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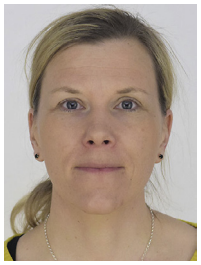
ARTICLE

Pregnancy potential and perinatal outcomes of embryos cryopreserved twice: a case–control study



BIOGRAPHY

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KEY MESSAGE

Survival rate is high after repeated cryopreservation by vitrification. Transfers of twice-cryopreserved embryos result in uncompromised pregnancy results. When more than one good quality cryopreserved embryo is available for transfer, repeated cryopreservation avoids double embryo transfer and wastage of embryos. Good quality repeat cryopreserved embryos should be considered suitable for transfer.

ABSTRACT

Research question: What are the pregnancy and perinatal outcomes of twice-cryopreserved embryos compared with embryos cryopreserved once?

Design: Retrospective register-based case–control study. The case group consisted of transfers of twice-cryopreserved embryos (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 35 years or 35 and greater.

Results: The survival rate of twice-cryopreserved embryos was 92.2%, and 93.7% of the planned frozen embryo transfers (FET) 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% versus 31.9%, adjusted odds ratio [OR] 0.70, 95% CI 0.40–1.22, P = 0.21), clinical pregnancy (31.5% versus 36.8%, adjusted OR 0.71, 95% CI 0.42–1.21, P = 0.21) and miscarriage (4.5% versus 3.9%, adjusted OR 1.10, 95% CI 0.33–3.60, P = 0.88) in the case and the control groups were comparable. No difference was seen in the preterm delivery rate (cases 4.2% versus controls 10.3%, P = 0.69). Twenty-five children were born in the case group and 100 in the control group. No difference in birthweight 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 twice-cryopreserved embryos. To avoid embryo wastage and transfer of multiple embryos, good quality surplus embryos from FET cycles may be cryopreserved again by vitrification.

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Declaration: The authors report no financial or commercial conflicts of interest.

KEYWORDS

Double cryopreservation
Frozen embryo transfer
Live birth rate
Pregnancy outcome
Repeated cryopreservation
Vitrification

INTRODUCTION

Embryo cryopreservation is a compelling option for increasing the cumulative pregnancy rate of treatments with assisted reproductive technologies (ART). Several studies on global trends in ART have demonstrated the live birth rate (LBR) in frozen embryo transfer (FET) cycles to be equal or even superior 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 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 with repetitive IVF cycles, FET provides a safe, cost-effective and patient-friendly way of achieving 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 (*Practice Committee of the Society for Assisted Reproductive Technology and Practice 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 favourable that double, not to mention multiple, embryo transfers are becoming difficult to justify (*Grady et al., 2012*; *Veleva et al., 2009*). In FET cycles, however, the eSET strategy may result in surplus good quality embryos after thawing/warming.

Repeated cryopreservation of viable surplus embryos in FET cycles is a potential way of further increasing the cumulative pregnancy rate as well as reducing 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 repeated cryopreservation by vitrification seems to achieve similar clinical pregnancy rates (CPR) compared with blastocysts vitrified once (*Kumasako et al., 2009*; *Zheng et al., 2017*). Currently, fewer than 100 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 from repeated cryopreservation of embryos is yet to be determined. 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 birthweight (*Berntsen and 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 cryopreserved twice by vitrification.

MATERIALS AND METHODS

Study design

This study was a retrospective register-based case–control study consisting of transfers of cryopreserved embryos carried out at two centres, Turku University Hospital (Centre A) and the Central Hospital of Central Finland (Centre B), both in Finland, between January 2012 and December 2019. The case group consisted 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 a freeze-all strategy to avoid ovarian stimulation 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 centre. 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

TABLE 1 SUMMARY OF PREVIOUSLY PUBLISHED STUDIES ON EMBRYO TRANSFER CYCLE OUTCOMES AFTER REPEATED CRYOPRESERVATION

Author	Years	Country	Freezing method	No. of FET (cases / controls)	No. of embryos transferred (cases / controls)	Survival rate (%)	CPR (cases / controls)	LBR (cases / controls)	No. of newborns in the case group
Koch et al. (2011)	2003–2009	Australia	slow × 2	52/40	55/43	82.0	17/27.5% (p=0.24)	13/15% (p=0.83)	7
Farhi et al. (2019)	2011–2016	Israel	slow × 2	25/50	27/50	96.4	16/44.2% (p<0.01)	12%/N/A	5
Murakami et al. (2011)	2000–2009	Japan	1. slow 2. vitrif	92/335	105/474	98.1	66.3/59.6% (p>0.05)	50/56.3% (p>0.05)	46
Zheng et al. (2017)	2009–2012	China	1. slow 2. vitrif	127/444	179/637	98.9	44.1/48.4% (p>0.05)	29.1/39.2% (p=0.04)	37
Kumasako et al. (2009)	2001–2007	Japan	vitrif × 2	50/201	N/A	84.1	27.8/25.9% (p>0.05)	N/A	N/A
Taylor et al. (2014)	2009–2013	USA	1. slow/vitrif 2. vitrif	16/85	21/113	87.5	56.3/61.2% (p>0.05)	47.6/54.0% (p>0.05)	N/A

CPR = clinical pregnancy rate; FET = frozen embryo transfer; LBR = live birth rate; N/A = not available; vitrif = vitrification.

TABLE 2 CLINICAL CHARACTERISTICS OF THE STUDY GROUPS WITH UNIVARIATE ANALYSIS

Characteristic	Case	Control	*P*-value
	(*n* = 89)	(*n* = 304)	
Age at oocyte retrieval[a] (years)	32 (4.2)	33 (4.1)	0.17
BMI[b] (kg/m^2)	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 transfers (%)	100	86[d]	
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-squared test.
[d] 14% double embryo transfers.

35 years and 35 and greater. Factors known to contribute to the success of 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).

The embryos were initially cryopreserved by either slow freezing or vitrification. All repeat cryopreservations 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 up until 2013; FreezeKit™ Cleave, Vitrolife Sweden AB, 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 up until 2013; ThawKit Cleave™, Vitrolife Sweden, from 2014 onwards). In Centre A, the Rapid-i™ Vitrification System (Vitrolife Sweden) was used for vitrification and warming of the embryos in a closed system, as described by the manufacturer. In Centre B, a VitriFreeze ES/VitriThaw ES system (FertiPro) with HSV® straws (Cryo Bio Systems) was used. The embryos were cultured in G1-PLUS™/G2-PLUS™ sequential media (Vitrolife Sweden) or SAGE 1-Step™ media (Origio) in 7% CO_2, 8% O_2, 85% N_2 (Centre A) or 6% CO_2, 10% O_2, 84% N_2 (Centre B) at 37 ± 0.1°C. Embryo transfers were performed under ultrasound guidance in natural or artificial cycles. Single embryo transfer was preferred, with double embryo transfer only in cases of poor embryo quality. Pregnancy tests were performed at an embryonic age of 14–16 days. Ultrasound examination was performed at the 7th gestational week to confirm a clinical pregnancy.

Outcome measures

The primary outcome was LBR, defined by the birth of at least one liveborn infant, and the secondary outcomes were CPR (a pregnancy visible with sonography at a minimum of 6 gestational weeks or an extrauterine pregnancy) and miscarriage rate (a spontaneous abortion of a detected clinical pregnancy up to 22 gestational weeks). The definition of a pregnancy was a positive pregnancy test. Biochemical pregnancies were defined as wastage of an early pregnancy not yet sonographically visible and recorded to analyse the total early pregnancy wastage rate. The gestational age at birth, birthweight and the sex ratio of the newborns were analysed. Any malformations were also recorded.

Statistical analysis

The data are 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 retrieval was tested with Student's *t*-test. Categorical data were analysed using a chi-squared test. Fisher's exact test was used if the variable had low group frequencies.

Statistical associations for the outcome of pregnancy with study groups and relevant explanatory variables were examined using mixed-effects logistic regression (GLIMMIX Procedure, SAS Institute Inc., Cary, NC, USA). The statistical models were made separately for live birth, pregnancy, clinical pregnancy, miscarriage and biochemical pregnancy rates as response variables. Female age at oocyte retrieval, 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 centre 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 birthweight 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 body mass index (BMI) and female parity were included in the model as explanatory variables and were treated as fixed effects. Study centre 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, Finnish law does not require approval by an ethical committee for register studies.

RESULTS

Altogether, 2834 FET cycles were performed during the study period. Of these, 89 FET 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 FET 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 retrieval and BMI were similar between the groups; nevertheless, the duration of infertility was significantly longer ($P < 0.001$) and the proportion of primary infertility was significantly lower among the cases ($P = 0.01$). There was a significant difference between the first cryopreservation method between the case and control groups, as slow freezing was used in 58% of the case group FET cycles versus 40% in the control group ($P = 0.002$). Also, FET was performed more often during an artificial cycle in the case group ($P = 0.029$) (TABLE 2).

The embryo transfer cycle outcomes are presented in TABLE 3. In univariate analysis, there were no statistical differences in LBR, CPR or miscarriage rates between the groups (27.0% versus 31.9%, $P = 0.35$; 31.5% versus 36.8%, $P = 0.35$; 4.5% versus 3.9%, $P = 0.77$, respectively). The results remained insignificant in multivariate analysis, which adjusted for female age at oocyte retrieval, the duration of infertility, cycle type (natural or artificial), and study group (TABLE 4). There was no difference in the cycle outcomes between slow freezing and vitrification as the primary cryopreservation method (Supplementary Table 1) and furthermore, the primary freezing method did not become statistically significant in the adjusted mixed-effects logistic regression models (data not shown). 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

TABLE 3 EMBRYO TRANSFER CYCLE OUTCOMES OF THE STUDY GROUPS WITH UNIVARIATE ANALYSIS

Outcome	Case	Control	*P*-value
	(*n* = 89)	(*n* = 304)	
Pregnancy (%)	33 (37.1)	131 (43.1)	0.34
Clinical pregnancy (%)	28 (31.5)	112 (36.8)	0.35
Miscarriage[b] (%)	4 (4.5)	12 (3.9)	0.77
Extrauterine pregnancy (%)		2 (0.7)	
Termination of pregnancy[a] (%)		1[d] (0.3)	
Biochemical pregnancy[a] (%)	5 (5.6)	12 (3.9)	0.81
Live birth (%)	24 (27.0)	97 (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] (<37 gestational weeks, %)	1 (4.2)	10 (10.3)	0.69
Weight[c] (g, singleton pregnancies)	3730 (3500, 4050)	3490 (3150, 3900)	0.064
Weight group[a]			
AGA (%)	25 (100.0)	90 (90.0)	
LGA (%)	0	8 (8.0)	
SGA (%)	0	2 (2.0)	
Sex, boys[a] (%)	12 (48.0)	45 (45.0)	0.77

AGA = appropriate for gestational age; LGA = large for gestational age; SGA = small for gestational age.

[a] Analysed with chi-squared test.

[b] Analysed with Fisher's exact test.

[c] Analysed with Wilcoxon rank-sum test; results presented as median (quartiles, Q1, Q3).

[d] Pregnancy terminated due to aneuploidy.

TABLE 4 THE OR FOR EMBRYO TRANSFER CYCLE OUTCOMES WITH TWICE-CRYOPRESERVED EMBRYOS COMPARED WITH ONCE-CRYOPRESERVED EMBRYOS

Outcome	Adjusted	Adjusted *P*-value
	OR (95% CI)	
Live birth	0.70 (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	1.10 (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 oocyte pickup, the duration of infertility and cycle type (natural or artificial) as explanatory factors (results not shown).

CI = confidence interval; OR = odds ratio.

3150 and 3900 g) in the control group and were not significantly different ($P = 0.064$). The embryonic age at transfer had no effect on birthweight (data not shown). 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.

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 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 not all risks can 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 (THL Medical Birth Register 2020, https://thl.fi/en/web/thlfi-en/statistics/information-on-statistics/register-descriptions/newborns).

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 to waste. In addition, the majority of infertility clinics have today shifted entirely from slow freezing cleavage-stage embryos in multiples into vitrifying single embryos that will be successfully warmed one by one, with little need for repeated cryopreservation. However, there are certain situations, such as cancellation of a FET 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 repeated cryopreservation is preimplantation genetic testing (PGT), which has only recently become widely available. Thawing previously frozen embryos for biopsy for aneuploidy screening or diagnosis of a known hereditary disease requires repeated cryopreservation while the analysis is being completed, followed by the transfer of a putatively healthy blastocyst, such as described by *Wilding et al. (2019)*. In many clinics, due to limited personnel and financial resources, a relatively large proportion of embryos are still cryopreserved at the cleavage stage and, because this was previously 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; but again, the fate of prospective surviving surplus blastocysts must be considered.

The current 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.

There are still some concerns regarding the possible health consequences of FET, such as an increased risk of large for gestational age fetuses as compared with fresh embryo transfers (*Berntsen and 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 those 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 birthweight was seen after the transfer of repeat vitrified embryos, although the difference was not statistically significant (2994 g versus 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 twice-cryopreserved embryos and the difference in birthweight compared with once-cryopreserved vitrified–warmed embryos was non-significant (3417 g versus 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 on 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 and 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 (*Heber and Ptak, 2021*; *La Rovere et al., 2019*) 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 and Bartolomei, 2017*). There are no studies considering the safety of repeated freeze–thaw cycles in the long term.

The main limitation of this 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, although all repeated cryopreservations were performed by vitrification, both slow freezing and vitrification were utilized as a first cryopreservation method in both groups. The variation reported in the first cryopreservation methods represents clinical practice in many clinics; 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 women in the case group, explained by the fact that the twice-cryopreserved embryos were transferred as a final 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 avoids double embryo transfer and wastage of embryos when there is more than one good quality embryo 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.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Petri Valtonen and Hanna Parikka for their contribution to data collection. This study received Finnish State Research Funding (to VJ).

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2021.06.028.

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Received 24 March 2021; received in revised form 27 May 2021; accepted 29 June 2021.