulating hormone, and anti-Mullerian hormone), causal factors of infertility, and fertilization methods were not correlated with cryostorage duration. The cryostorage duration groups exhibited no differences in the transferred blastocyst grade (Table 2).

No differences were observed in the vitrification days and grade of transferred blastocysts. Furthermore, there are insignificant differences between clinical outcomes, including the clinical pregnancy rate, implantation rate, live birth rates, and cryostorage duration groups. Additionally, neonatal outcomes, such as gestational week, low birth weight, macrosomia, birth length, infant sex and twin births, showed no significant differences ($p > 0.05$, Table 3).

After adjusting for potential confounding factors, including patient age at oocyte pickup and blastocyst transfer, BMI, cause of infertility diagnosis, and insemination methods, multivariate logistic regression analysis revealed that cryostorage duration did not significantly correlate with the age at oocyte pickup, age at blastocyst transfer, blastocyst grade, or vitrification days. Furthermore, cryostorage duration showed insignificance concerning the clinical pregnancy (group 2 *aOR* 1.07, 95% *CI* 0.83–1.39, group 3 *aOR* 1.31, 95% *CI* 0.86–2.01, group 4 *aOR* 1.45, 95% *CI* 0.89–2.37, and group 5 *aOR* 1.25, 95% *CI* 0.77–2.03, $p > 0.05$), implantation (group 2 *aOR* 1.1, 95% *CI* 0.85–1.43, group 3 *aOR* 1.31, 95% *CI* 0.86–2.01, group 4 *aOR* 1.09, 95% *CI* 0.68–1.74, and group 5 *aOR* 1.27, 95% *CI* 0.79–2.03, $p > 0.05$), live birth (group 2 *aOR* 0.97, 95% *CI* 0.74–1.01, group 3 *aOR* 1.01, 95% *CI* 0.67–1.54, group 4 *aOR* 1.14, 95% *CI* 0.79–1.83, and group 5 *aOR* 1.23, 95% *CI* 0.76–1.98, $p > 0.05$), and miscarriage (group 2 *aOR* 1.12, 95% *CI* 0.66–1.42, group 3 *aOR* 1.25, 95% *CI* 0.87–2.5, group 4 *aOR* 1.35, 95% *CI* 0.71–1.87, and group 5 *aOR* 1.31, 95% *CI* 0.77–2.87, $p > 0.05$) when compared to group 1 (Table 4).

Discussion

The cryopreservation of blastocysts has become a trending method in current IVF treatment. However, during the FET cycle, optimal storage duration for blastocysts has not been determined with clinical and neonatal outcomes. Recent studies have reported that prolonged cryostorage of embryos does not affect clinical outcomes. Our study result was similar to previous studies, and cryostorage duration has no impact on clinical outcomes. However, storage duration does not appear to influence neonatal outcomes.

Table 3 Patient's characteristics divided by clinical and neonatal outcome

	0–2 months Group 1	3–6 months Group 2	7–12 months Group 3	13–24 months Group 4	25–81 months Group 5	*P*-value
Day of blastocyst n, (%)						
5 days	421 (83.86)	371 (83.75)	93 (83.04)	72 (85.71)	76 (83.52)	0.99
6 days	81 (16.14)	72 (16.25)	19 (16.96)	12 (14.29)	15 (16.48)	
Blastocyst quality of transfer n, (%)						
Excellent (AA, AB, BA)	242 (48.21)	214 (48.31)	59 (52.68)	36 (42.86)	44 (48.35)	0.35
Good (BB, AC, CA)	141 (28.09)	120 (27.09)	28 (25)	25 (29.76)	32 (35.16)	
Poor (BC, CB, CC)	119 (23.71)	109 (24.06)	25 (22.32)	23 (27.38)	15 (16.49)	
Clinical outcomes						
Clinical pregnancy n, (%)	281 (55.98)	254 (57.34)	70 (62.5)	55 (65.48)	57 (62.64)	0.33
Implantation n, (%)	250 (49.8)	230 (51.92)	63 (56.25)	44 (52.38)	51 (56.04)	0.28
Live birth n, (%)	223 (44.42)	198 (44.7)	49 (43.75)	35 (41.67)	36 (39.56)	0.91
Miscarriage n, (%)	28 (11.16)	36 (15.38)	13 (20.63)	9 (20.45)	10 (19.61)	0.17
Neonatal outcomes						
Gestational week (mean ± day)	38.7 ± 4.14	38.90 ± 1.51	38.75 ± 1.27	38.56 ± 1.41	37.59 ± 2.68	0.54
Neonatal male gender n, (%)	115 (52.27)	108 (56.84)	27 (58.7)	17 (50)	19 (55.88)	0.88
Birth weight (2500–4500 g) n, (%)	201 (92.2)	180 (95.24)	45 (97.83)	32 (94.12)	27 (79.41)	0.54
Low weight (< 2500 g) n, (%)	15 (6.88)	8 (4.23)	1 (2.17)	5 (5.88)	7 (20.59)	
Macrosomia (> 4500 g) n, (%)	2 (0.92)	1 (0.53)	0	0	0	
Birth length (mean ± cm)	50.19 ± 2.43	50.61 ± 2.56	49.65 ± 1.99	50.03 ± 2.46	49.14 ± 3.11	0.18
Singleton birth n, (%)	97.76 (218)	191 (96.65)	48 (100)	34 (100)	32 (91.43)	0.11
Twin birth n, (%)	5 (2.24)	6 (3.05)	0	0	3 (8.57)	

P < 0.05 statistically significant

Table 4 Multivariable regression analysis of miscarriage

	Clinical pregnancy		Implantation		Live birth		Miscarriage	
	aOR (95% *CI*)	*p*-value	aOR (95% *CI*)	*p*-value	aOR (95% *CI*)	*p*-value	aOR (95% *CI*)	*p*-value
Cryostorage duration								
1 group	Reference		Reference		Reference		Reference	
2 groups	1.07 (0.83–1.39)	0.51	1.1 (0.85–1.43)	0.39	0.97 (0.74–1.25)	0.81	1.12 (0.66–1.42)	0.12
3 groups	1.31 (0.86–2.01)	0.27	1.31 (0.86–1.97)	0.26	1.01 (0.67–1.54)	0.80	1.25 (0.87–2.5)	0.26
4 groups	1.45 (0.89–2.37)	0.08	1.09 (0.68–1.74)	0.55	1.14 (0.71–1.83)	0.71	1.35 (0.71–1.87)	0.07
5 groups	1.25 (0.77–2.03)	0.37	1.27 (0.79–2.03)	0.31	1.23 (0.76–1.98)	0.45	1.31 (0.77–2.37)	0.16

Multivariable logistic regression analysis with *aOR* adjusted odds ratio and *CI* confidence interval adjusted by patient age at OPU and BT, fertilization methods, repeated cycles, *BMI* Body mass index, and cause of infertility diagnosis

Previous studies have debated whether the duration of cryopreservation negatively affects clinical and perinatal outcomes. A study involving 603 frozen embryo transfer (FET) cycles in a closed system reported that prolonged storage was not correlated with survival rate, pregnancy rate, and live birth rate. However, another study suggested that storage duration could influence macrosomia birth and weight [19]. Using a similar open device system, such as the Cryotop, S. Ueno and colleagues reported that the cryostorage duration of blastocysts does not affect the live birth rate. Additionally, the weight and malformation rate showed a decreasing trend with increasing duration, although this difference was not statistically significant [20]. Other studies have reported no significant differences in cryostorage duration with respect to survival rate or clinical outcomes, including clinical pregnancy, live birth, and neonatal outcomes [21–24]. Although studies have involved the transfer of one or double embryos at different stages during FET, the prolonged storage duration was correlated with birth weight. Our study's results are similar to those of other researchers after SVBT, as they did not differ from

neonatal outcomes and did not negatively affect clinical outcomes.

In contrast, recent studies in which a higher number of participants had a longer duration of cryostorage have shown that this duration negatively affects clinical outcomes. Li and colleagues performed a large retrospective study on the first cycles of 24,698 patients using a vitrification open system after 3 months or more before storage. Biochemical pregnancy, clinical pregnancy, and live birth rates decreased with increasing cryostorage duration. Additionally, long-term storage increases the miscarriage rate [25]. In another study of 31,143 FET cycles, the implantation and live birth rates decreased as the storage time increased. Additionally, compared with those of storage groups, the number of births increased as the embryos were vitrified at different stages [26]. Cimadomo and colleagues reported for 90–180 days (42.9%), 181–360 days (41.7%), and 361–702 days (39.6%) longer than 60 days (49.4%) had undergone a single euploid blastocyst transfer [27]. Another study reported that the first vitrified, warmed blastocyst cycle before 24 months of age did not affect the outcomes and negatively affected the duration of cryostorage [28]. A study of multicenter cohorts and large participants showed that duration of storage longer than 3 months was associated with significantly lower rates of pregnancy and live birth [29].

Previous studies have shown that the miscarriage rate could be greater in patients who underwent FET (14.5%) than in those who underwent fresh cycles (9%) [30]. An increased risk of miscarriage is known for women aged over 35 years and with higher/lower BMI [31]. Other studies have reported women aged over 35 years, with endometrial thickness < 15 mm, frozen embryo transfer, and previous miscarriage, are increasing miscarriage rate [32]. Dayuan Shi and colleagues reported that the miscarriage rate increased when the trophectoderm (TE) grade decreased (from A to C), and the aneuploid rate was higher in B grade of inner cell mass (ICM) than in A grade (73.6 vs 26.4, $p < 0.01$, respectively). Moreover, compared with fresh cycles, FET cycles were associated with 1.6 times (95% *CI* 1.2–2.1, $p < 0.01$) higher risk of miscarriage, and 6-day blastocysts were associated with a higher risk of miscarriage than 5-day blastocysts (*OR* 1.5, 95% *CI* 1.07–2.14, $p = 0.01$) [33]. Research has shown that maternal age, a blastocyst with a poor grade of ICM and a poor grade of TE, correlated with miscarriage rate and could be additional factors [34, 35].

The mechanisms elucidating the correlation between prolonged embryo storage and adverse pregnancy outcomes remain unclear, exacerbating the complexity of understanding the deleterious impact on pregnancy outcomes after FET. Several possible mechanisms have been suggested. First, temperature changes may affect cryostorage embryos. Prolonged cryostorage, repeated openings of cryotanks, changes in storage systems, and laboratory procedures over time can impact the clinical outcomes of SVBT [20]. Second, DNA integrity was assessed after both slow freezing and vitrification of blastocysts in an animal study. Cryoprotectants such as ethylene glycol,1,2-propanediol or glycerol have been shown to increase DNA fragmentation. However, the incidence of aneuploidy remained unaffected regardless of the freezing method used for two-cell mouse embryos [36]. During vitrification, embryos are exposed to a cold cryoprotectant and are directly immersed in liquid nitrogen within an open vitrification system. Extended durations of exposure in such open vitrification systems have the potential to modify early embryonic development patterns and impact post-thaw survival outcomes [26].

Conclusion

In the present study, participants who underwent the first single blastocyst transfer were categorized into five groups based on cryostorage duration. However, statistical analysis revealed that cryostorage duration was not significantly related to clinical pregnancy, implantation pregnancy, live birth rate, and miscarriage. Notably, logistic regression analysis, adjusted for potential confounding factors, revealed no correlation between clinical outcomes and cryostorage duration. To investigate the correlation between clinical outcomes and cryostorage duration, we applied a comprehensive statistical approach, considering all potential factors. In future studies, adjustments may be considered, such as analyzing a wider range of cryostorage durations for blastocysts and increasing the sample size. In previous studies, reported neonatal outcomes, including birth weight and offspring sex, were correlated with cryostorage duration. Although we did not find any association between clinical/neonatal outcomes and cryostorage duration, the prolonged cryostorage groups exhibited no correlation.

Abbreviations

FET	Frozen embryo transfer
ART	Assisted reproductive technology
SVBT	Single vitrified-warmed blastocyst transfer
IVF	In vitro fertilization
BMI	Body mass index
AMH	Anti-Mullerian hormone

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Authors' contributions

MB conducted data analysis, revision, and designed the research. MB and JJ wrote and revised the manuscript. ChG, DS, BE, SE, NM, and LkhB contributed to the collection of data from IVF and clinical laboratories. GT, AA, AD, GG, TsB, and AKh were involved in IVF treatment, conceptualization, and management. All authors have read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are available from correspondence on request.

Declarations

Ethics approval and consent to participate
All procedures were performed in accordance with the ethical standards of the ethics committee of our hospital (approval number: EA2023080502). All procedures performed in studies involving human participants were in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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