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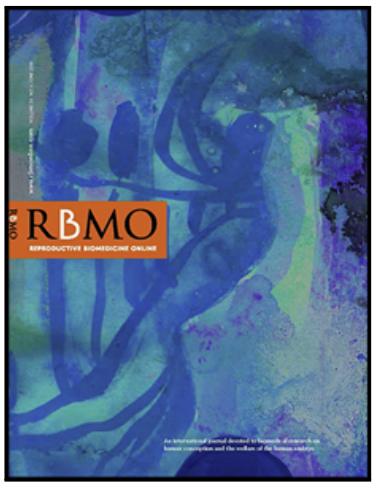

Pregnancy potential of embryos cryopreserved and thawed twice. A case-control study

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Highlights

- An effective way to decrease perinatal risks is to avoid multiple pregnancies
- Elective single frozen embryo transfer may result in surplus thawed good embryos
- Recryopreservation cycle with vitrification results in good embryo survival rates
- Transfers of twice-frozen embryos result in favorable pregnancy outcomes
- Perinatal outcome after recryopreservation with vitrification is uncompromised

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Pregnancy potential of embryos cryopreserved and thawed twice.

A case-control study

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

ABSTRACT

Research Question

What are the pregnancy and perinatal outcomes of embryos cryopreserved twice, compared to embryos cryopreserved once?

Design

Retrospective register-based case-control study. The case group consisted of transfers of embryos cryopreserved twice (n=89), and the control group of transfers of embryos cryopreserved once (n=304). Matching criteria were embryonic age at transfer and female age category of less than or greater than 35 years.

Results

The survival rate of embryos cryopreserved twice was 92.2 %, and 93.7% of the planned frozen embryo transfers (FETs) could be completed. FET was performed with cleavage stage embryos in 17 cases and 68 controls and with blastocysts in 72 cases and 238 controls. The rates of live birth (27.0% vs 31.9%, adjusted OR 0.7, 95% CI 0.4-1.22, *p*=0.21), clinical pregnancy (31.4% vs 36.8%, adjusted OR 0.71, 95% CI 0.42-1.21, *p*=0.21) and miscarriage (4.5% vs. 3.9%, adjusted OR 1.1, 95% CI 0.33-3.6, *p*=0.88) in the case and the control groups were comparable. No difference was seen in the preterm delivery rate (cases 4.2% vs controls 10.3%, *p*=0.69). Twenty-five children were born in the case group and 100 in the control group. No difference in birth weight was detected between the groups and there were no large for gestational age fetuses or congenital malformations in the case group.

Conclusions

Uncompromised live birth rates and neonatal outcomes may be expected after the transfer of embryos cryopreserved twice. To avoid embryo wastage and transfer of multiple embryos, good quality surplus embryos from FET cycles may be recryopreserved by vitrification.

Abbreviations

ART= Assisted reproduction technique, FET= Frozen embryo transfer, ET= embryo transfer, SET= single embryo transfer, eSET= elective single embryo transfer, CPR= clinical pregnancy rate, LBR= live birth rate

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INTRODUCTION

Embryo cryopreservation is a compelling option for increasing the cumulative pregnancy rate of assisted reproductive treatments (ART). Several studies on global trends in ART have demonstrated the live-birth rate in frozen embryo transfer (FET) cycles to be equal or even superior compared to fresh embryo transfer (Acharya et al., 2018; De Geyter et al., 2020; Zhang et al., 2018). Reassuring outcomes of FET have encouraged the practice of freezing all good-quality embryos in the initial cycle, followed by elective FET (eFET) in subsequent cycles (Roque et al., 2019). Consequently, the number of FET cycles has steadily increased worldwide (Ferraretti et al., 2017). Simultaneously, the advanced cryopreservation technique of vitrification has improved embryo survival rates, providing more viable embryos for utilization (Edgar et al., 2000).

Compared to repetitive IVF cycles, FET provides a safe, cost-effective, and patient friendly method to achieve a pregnancy. Elective single embryo transfer (eSET) is a widely accepted method to decrease risks related to multiple pregnancies, supported by several national and professional guidelines (Committee of the Society for Assisted Reproductive Technology & Committee of the American Society for Reproductive Medicine, 2012; Tiitinen, 2012). Important factors in the successful implementation of eSET protocols are adequate patient information and criteria for embryo selection. The pregnancy outcomes reached with modern embryo culture and selection methods are so favorable that double, not to mention multiple, embryo transfers are becoming difficult to justify (Veleva et al., 2009, Grady et al., 2012). In FET cycles, however, the eSET strategy may result in surplus good-quality embryos after thawing.

Recryopreservation of viable surplus embryos in FET cycles is a potential method to further increase the cumulative pregnancy rate as well as reduce the risk of multiple pregnancies and the burden of repeated IVF treatments. However, there are only limited and somewhat-conflicting reports on the effectiveness of repeated embryo cryopreservation (summarized in Table 1). Reports on the pregnancy potential of embryos frozen twice by slow freezing methods are contradictory (Farhi et al., 2019; Koch et al., 2011), whereas recryopreservation by vitrification seems to achieve similar clinical pregnancy rates (CPR) compared to blastocysts vitrified once (Kumasako et al., 2009; Zheng et al., 2017). Presently, less than a hundred babies have been reported to be born from repeatedly cryopreserved embryos.

All in all, due to the limited number of comparative studies and variable cryopreservation methods used, the clinical benefit gained with repeated cryopreservation of embryos is yet to be determined.

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Moreover, it is recognized that FET is not without complications: FET has been found to be associated with an increased risk of hypertensive disorders and pre-eclampsia during pregnancy, as well as increased neonatal birth weight (Berntsen & Pinborg, 2018; Ginström Ernstad et al., 2019; Sha et al., 2018). It is therefore of concern whether repeated cryopreservation of embryos would increase the risk of perinatal complications and affect fetal growth and neonatal health. The aim of this study was to assess the pregnancy potential and perinatal outcome of embryos refrozen by vitrification.

MATERIALS AND METHODS

Study design

This study is a retrospective register-based case-control study that consists of transfers of cryopreserved embryos carried out at two centers, the University Hospital of Turku (Center A) and the Central Hospital of Central Finland (Center B), both in Finland, between January 2012 and December 2019. The case group consists of transfers of embryos cryopreserved twice, and the control group of transfers of embryos cryopreserved once. The indication for cryopreservation in both groups was the clinical practice of eSET followed by cryopreservation of surplus embryos or freeze all- strategy to avoid ovarian hyperstimulation in some patients. The cases were manually searched in laboratory documents, and for each case, the chronologically most proximate controls were searched from the same center. Repeated transfers were excluded from the controls. The targeted number of controls was four, and the matching criteria were embryonic age at transfer and female age categories of less than and greater than 35 years. Factors known to contribute to the success of assisted reproductive technologies (ART) were recorded from patient charts. Characteristics of the study population are presented in Table 2.

Embryo cryopreservation

At the cleavage stage, surplus embryos with less than 25% fragmentation or difference in blastomere size were selected for cryopreservation. At the morula stage, well-compacted embryos with less than 25% fragmentation were cryopreserved, and at the blastocyst stage, an adequate number of cells in the trophectoderm and inner cell mass was required (at least Gardner class BC or CB).

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The embryos were initially cryopreserved by either slow freezing or vitrification. All recryopreservations were carried out by vitrification. Slow freezing of the embryos was performed according to the manufacturer's protocol (Sydney IVF Cryopreservation kit, K-SICS-5000, Cook Australia until the year 2013 and Freeze Kit Cleave, Vitrolife Sweden from 2014 onwards). The cooling rate was controlled with a freezer (Planer Kryo 10-MRV or Planer Kryo 360, Planer PLC, Sunbury on Thames, UK). Thawing was carried out by rapid warming in a 30°C water bath, and rehydration was carried out in a series of media with decreasing cryoprotectant concentrations according to the manufacturer's protocol (Sydney IVF Thawing Kit, K-SITS-5000, Cook Australia until year 2013 and later, Thaw Kit Cleave, Vitrolife Sweden). In Center A, Rapid-i vitrification systems (Vitrolife Sweden) were used for vitrification and warming of the embryos in a closed system as described by the manufacturer. In Center B, a VitriFreeze ES/VitriThaw ES system (FertiPro) with HSV straws (Cryo Bio system) was used. The embryos were cultured in G1-PLUS™/G2-PLUS™ sequential media (Vitrolife Sweden) or Sage one-step media (Origio) in 7% CO_2, 8% O_2, 85% N_2 (Center A) or 6% CO_2, 10% O_2, 84% N_2 (Center B) at 37°C ± 0.1°C. Embryo transfers were performed under ultrasound guidance. Pregnancy tests were made at the embryonic age of 14–16 days. Ultrasound examination was performed at the 7th gestational week to confirm a clinical pregnancy.

The outcome measures

The primary outcome was birth rate (LBR), and the secondary outcomes were CPR and miscarriage rates. Biochemical pregnancies were recorded to analyze the total early pregnancy wastage rate. The gestational age at birth, birth weight, and the sex ratio of the newborns were analyzed. Any malformations were also recorded.

Statistical analysis

The data were presented as a median with quartiles (Q1, Q3) for continuous variables and percentages for categorical variables. For continuous variables with normal distribution, the differences between the study groups were examined using Student's *t* test, and for those with a non-normal distribution, a Wilcoxon rank-sum test was used. Female age at oocyte pickup was tested with Student's *t* test. Categorical data were analyzed using a chi-squared test. A Fisher's exact test was utilized if the variable had low group frequencies.

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Statistical associations for the outcome of pregnancy with study groups and relevant explanatory variables were examined using mixed-effects logistic regression (GLIMMIX-procedure of SAS). The statistical models were made separately for live birth, pregnancy, clinical pregnancy, miscarriage, and biochemical pregnancy rates as response variables. Female age at oocyte pickup, the duration of infertility, cycle type (natural or artificial), and study group were included in the models as explanatory variables and were treated as fixed effects. Study center and case-control id were treated as random effects in the models. The matching of cases with controls was taken into account by adding them as random effects in the model.

For the multivariate analysis of birth weight between the study groups, a linear mixed model was used. Outliers were removed to achieve a better fit for the model. Twins were also removed from the data. The child's sex, gestational age, maternal BMI, and female parity were included in the model as explanatory variables and were treated as fixed effects. Study center and case control id were also treated as random effects in this model. The level of significance was set at a p value of < 0.05 (two-tailed). All of the statistical analyses were performed using SAS version 9.4 (SAS Institute Inc, Cary, NC, USA).

Ethics

The study was reviewed and approved by the institutional review board of Turku University Hospital, Turku, Finland, and by the chief medical director of the Central Finland Health Care District. Based on EU General Data Protection Regulation 2016/679 (GDPR), Article 6(1)(e) and Article 9(2)(j); Data Protection Act, Sections 4 and 6, the Finnish law does not require approval by the ethical committee for register studies.

RESULTS

Altogether, 2834 FET cycles were performed during the study period. Of these, 89 FETs were carried out with twice-cryopreserved embryos (case group). The survival rate of the twice-cryopreserved embryos was 92.2 % (94/102), and 93.7% (89/95) of the planned FETs could be carried out. A total of 304 FET cycles formed the control group. The targeted number of controls per case was 4, however this was not reached for day 5 and 6 embryos (mean of 3.6 and 2.4 controls per FET, respectively). FET was performed with cleavage stage (day 3 and 4) embryos in 17 cases and 68 controls and with blastocysts (day 5 and 6 embryos) in 72 cases and 238 controls. The female age at oocyte pickup and BMI were similar between the groups; nevertheless, the duration of

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infertility was significantly longer and the proportion of primary infertility was significantly lower among the cases. Also, FET was performed significantly more often during an artificial cycle in the case group (Table 2).

The results are presented in Tables 3 and 4. In univariate analysis, there were no statistical differences in LBR, CPR, or miscarriage rates between the groups (27.0% vs 31.9%, p = 0.35; 31.4% vs 36.8%, p = 0.35; 4.5% vs 3.9%, p = 0.77, respectively). The results remained insignificant in multivariate analysis. There was no difference in the pregnancy outcomes between slow freezing and vitrification as the primary cryopreservation method (Suppl 1) and furthermore, the primary freezing method did not become statistically significant in the adjusted mixed effects logistic regression models. The results were therefore reported regardless of the primary cryopreservation method, due to the limited study size.

In the case and control groups, 25 and 100 children were born, respectively. All newborns in the case group were of appropriate weight for their gestational age. The median weight of the newborns was 3730 g (quartiles 3500 and 4050 g) in the case group and 3490 g (quartiles 3150 and 3900 g) in the control group, representing an insignificant statistical difference (p =0.064). The embryonic age at transfer had no effect on birth weight. Only singleton, full-term deliveries were included in the analysis. Adjustment for gestational age, sex of the child, female parity, and BMI did not change the results in the linear mixed model (adjusted p = 0.28).

There were no reports of congenital malformations among the newborns in the case group. In the control group, there was one termination of pregnancy due to aneuploidy, one case of undescended testicle, one child with a hypoplastic aortic valve without stenosis, and one child with trigonocephaly.

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DISCUSSION

In the present case-control study, LBR, CPR, and miscarriage rates of twice-cryopreserved embryos were comparable to those of embryos cryopreserved once, suggesting that repeated cryopreservation did not have a deleterious effect on the pregnancy potential of the embryos. Moreover, all children originating from twice-cryopreserved embryos were appropriate for gestational age in weight, and no malformations were reported. However, the small study material

limits the interpretation of the results, and larger studies are warranted to address the safety of repeated cryopreservation.

The goal of all infertility treatments is the birth of a healthy child and a new mother on her feet. Although all risks cannot be avoided, the simplest way to reduce the number of complications is to avoid multiple gestations. In 2018 in Finland, 95.3% of all embryo transfers were completed as single embryo transfers, and only 3.2% of the embryo transfers resulted in twin pregnancy (Medical Birth register, THL 2020).

The development of culture media and cryopreservation methods, as well as the improvement of embryo scoring systems, have greatly improved embryo selection processes and reduced the number of supernumerary embryos that go into wastage. In addition, the majority of infertility clinics have nowadays shifted entirely from slow freezing cleavage stage embryos in multiples into vitrificating single embryos that will be successfully thawed one-by-one, with little need for recryopreservation. However, there are certain situations, such cancellation of a frozen embryo transfer cycle for patient-related reasons, when a clinician needs to face the question of whether an embryo should be cryopreserved a second time and whether the subsequent outcome will be compromised. An increasingly important indication for recryopreservation is preimplantation genetic testing (PGT) that has not been widely available until recently. Thawing previously frozen embryos for biopsy for aneuploidy screening or diagnosis of a known hereditary disease requires recryopreservation while the analysis is being completed, followed by the transfer of a putatively healthy blastocyst, such as described by Wilding et al. in 2019. In many clinics, due to limited personnel and financial resources, a relatively large proportion of embryos are still frozen at the cleavage stage, and previously this having been the standard of care in even more clinics, several patients still have numerous cleavage stage embryos cryopreserved. Women whose fertility is threatened by age or a progressive disease would especially benefit from enhancing the fertility treatment process with blastocyst culture, followed by a lower number of good-prognosis embryo transfers; yet, again the fate of prospective surviving surplus blastocysts must be considered.

Our study strengthens the limited evidence from previous studies (Murakami et al., 2011; Taylor et al., 2014; Zheng et al., 2017) that repeated cryopreservation is a viable option to avoid embryo wastage or transfer of multiple embryos, and thus to optimize the utilization of cryopreserved embryos.

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There are still some concerns regarding the possible health consequences of frozen embryo transfers, such as an increased risk of large gestational-age fetuses as compared with fresh embryo transfers (Berntsen & Pinborg, 2018; Pelkonen et al., 2010), and additional studies are needed to evaluate whether this tendency is further increased in repetitive cryopreservation cycles. The neonatal outcome of twice-cryopreserved embryos has been evaluated in two previous studies whose results are in line with the results of the present study. In a study by Murakami et al. (2011), no congenital anomalies were reported in 46 neonates born after the transfer of twice-cryopreserved embryos. A tendency towards a slightly higher birth weight was seen after the transfer of re-vitrified embryos, although the difference was not statistically significant (2994 g vs. 2876 g in the twice-cryopreserved and once-cryopreserved groups, respectively). Additionally, a lower preterm delivery rate was noted in the deliveries deriving from transfers of twice-cryopreserved embryos. However, it is noteworthy that the twin rate was significantly higher among pregnancies deriving from embryos cryopreserved once and that both singleton and twin deliveries were included in the analysis of neonatal outcomes. Parallel to the present study, only singleton deliveries were included in the analysis of perinatal outcomes in a study by Zheng et al. (2017). Accordingly, no congenital anomalies were detected in the 29 neonates born after the transfer of slow-frozen vitrified embryos, and the difference in birth weight compared to controls was nonsignificant (3417 g vs 3338 g in the twice-cryopreserved and once-cryopreserved groups, respectively).

Although the vast majority of children conceived with ART treatments are born completely healthy, the long-term safety and eventual epigenetic effects to the offspring may raise concerns. As epigenetic reprogramming is thought to occur in two waves, the first during gametogenesis and the second around the preimplantation time (Reik & Dean, 2001), ART techniques may cause epigenomic alterations. Epimutations are believed to increase the risk of metabolic, cardiovascular, and neuropsychiatric diseases manifesting later in the life of an individual and are therefore a difficult subject to study, with many confounding factors and decades of follow-up time required. To date, some studies have suggested an increased risk of the conditions mentioned above, as well as of some types of cancer, such as acute lymphoblast leukaemia and retinoblastoma, in children born after ART. (Feuer et al., 2013; Meister et al., 2018; Vrooman & Bartolomei, 2017) There are no studies considering the safety of repeated freeze-thaw cycles in the long term.

The main limitation of our study is its retrospective design. There might be ethical concerns in carrying out a prospective study on repeated cryopreservation in humans, but valuable data might be gained by animal studies. The other important limitation is the small sample size, which is still

comparable to that of previous studies. Also, the embryos in both groups were either slow frozen or vitrified at the time of the first cryopreservation, but the recryopreservation method in the case group was exclusively vitrification. The variation reported in the first cryopreservation methods represents clinical practice in many clinics, and nevertheless, the results did not differ between primarily vitrified or slow frozen embryos. The variation of the embryonic age at transfer was taken into consideration in the study design by using it as a matching criterion. Furthermore, there were some differences between the study groups. Primary infertility was less prevalent and the duration of infertility longer among the women in the case group, explained by the fact that the twice-cryopreserved embryos were transferred as a last option. Nevertheless, we consider that the results published so far provide the clinician with sufficient evidence to proceed with this method when indicated.

In conclusion, repeated cryopreservation presents as a cost-effective method to avoid double embryo transfer and wastage of embryos when there are more than one good-quality embryos available for FET. It also enables PGT of previously cryopreserved embryos. The possible long-term consequences to the health of the offspring warrant further studies.

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Table 1. Summary of previously published studies on pregnancy outcome after repeated cryopreservation.

Author	Years	Country	Freezing method	#FET (cases / controls)	# of embryos transferred (cases/controls)	#2 Survival rate	CPR (cases/ controls)	LBR (cases/controls)	# of newborns in the case group
Koch et al.	2003-2009	Australia	slow x2	52 / 40	55 / 43	82%	17/27.5% (NS)	13/15% (NS)	7
Farhi et al.	2011-2016	Israel	slow x2	25 / 50	27 / 50	96.4 %	16/44.2% (p<0.01)	12%/ N/A	5
Murakami et al.	2000-2009	Japan	1.slow 2.vitrif	92 / 335	105 / 474	98.1 %	66.3/59.6% (NS)	50/56.3% (NS)	46
Zheng et al.	2009-2012	China	1.slow 2.vitrif	127 / 444	179 / 637	98.9 %	44.1/48.4% (NS)	29.1/39.2% (p=0.04)	37
Kumasako et al.	2001-2007	Japan	vitrif x2	50 / 201	N/A	84.1 %	27.8/25.9% (NS)	N/A	N/A
Taylor et al.	2009-2013	USA	1.slow/vitrif 2.vitrif	16 / 85	21 / 113	87.5 %	56.3/61.2% (NS)	47.6/54.0% (NS)	N/A

vitrif= vitrification, FET= Frozen embryo transfer, CPR= clinical pregnancy rate, LBR= live birth rate

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Table 2. Clinical characteristics of the study groups with univariate analysis.

	Case (n = 89)	Control (n = 304)	p-value
Age at oocyte pickup [a] (years)	32 (4.2)	33 (4.1)	0.17
BMI [b] (kg/m2)	23 (21, 26)	23 (21, 26)	0.86
Infertility duration [b] (months)	64 (45, 94)	49 (35, 75)	<0.001
Primary infertility c (%)	28	43	0.01
Smoking [c] (%)	7	4	0.35
First Cryo-method			0.002
Slow freezing [c] (%)	58	40	
Vitrification [c] (%)	42	60	
Embryonic age at the first cryo [b] (days)	2 (2, 3)	3 (2, 5)	<0.001
Single embryo transfer (%)	100	86	
Protocol			0.029
Artificial cycle [c] (%)	40	28	
Natural cycle [c] (%)	60	72	

[a]Analysed with Student's t-test; results expressed as mean (SD)
[b]Analysed with Wilcoxon rank sum test; results presented as median (quartiles, Q1,Q3)
[c]Analysed with Chi square test

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Table 3. Pregnancy outcomes of the study groups with univariate analysis.

	Case	Control	P-value
	(n = 89)	(n = 304)	
Pregnancy rate[a] (%)	37.1	42.8	0.34
Clinical pregnancy rate[a] (%)	31.4	36.8	0.35
Miscarriage[b] (%)	4.5	3.9	0.77
Extrauterine pregnancy (%)		0.7	
Termination of pregnancy[a] (%)		0.3	
Biochemical pregnancy[a] (%)	5.6	3.9	0.81
Live birth rate[a] (%)	27.0	31.9	0.35
Singleton (n)	23	94	
Twin (n)	1	3	
Newborns (n)	25	100	
Gestational age [c] (weeks)	40 (39, 41)	39 (38, 40)	0.065
Preterm deliveries [b] (<37gwk, %)	4.2	10.3	0.69
Weight [c] (grams, singleton pregnancies)	3730	3490	0.064
	(3500, 4050)	(3150, 3900)	
Weight group [a]			
AGA (%)	100.0	90.0	
LGA (%)	0	8.0	
SGA (%)	0	2.0	
Sex, boys[a] (%)	48.0	45.0	0.77

[a]Analysed with Chi square test
[b]Analysed wih Fisher´s exact test
[c]Analysed with Wilcoxon rank-sum test; results presented as median (quartiles, Q1, Q3)
AGA = Appropiate for gestational age, LGA = Large for gestational age, SGA = Small for gestational age

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Table 4. The odds ratios for pregnancies with twice cryopreserved embryos compared to once cryopreserved embryos

	Adjusted OR (95% CI)	Adjusted p-value
Live Birth	0.7 (0.40, 1.22)	0.21
Pregnancy	0.74 (0.44, 1.22)	0.24
Clinical pregnancy	0.71 (0.42, 1.21)	0.21
Miscarriage	1.1 (0.33, 3.60)	0.88
Biochemical pregnancy	0.97 (0.33, 2.86)	0.96

The multivariate models (mixed effects logistic regression) included the study group, female age at ovum pickup, the duration of infertility and cycle type (natural or artificial) as explanatory factors (results not shown). OR = Odds ratio, CI = Confidence interval

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Key Message

Our study shows that embryo transfers after a second cryopreservation by vitrification result in uncompromised pregnancy results. Repeated cryopreservation appears to be a cost-effective method to avoid double embryo transfer and wastage of embryos when there is more than one good quality embryo available for frozen embryo transfer.

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