

The effect of repeated cryopreservation and thawing using cryotip on the clinical outcomes of embryos

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Abstract

Purpose: To compare the clinical outcomes of embryo transfers that were cryopreserved and thawed two or three times with those cryopreserved and thawed once by CryoTip.

Methods: Data for 388 single cryopreserved-thawed blastocyst transfer cycles, performed from April 2012 to March 2014, were assessed. The blastocysts were classified into three groups: blastocysts (A) cryopreserved once, (B) cryopreserved twice, and (C) cryopreserved three times.

Results: The pregnancy rate was 43.8% (134/306) in group A and 46.3% (38/82) in group B, while the miscarriage rate was 29.1% (39/134) in group A and 23.7% (9/38) in group B. The rate of improvement/maintenance of blastocyst grade was 84.0% (257/306) in group A and 80.5% (66/82) in group B. The pregnancy and miscarriage rates of the blastocysts that showed improvement/maintenance in the grade were 45.9% (118/257) and 29.7% (35/118) in group A and 48.5% (32/66) and 21.9% (7/32) in group B, respectively. The pregnancy rate was 33.3% (2/6), while the miscarriage rate was 0.0% (0/2) in group C.

Conclusions: Pregnancy rates achieved with re-cryopreserved and rethawed blastocyst transfer were comparable to those achieved with single cryopreserved-thawed blastocyst transfer.

KEYWORDS

birth weight, blastocyst, closed carriers, CryoTip, re-cryopreserved

1 | INTRODUCTION

In Japan, the number of pregnancies with cryopreserved-thawed embryo transfer is overwhelmingly greater than that by fresh embryo transfer.¹ Therefore, cryopreserved-thawed embryo transfer has become an essential technique in assisted reproductive technologies

(ART); the number of treatment cycles has been increasing. Vitrification using various types of carriers, such as Cryoloop,²⁻⁴ Cryotop,^{5,6} and CryoTip,^{7,8} was developed for embryo cryopreservation and has provided high embryo survival and pregnancy rates. The use of an artificial shrinkage procedure, in which the fluid in the blastocoel is artificially removed at the time of the vitrification of blastocysts, further

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improves the survival rate.⁹ There are two types of carriers used in embryo vitrification: open carriers, such as Cryoloop and Cryotop, and closed carriers, such as CryoTip. Open carriers can achieve ultrafast vitrification and warming due to the minimal volume of vitrification fluid at the time of cryopreservation, combined with the direct contact of the embryo samples with liquid nitrogen, which makes it possible to achieve good embryo survival and pregnancy rates. However, with vitrification, several concerns have been raised regarding the potential risk to human embryos from exposure to contaminants, already present in liquid nitrogen at the time of vitrification or potentially introduced to the embryos during storage in open containers.^{10,11} While no studies have demonstrated unintentional uptake of any pathogen by human embryos during vitrification or storage, under experimental conditions, such contaminations may occur. Therefore, closed carriers, in which embryo samples do not directly come into contact with liquid nitrogen, are thought to be able to reduce the infection risk for embryos. Clinical outcomes of CryoTip are comparable to those of Cryoloop and Cryotop,^{7,8} making CryoTip efficient enough as a closed carrier for embryo cryopreservation. Open carriers are not approved in countries outside Japan¹²; however, in Japan, Cryotop (an open carrier) is the standard method for cryopreservation. Samples with infections are stored in a different tank.¹³ For these reasons, our clinic uses closed CryoTip for vitrification-cryopreservation, resulting in good clinical outcomes.

Some studies reported the prognosis of re-cryopreserved embryos. In one study, pronuclear embryos that had been cryopreserved, thawed, and developed to morula embryos were re-cryopreserved, thawed, and developed to blastocysts. These blastocysts have been transferred and resulted in live births.¹⁴ In another study, the transfer of blastocysts that had been cryopreserved and thawed twice achieved pregnancy.¹⁵ However, there are only a small number of reports on the prognosis of re-cryopreserved embryos, so their use has remained unclear. This prompted us to compare blastocyst transfers that were cryopreserved and thawed two or three times with those cryopreserved and thawed just once using CryoTip. We examined the clinical outcomes, birth weight, and birth week. With cryopreserved-thawed embryo transfer, the implantation ability is sometimes estimated by the morphological assessment of the embryos at the time of cryopreservation. However, morphological assessment of blastocysts after thawing is not always consistent with that at the time of cryopreservation. Repeated cryopreservation and thawing may have an impact on the morphological assessment of embryos. Therefore, we also examined the effect of changes in morphological assessment due to re-cryopreservation on clinical outcomes.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Between April 2012 and March 2014, we examined the outcomes of 240 patients for 388 cycles.

The embryos were classified into the following three groups: (A) blastocysts cryopreserved once, (B) blastocysts cryopreserved twice, and (C) blastocysts cryopreserved three times. We compared the following parameters between groups A and B: age, number of blastocyst transfers cycles, pregnancy rate, miscarriage rate, birth weight, week of delivery, and changes in blastocyst grade after thawing. We also compared the pregnancy and miscarriage rates among those blastocysts with grades that had been improved, maintained, or reduced after thawing. Due to the small number of cases in group C, only the pregnancy and miscarriage rates and the data at the time of delivery were shown. The blastocysts were graded based on the morphological characteristics of the inner cell mass (ICM) and the trophectoderm (TE) as A, B, and C, from higher to lower grades.

2.2 | Ovarian stimulation and culture condition

In the oocyte retrieval cycle, ovarian stimulation was carried out using standard gonadotropin-releasing hormone agonist (GnRHa) follicle-stimulating hormone (FSH) protocols or an antagonist FSH protocol. Oocytes were retrieved under transvaginal ultrasound guidance 36 hours after human chorionic gonadotropin (hCG; Fuji Pharmaceutical Company, Ltd.) or leuprolide were injected. The retrieved oocytes were subjected to conventional *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) procedures. After insemination, the oocytes were cultured individually in 25 μ L droplets of Global medium (Life Global) at 37°C, in an atmosphere of 5% O₂, 6% CO₂, and 89% N₂, for a maximum of 6 days. All the embryos were cultured in Embryo GPS[®] dishes (Sun IVF) after insemination. After culturing, the blastocysts were cryopreserved using CryoTip[®] (Kitazato, Shizuoka). After thawing, single cryopreserved blastocyst transfer was performed during the hormone replacement therapy cycles.

2.3 | Vitrification and thawing method

Cryopreservation of the blastocysts was performed by established methods using a CryoTip[®]. The blastocysts cultured in a Global medium were transferred into a 50 μ L drop of equilibration solution for 14-15 minutes. The cells were allowed to spontaneously shrink and return to their original size through the infiltration of the equilibration solution. The blastocysts were then transferred to four vitrification solution (VS) drops (50 μ L each) for 10 seconds in VS1 and VS2, 5 seconds in VS3, and for a maximum of 60 seconds in VS4. The blastocysts were charged into the CryoTip straw with a minimal VS volume; the CryoTip was sealed on both ends and quickly plunged into fresh liquid nitrogen. To thaw the blastocysts, the CryoTip straw was immersed quickly into 37°C of water for 2 seconds, and the straw ends were cut to push the blastocysts into a dish. The resulting drop with the blastocysts was mixed with another similarly sized drop of thawing solution for 1 minutes to be transferred to a thawing solution drop for 1 minutes. The blastocysts were transferred to two diluent solution drops (2 minutes

each), moved to three washing solution (WS) drops (3 minutes each), and finally transferred to the culture medium. The embryos that had developed to the blastocyst stage were scored depending on the developmental stage and graded according to quality using published criteria¹⁶ with slight modifications.

2.4 | Culture of blastocyst and transfer

After thawing, the blastocysts were cultured separately in Embryo GPS dishes with Global medium. The blastocysts were monitored using a time-lapse incubator (CCM-IVF and iBIS; Astec). Approximately 2-3 hours after thawing, a single blastocyst transfer was performed. The blastocysts were transferred after the succeeding hormone replacement therapy cycle. Three weeks after the transfer, the clinical pregnancy rates were determined using ultrasound to detect the presence of a gestational sac. In this study, the clinical pregnancy rates in the three groups were summarized when only a single blastocyst was transferred.

2.5 | Statistical analysis

Statistical analysis was performed using the chi-square (χ^2) test with continuity correction (experiments 1 and 2) or Kruskal-Wallis analysis of variance (experiment 1). A *P*-value < .05 was considered statistically significant.

3 | RESULTS

3.1 | Blastocysts cryopreserved once vs. blastocysts cryopreserved twice

As shown in Table 1, the blastocyst transfer cycles in group B (2.51 ± 1.82) were significantly higher than in group A (1.81 ± 1.23) ($P < .01$). There were no significant differences in the pregnancy rates (43.7%-46.3%), miscarriage rates (23.7%-29.1%), birth weights (3002-3079 g), and week of delivery (37.62-38.01) between the two groups.

As shown in Table 2, the pre-cryopreserved blastocyst grade was compared with the post-cryopreserved blastocyst grade, and the rate of improvement or maintenance in the blastocyst grade was 84.0% (257/306) in group A and 80.5% (66/82) in group B. The pre-cryopreserved blastocyst grade was compared with the post-cryopreserved blastocyst grade, and the rate of decline in the blastocyst grade was 16.0% (49/306) in group A and 19.5% (16/82) in group B. The pregnancy rates of the blastocysts that showed improvement or maintenance in the grade were 45.9% (118/257) in group A and 48.5% (32/66) in group B. The miscarriage rates of the blastocysts that showed improvement or maintenance in the grade were 29.7% (35/118) in group A and 21.9% (7/32) in group B. The pregnancy rates of the blastocysts that showed a decline in

TABLE 1 Clinical background and characteristics of once and twice cryopreservation and thawing groups

Groups	A	B
Age ^a	34.46 ± 4.32	34.91 ± 3.89
No. of blastocyst transfer cycles ^a	1.81 ± 1.23*	2.51 ± 1.82**
No. of blastocyst transfer, n	306	82
No. of pregnancies, n (%)	134 (43.7)	38 (46.3)
No. of miscarriages, n (%) ^b	39 (29.1)	9 (23.7)
No. of newborns, n	92 ^c	28 ^d
Birth weight (g) ^a	3002 ± 484	3079 ± 434
Week of delivery ^a	38.01 ± 2.75	37.62 ± 3.73
No. of preterm births (<37 gestational weeks), n (%) ^e	10 (10.9)	3 (10.7)

Note: A: cryopreservation and thawing once, B: cryopreservation and thawing twice.

*, **Values with different superscript letters signify statistically significant difference ($P < .01$) within each column.

^aData are presented as mean ± standard deviation.

^bPercentage per pregnancy.

^cBecause the number of newborns was not recorded in three patients, data derived from only 92 patients were shown in group A.

^dBecause the number of newborns was not recorded in one patient, data derived from only 28 patients were shown in group B.

^ePercentage per newborn.

TABLE 2 Effects of the number of repetitions of cryopreservation and thawing on blastocyst grade, and pregnancy and miscarriage rates with single blastocyst transfer

Groups	A	B
No. of blastocysts, n	306	82
High-quality ^a , n (%)	257 (84.0)	66 (80.5)
No. of pregnancies, n (%)	118 (45.9)	32 (48.5)
No. of miscarriages, n (%) ^c	35 (29.7)	7 (21.9)
Low-quality ^b , n (%)	49 (16.0)	16 (19.5)
No. of pregnancies, n (%)	16 (32.7)	6 (37.5)
No. of miscarriages, n (%) ^c	3 (18.8)	2 (33.3)

^aHigh-quality: blastocysts whose grade improved or was maintained after thawing.

^bLow-quality: blastocysts whose grade declined after thawing.

^cPercentage per pregnancy.

the grade were 32.7% (16/49) in group A and 37.5% (6/16) in group B. The miscarriage rates of the blastocysts that showed a decline in the grade were 18.8% (3/16) in group A and 33.3% (2/6) in group B.

3.2 | Embryos cryopreserved three times

As shown in Table 3, in group C, the pregnancy rate was 33.3% (2/6), and the miscarriage rate was 0.0% (0/2). The two patients delivered babies.

TABLE 3 Clinical background and characteristics of cryopreservation and thawing three times

Group	C	
No. of blastocysts, n	6	
No. of pregnancies, n (%)	2 (33.3)	
No. of miscarriages, n (%)	0 (0.0)	
Newborn number	No. 1	No. 2
Age of the mother (y)	35	38
Birth weight (g)	3534	2588
Week of delivery	39	37

4 | DISCUSSION

Although cryopreservation of embryos is part of most IVF programs, there are only limited studies on the perinatal outcome of the children born after the replacement of cryopreserved embryos. In this study, the rate of pregnancy achieved by re-cryopreserved-rethawed embryo transfer was comparable to that of pregnancy achieved by cryopreserved-thawed embryo transfer. Also, no difference was observed in terms of the miscarriage rate between the two treatments. There was no intergroup difference in terms of birth weight, birth week, or preterm birth rate, suggesting safety for newborns. When multiple embryos are thawed for cryopreserved-thawed embryo transfer, surplus embryos sometimes need to be cryopreserved again. It is now possible for the re-cryopreserved embryos to be used in the next cycle rather than being disposed.

The speed of vitrification and warming has a significant impact on embryo survival in the vitrification-cryopreservation of embryos. To increase these speeds, open carriers have been developed.²⁻⁶ However, cross-contamination during vitrification and liquid nitrogen storage cannot be excluded. A study reported that there was no evidence of these risks.¹⁷ Physical impacts due to the replenishment of liquid nitrogen are inevitable (eg, the submerging of canes and withdrawal from liquid nitrogen and relocation of the tank). Embryos placed on an open carrier are directly exposed to liquid nitrogen, and, therefore, may be affected in some form. On the other hand, closed carriers reportedly preserve the quality of embryos, even if the storage period is extended.¹⁸ Closed carriers are, therefore, thought not to be affected by liquid nitrogen. If embryos are re-cryopreserved, the storage period is likely to be prolonged; thus, closed carriers may be more suitable than open carriers for cryopreservation of embryos. Some studies reported that despite the lower vitrification and warming speeds with closed carriers compared to those with open carriers, both types of carriers yield similar clinical results.^{7,19,20} This study showed the results of the re-cryopreserved embryo transfer to be equivalent to those of cryopreserved embryo transfer. Although the number of cases subjected to cryopreservation three times was small, two cases of pregnancy were recorded. Three-time cryopreservation is thought to be rare (eg, when patients have a strong desire for pregnancy); however, it is a feasible alternative.

The rate of low birth weight for infants with ART treatment was reported to be higher than those with non-ART treatments in singleton pregnancies. It was postulated that the mean birth weight after cryopreserved-thawed embryo transfer was higher than that of fresh embryo transfer.²¹⁻²³ The duration of embryonic culture may also be a factor that affects the birth weight of neonates.²¹ Differences in hormone supplementation therapy in cryopreserved-thawed embryo transfer also affect the birth weight. The supplementation of estrogen and progesterone suggests the possible improvement of the uterine environment, leading to the development of the placenta, subsequent fetal growth, and heavier birth weight after implantation.^{21,24} On the other hand, some studies indicate that inherent pathologic factors associated with infertility itself are likely to have a greater impact on fetal growth than infertility therapies.^{25,26} Our study found no difference in birth weight between groups that had been cryopreserved once or twice. This suggests that repeated ART treatment, such as cryopreservation and thawing, does not necessarily have a greater impact on fetuses. The differences in the culture period did not affect the fetuses because the blastocysts were used in both groups. Therefore, embryo transfer during a hormone supplementation cycle is likely to affect the birth weight. Repeated cryopreservation and thawing did not affect birth weight and was, therefore, thought to be an effective method. The rate of preterm birth was approximately 10% in both groups, suggesting no impact of re-cryopreservation.

No significant difference in the decline of the blastocyst grade was found between groups; repeated cryopreservation and thawing did not cause a decline in the grade of the blastocysts. There was also no significant difference in the pregnancy rate among the groups in terms of the blastocyst grade (improved, maintained, or reduced). Therefore, re-cryopreservation and thawing were found to be effective.

It is important to identify abnormal chromosomes and transfer euploid embryos to improve the success rate of IVF. Preimplantation genetic testing for aneuploidy (PGT-A) is currently considered to be the most reliable method of selecting euploid embryos for transfer. In 2019, the Japan Society of Obstetrics and Gynecology started a clinical study in Japan. Our clinic began the clinical application of PGT using cleavage stage embryos and fluorescent in situ hybridization since 2008. In 2014, we changed to TE-biopsy and analysis by next-generation sequencing. Grade reduction of the re-cryopreserved-thawed blastocysts is minor, and the pregnancy rate in re-cryopreserved-thawed embryo transfer is equivalent to that in cryopreserved-thawed embryo transfer. These findings will enable us to perform PGT-A using cryopreserved embryos in patients whose blastocysts have been cryopreserved, depending on the treatment results. Cryopreserved blastocysts are thawed and biopsied. After which, the blastocysts with euploid chromosomes that have been re-cryopreserved and thawed are transferred, which can lead to pregnancy. This method is minimally invasive for patients because repeated oocyte retrieval is not necessary. This method can also reduce the miscarriage rate and avoid the embryo transfer process, which is highly

likely to fail in pregnancy, making it possible to proceed rapidly to the next treatment cycle.

In this study, the rate of pregnancy with re-cryopreserved-rethawed blastocyst transfer was comparable to that of pregnancy with single cryopreserved-thawed blastocyst transfer. Also, there were no differences in the miscarriage rate, birth weight, and week of delivery. Therefore, the usefulness of re-cryopreserved-rethawed embryo transfer using CryoTip was suggested. Pregnancy was achieved in two patients, which resulted in live births after three-time-cryopreserved-thawed embryo transfer. This suggests that repeated cryopreserved-thawed embryos remain eligible for embryo transfer.

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ETHICAL APPROVAL

This study was approved by the Institutional Review Board of Takeuchi Ladies Clinic/Center for Reproductive medicine. All procedures performed in the study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

DISCLOSURES

Conflict of interest: The authors declare that they have no conflict of interest.

Human rights statements and informed consent and Animal studies: All the procedures were followed in accordance with the ethical standard of the responsible committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all the patients in the study.

CONSENT TO PARTICIPATE

All study participants provided written informed consent for their treatment.

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