

Article

Blastocyst vitrification, cryostorage and warming does not affect live birth rate, infant birth weight or timing of delivery



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KEY MESSAGE

We found no evidence of an effect of blastocyst vitrification and cryostorage on the likelihood of implantation, clinical pregnancy, early pregnancy loss, live birth, low birth weight or preterm delivery.

ABSTRACT

Research question: Does vitrification and warming affect live birth rate, infant birth weight and timing of delivery?

Design: Retrospective, cohort study comparing outcomes of donor oocyte recipient fresh ($n = 25$) versus vitrified ($n = 86$) euploid blastocyst transfers; donor oocyte recipient singleton live births from fresh ($n = 100$) versus vitrified ($n = 102$) single embryo transfers (SET); and autologous vitrified euploid SET ($n = 1760$) [cryostored 21–1671 days].

Results: Group 1: fresh and vitrified–warmed blastocysts had similar live birth (OR 1.7; 95% CI 0.5 to 5.9), implantation (OR 0.9; 95% CI 0.2 to 3.9), clinical pregnancy (OR 3.4; 95% CI 0.9 to 13.0) and pregnancy loss (OR 1.2; 95% CI 0.98 to 1.4); group 2: low birth weight (OR 0.44; 95% CI 0.1 to 1.6) and preterm delivery (OR 0.99; 95% CI 0.4 to 2.3) rates were similar in fresh and vitrified–warmed blastocyst transfers; group 3: cryostorage duration did not affect live birth (OR 1.0; 95% CI 1.0 to 1.0), implantation (OR 1.0; 95% CI 0.99 to 1.01), clinical pregnancy (OR 1.0; 95% CI 1.0 to 1.0), pregnancy loss (OR 0.99; 95% CI 1.0 to 1.0), birth weight ($\beta = -15.7$) or gestational age at delivery ($\beta = -0.996$).

Conclusions: Vitrification and cryostorage (up to 4 years) are safe and effective practices that do not significantly affect clinical outcome after embryo transfer.

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Introduction

Since the first live birth from a cryopreserved human embryo almost 40 years ago (Downing et al., 1985), embryo cryopreservation techniques have greatly improved in efficacy and efficiency. The past decade has seen a shift from slow-freezing to vitrification, which involves ultra-rapid freezing and suspension of embryos in a glass-like state. Compared with slow-freezing, vitrification reduces the formation of ice crystals (Liebermann and Tucker, 2006; Son and Tan, 2009; Zegers-Hochschild et al., 2009) and results in increased embryo cryo-survival (Kolibianakis et al., 2009; Loutradi et al., 2008), clinical pregnancy (AbdelHafez et al., 2010; Stehlik et al., 2005; Wong and Wong, 2011), and live birth rates (Li et al., 2014). Embryo cryopreservation facilitates ovarian hyperstimulation syndrome prevention through freeze-all; elective fertility preservation for social and medical reasons; embryo transfer in a physiologic endometrial hormonal milieu; and preimplantation genetic testing (PGT) before transfer (Pandian et al., 2009; Wang et al., 2017). Compared with fresh embryo transfer, the more physiologic uterine environment of frozen embryo transfer (FET) might be more favourable for implantation and placentation (Amor et al., 2009; Healy et al., 2010; Kansal Kalra et al., 2011; Zeilmaker et al., 1984). The finding of improved pregnancy and live birth rates in FET cycles (Roque et al., 2013; Shapiro et al., 2011) was confirmed by a recent randomized controlled trial in which patients undergoing PGT were randomized to fresh embryo transfer or freeze-only cycles followed by subsequent FET (Coates et al., 2017). Live births achieved using FET have been associated with a decreased incidence of low birth weight (Pelkonen et al., 2010; Schwarze et al., 2015) and preterm delivery (Wennerholm et al., 2013).

The rapid adoption of vitrification into IVF practice and the growing proportion of IVF live births arising from FET warrants the evaluation of the effect of this technology on peri-implantation, i.e. short-term, and perinatal, i.e. long-term, outcomes to ensure its safety and efficacy. During vitrification, embryos are exposed to cryoprotectants and, in open-vitrification systems, are directly in contact with liquid nitrogen (Bielanski et al., 2003; Gosden, 2011). One or more of these exposures might alter early embryo development and affect implantation and growth potential. Compared with naturally conceived pregnancies, FET has been reported to lead to an increased risk of large for gestational age or macrosomic (Pinborg et al., 2014; Sazonova et al., 2012) infants, suggesting that the cryopreservation process may influence placentation and fetal growth. Most studies of FET cycles to date have not been appropriately designed to isolate for the independent effects of embryo cryopreservation and warming on clinical outcome. Studies comparing pregnancies from FET and natural conception are confounded by laboratory handling and programmed hormonal preparation of endometria, whereas studies comparing pregnancies from FET and fresh embryo transfer are confounded by the effect of ovarian stimulation on endometrial receptivity in fresh transfers. Furthermore, prior studies have not accounted for embryo ploidy status, a key factor affecting implantation potential.

Donor oocyte IVF provides a unique model for evaluating whether vitrification and warming of embryos has an independent effect on embryo implantation and placentation. In donor cycles, the ovarian stimulation of the donors is separated from the endometrial environment of the recipients (Navot et al., 1991). Initial studies comparing fresh and frozen embryo transfers in donor oocyte recipients reported reduced pregnancy rates after FET (Check et al., 1995; Tatpati et al., 2010). These studies, however, involved the transfer of pronu-

clear and cleavage-stage embryos that underwent slow-freezing. Therefore, the results cannot be applied to the current treatment paradigm of blastocyst culture and vitrification. Recent studies using the donor oocyte IVF model have focused on perinatal outcome, reporting no effect of embryo vitrification on infant birth weight or gestational age at delivery (Galliano et al., 2015; Kalra et al., 2011). Interpretation of these results is limited as these studies pooled data from multiple IVF centres and included mixed cohorts of blastocyst and cleavage-stage embryo transfers.

Few studies have explored a possible effect of cryostorage duration on embryo viability, implantation potential and perinatal outcome. Although cryopreservation is thought to halt metabolism and ageing, it is reasonable to question the stability of vitrified embryos over time. Vitrification involves rapid solidification of fluid into a glassy, disorganized, unstable state. As the temperature decreases below the threshold for glass transition, the disordered molecular pattern of a liquid is maintained despite the physical transition to a solid (Wowk, 2010). Within this state, cooling by only 10°C can induce an increase in viscosity by a factor of 1000 (Wowk, 2010). Therefore, the molecular structure of vitrified cells may be sensitive to storage temperature variations and affected by the duration of cryostorage in liquid nitrogen (Wirleitner et al., 2013). Most studies have failed to demonstrate a time-related effect of cryostorage on pregnancy and live birth rates (Aflatoonian et al., 2013; Riggs et al., 2010; Wirleitner et al., 2013); however, few have evaluated the effect of cryostorage duration on perinatal outcome.

Given the widespread clinical use of vitrification, a robust, continued evaluation of this technology is necessary to confirm whether blastocyst vitrification has independent effects on embryo–endometrial interaction and implantation, and whether this translates to any downstream effects influencing perinatal outcome. This study provides a comprehensive assessment of whether blastocyst vitrification, storage and warming affect reproductive and perinatal outcome after vitrified–warmed embryo transfer. To assess the effect of vitrification on embryonic implantation potential, donor oocyte recipients that underwent transfer of single, euploid fresh and vitrified–warmed blastocyst were evaluated. To assess the effect of blastocyst vitrification on birth weight and gestational age at delivery, donor oocyte recipients that achieved a singleton live birth after fresh and vitrified–warmed SET were compared. Finally, to assess the effect of cryostorage duration on IVF and perinatal outcome, we evaluated a cohort of patients with euploid blastocysts derived from autologous oocytes, who underwent PGT, vitrification and warming before SET.

Materials and methods

Study design and patient population

A single-centre, retrospective, cohort analysis of three distinct patient groups was conducted, analysing blastocyst transfers carried out between 2011 and 2016. All embryo transfers involving blastocysts derived from previously cryopreserved oocytes were excluded. Patients were identified from an electronic medical records database.

Group 1: Donor oocyte recipients undergoing transfer of fresh versus vitrified–warmed, PGT-screened blastocysts

To evaluate the effect of blastocyst vitrification on IVF and embryo transfer cycle outcome, donor oocyte recipients who underwent a fresh

(25 cycles in 24 patients) or vitrified–warmed embryo transfer (86 cycles in 59 patients) of an euploid blastocyst were compared. Blastocysts were derived from fresh donor oocytes and underwent trophectoderm biopsy for aneuploidy screening. A sub-analysis including only SET cycles was conducted.

Group 2: Donor oocyte recipients who achieved a singleton live birth after undergoing the transfer of fresh or vitrified–warmed, single blastocysts

To evaluate the effect of blastocyst vitrification on perinatal outcome, donor oocyte recipients who underwent transfer of a single, fresh ($n = 100$) or vitrified–warmed ($n = 102$) blastocyst resulting in a singleton live birth were included. Patients with monozygotic twins were excluded. The IVF and embryo transfer cycle demographics, characteristics and outcomes of the cohort from which these live births were derived were included. These cases included the first SET of donor oocyte recipients who underwent fresh ($n = 194$) and vitrified–warmed embryo transfers ($n = 230$) (of both PGT screened and unscreened embryos).

Group 3: Patients undergoing transfer of single, euploid vitrified–warmed blastocysts derived from autologous oocytes

To evaluate the effect of cryostorage duration on embryo transfer and perinatal outcome, patients ($n = 1297$) whose blastocysts underwent PGT, vitrification, warming and SET in autologous IVF and embryo transfer cycles ($n = 1760$) were included. Donor oocyte cycles were excluded from this analysis as they represented a small fraction of the cohort of patients undergoing single, euploid FET. Furthermore, their inclusion would introduce heterogeneity and confound the analysis as the use of donor oocytes lends itself to a favourable prognosis.

Stimulation protocol

Oocyte donors, patients, or both, underwent ovarian stimulation for IVF as previously described (Rodríguez-Purata et al., 2016). When at least two mature follicles 18 mm or wider were attained, final oocyte maturation was induced with recombinant HCG (Ovidrel®, EMD Serono, Rockland, MA, USA) alone (10000 IU) or, in high responders at risk of ovarian hyperstimulation syndrome, with 40 IU leuprolide acetate (Lupron®, AbbVie Laboratories, Chicago, IL, USA) combined with 1000 IU HCG (Novarel®, Ferring Pharmaceuticals, Parsippany, NJ, USA). Patients underwent vaginal oocyte retrieval under transvaginal ultrasound guidance 36 h after oocyte maturation was triggered.

Laboratory procedures (IVF, embryo culture, trophectoderm biopsy, embryo cryopreservation and warming)

Oocytes were fertilized by conventional insemination or intracytoplasmic sperm injection (ICSI) about 4 h after retrieval. In all cases in which PGT was planned, ICSI was used. Embryos were cultured to the blastocyst stage as previously described, with laser-assisted hatching on day 3 of development in cases in which PGT was planned (Rodríguez-Purata et al., 2016). Blastocyst trophectoderm biopsies were carried out, as previously described (Rodríguez-Purata et al., 2016), on day 5 or 6 of blastocyst development, depending on the timing of embryo expansion. In order to undergo biopsy, blastocysts were required to have a discernable inner cell mass, independent of the trophectoderm grade [consistent with a modified Gardner and Schoolcraft (Gardner and Schoolcraft, 1999) morphological grade of 4BC or better]. The presence of a multicelled trophectoderm herni-

ating out of the zona pellucida was considered ideal. Embryos failing to reach this developmental stage were deemed poor quality and incapable of further, normal development. Embryos biopsied on the morning of day 5 could be kept in culture overnight while PGT results were expedited to allow for fresh embryo transfer on the morning of day 6. Alternatively, blastocysts could be cryopreserved immediately after biopsy as part of a freeze-only treatment approach or in cases where slower developing embryos were not amenable to biopsy until day 6 or later. Cryopreserved blastocysts were individually vitrified using the modified Cryotop method, which has been previously described (Rodríguez-Purata et al., 2016).

Trophectoderm cells underwent 24 chromosome aneuploidy screening (at various laboratories) using one of the following platforms: quantitative-polymerase chain reaction, array comparative genomic hybridization, or targeted next-generation sequencing. In practice, patients using PGT are encouraged to undergo freeze-only cycles to allow availability of aneuploidy screening results for all blastocysts before transfer. For patients undergoing transfer of unscreened embryos, the decision of whether to carry out a fresh transfer, or to cryopreserve embryos for transfer in a subsequent FET cycle, was made based on clinical considerations and a discussion between the physician and the patient.

Endometrial preparation and embryo transfer

All donor oocyte recipients and autologous IVF patients who underwent transfer of vitrified–warmed embryos received synthetic hormonal preparation of their endometria before embryo transfer. To facilitate the transfer of fresh embryos in donor oocyte recipients, the recipient's cycle was synchronized with that of the oocyte donor. Synchronization was achieved by suppressing the recipient's hypothalamic–pituitary–ovarian axis with oral contraceptive pills for a minimum of 14 days, followed by down-regulation with daily-administered leuprolide acetate (Lupron®, AbbVie Laboratories, Chicago, IL, USA), continued for the duration of endometrial preparation. After menses and starting about 1 week before commencing ovarian stimulation in the oocyte donor, recipients began oral oestradiol (Estrace®; Teva Pharmaceuticals, Sellersville, PA, USA) 2 mg twice daily for 1 week, then 2 mg three times daily. After a minimum of 7 days of oestradiol administration, transvaginal ultrasounds were carried out weekly to assess recipients' endometrium, to ensure a thickness of at least 7 mm before transfer. Two days after the oocyte donor was administered HCG to trigger oocyte maturation, leuprolide acetate was discontinued and 50 mg of intramuscular progesterone (Progesterone injection®; Watson Pharma Inc., Parsippany, NJ, USA) was administered daily. Fresh embryo transfer was carried out after 6 days of progesterone supplementation. Preparation of donor oocyte recipients and patients using autologous oocytes who underwent FET involved the same protocol as described above for recipients of fresh embryos. A minimum of 12 days of oestradiol supplementation was administered before FET. Cryopreserved blastocysts were warmed using the modified Cryotop method as previously described by Rodríguez-Purata et al. (2016). Warming and transfer of the embryo was carried out on the sixth day of progesterone supplementation, regardless of the day of embryo development at time of cryopreservation. Embryos were selected for transfer based on PGT results, morphology grading, or both, according to a centre-modified Gardner and Schoolcraft scale (Gardner and Schoolcraft, 1999). The decision of how many embryos to transfer was made after a physician–patient discussion with extensive counselling regarding treatment prognosis, and an

individualized risk–benefit calculus of single versus multiple embryo transfer. Patients undergoing transfer of blastocysts determined to be euploid, based on PGT screening, were strongly encouraged to undergo elective SET.

Outcome measures

To examine the effect of blastocyst vitrification on IVF outcome, the primary outcomes analysed were live birth rate (LBR) (live birth of an infant ≥ 24 weeks gestation). Secondary outcomes included the rate of implantation (the number of intrauterine sacs divided by the number of embryos transferred), clinical pregnancy (CPR) (the proportion of patients with a fetal heart beat), and pregnancy loss (no gestational sac after serum beta-HCG 5 mIU/ml or more, or loss occurring after presence of an intrauterine gestational sac was confirmed). To investigate the effect of blastocyst vitrification on perinatal outcome, gestational age, infant birth weight and height and the rates of low birth weight (LBW) and preterm delivery were evaluated. Participants were categorized by whether their infants were of low (<2500 g), normal (between 2500 g and 4500 g) or macrosomic (>4500 g) birth weight. Preterm delivery was defined as birth at less than 37 weeks gestation. Whether cryostorage duration had an effect on the reproductive potential of vitrified blastocysts and the perinatal outcome of resulting pregnancies was assessed by analysing LBR, implantation rate, CPR, pregnancy loss, birth weight, gestational age at delivery and the rates of LBW and preterm delivery in relation to the duration of embryo cryostorage. The duration of cryostorage was calculated as the interval of time (days) elapsed from the date the blastocyst was vitrified to when it was warmed and transferred.

Statistical analysis

Statistical analyses were carried out using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). To examine the effect of blastocyst vitrification on IVF and embryo transfer outcome, the sample size of available participants provided 80% power to detect a 25% difference in live birth rate ($\alpha = 0.05$). In the analysis of the effect of blastocyst vitrification on perinatal outcome, the study had 80% power to detect a 1.5-week difference in gestational age at delivery and a 250 g difference in birth weight ($\alpha = 0.05$). Student's *t*-tests were carried out to compare continuous, normally distributed demographic and perinatal variables from donor oocyte recipients who underwent fresh compared with vitrified–warmed embryo transfer. Results are expressed as mean and SD with 95% confidence intervals. Chi-squared test and Fischer's exact test were used to compare the frequency of categorical clinical outcomes (implantation rate, CPR, LBR, pregnancy loss, preterm delivery, LBW) among donor oocyte recipients who underwent fresh and vitrified–warmed embryo transfers. Adjusted odds ratios with 95% CI were calculated using univariate and multivariate logistic regression analyses to assess the effect of embryo vitrification on the odds of implantation, clinical pregnancy, pregnancy loss, live birth, preterm delivery and LBW. Univariate and multivariate linear regression analyses were carried out to investigate whether blastocyst vitrification modified infant birth weight or gestational age at delivery. Univariate and multivariate logistic regression analyses were carried out to evaluate the effect of cryostorage duration on the odds of implantation, clinical pregnancy, pregnancy loss, live birth, preterm delivery and low birth weight. Univariate and multivariate linear regression analyses were carried out to assess the effect of cryostorage duration on infant birth weight and gesta-

tional age at delivery. Logistic and linear regression models were fit with generalized estimating equations to account for patients who underwent multiple fresh and vitrified–warmed embryo transfers. All demographic variables and cycle characteristics that were associated with the outcome in the univariate analysis ($P < 0.05$), and all variables thought to be clinically relevant were included as covariates in the model. All *P*-values are two-sided, with a critical significance level of $P < 0.05$.

Ethical approval

This retrospective cohort study was approved by the Western Institutional Review Board on 11 July 2017 (reference number: 1-1014319-1). The requirement for informed consent from patients was waived as patient data were anonymized and de-identified before analysis and inclusion in the study.

Results

The effects of blastocyst vitrification on IVF and embryo cycle outcomes

Donor oocyte derived blastocysts that underwent PGT for aneuploidy screening underwent fresh (25 cycles in 24 patients) and vitrified–warmed (86 cycles in 59 patients) transfers. Of the 91 blastocysts warmed for FET, 98.9% ($n = 90$) survived and were transferred. Demographic data and embryo transfer cycle characteristics are shown in **Table 1**. Recipients of fresh and vitrified–warmed blastocysts had similar baseline demographics. There was a significantly higher proportion of SET in the vitrified–warmed blastocyst transfer cohort ($P = 0.0006$), with a greater number of fresh blastocysts per transfer ($P = 0.02$). A greater proportion fresh blastocysts underwent trophectoderm biopsy on day 5 of development ($P < 0.0002$). The proportion of good-quality blastocysts (deemed amenable to trophectoderm biopsy, vitrification based on morphology grade criteria of 4BC or better, or both), however, was similar between the fresh and vitrified–warmed groups. Clinical outcomes of fresh versus vitrified–warmed SET of euploid blastocysts derived from donor oocytes are shown in **Table 2**. Patients who underwent fresh and vitrified–warmed euploid SET had similar rates of implantation, clinical pregnancy, pregnancy loss and live birth. After controlling for recipient age, oocyte donor age, recipient BMI, endometrial thickness at transfer, day of trophectoderm biopsy, and accounting for patients who underwent multiple transfer cycles, the odds of implantation (OR 0.93; 95% CI 0.22 to 3.94), clinical pregnancy (OR 3.39; 95% CI 0.88 to 13.0), pregnancy loss (OR 1.17; 95% CI 0.98 to 1.40), and live birth (OR 1.7; 95% CI 0.49 to 5.89) did not differ between the fresh or vitrified–warmed cohorts. To provide corroborating evidence using a larger sample size, the analysis was expanded to include all first fresh versus vitrified–warmed SET in donor oocyte recipients (including those that did and did not use PGT). Patient demographic data, embryo transfer cycle characteristics and outcomes are shown in **Table 3**. Fresh and vitrified–warmed SET resulted in similar rates of implantation, clinical pregnancy, pregnancy loss and live birth.

The effects of blastocyst vitrification on perinatal outcomes

Two hundred and two oocyte donor recipients achieved a singleton live birth from their first SET of a fresh ($n = 100$) or vitrified–

Table 1 – Donor oocyte recipient cycles using preimplantation genetic screening: baseline demographic and embryo transfer cycle characteristics.

	Fresh	Vitrified–warmed	P-value
Patients (n)	24	59	
Embryo transfer cycles (n)	25	86	
Recipient age (years) ^a	43.4 ± 4.0	44.9 ± 3.6	NS
Oocyte donor age (years) ^a	26.9 ± 3.7	27.6 ± 3.4	NS
Recipient BMI (kg/m ²) at transfer ^a	23.2 ± 3.6	23.5 ± 4.3	NS
Endometrial thickness at transfer (mm) ^a	8.8 ± 1.9	8.8 ± 1.7	NS
Peak serum estradiol (pg/ml) ^a	670.6 ± 440.9	535.2 ± 471.1	NS
Number of blastocysts/transfer ^a	1.28 ± 0.46	1.1 ± 0.2	0.02
Single-embryo transfers, % (n)	68.0 (17/25)	95.3 (82/86)	0.0006
Proportion of blastocysts that underwent trophectoderm biopsy on day 5 of development, % (n)	100.0 (25/25)	36.0 (31/86)	<0.0002
Blastocysts with morphology grade ≥4BC transferred, % (n)	92.0 (23/25)	93.0 (80/86)	NS

^a Results are expressed as mean ± SD with 95% CI.
BMI, body mass index.

warmed blastocyst (n = 102). These patients had similar baseline demographics and cycle characteristics, aside from the vitrified–warmed transfer group having a higher proportion of day-6 embryos (P < 0.0001) (Table 4). The perinatal outcomes of singleton live births from fresh versus vitrified–warmed SET were similar (Table 5). After controlling for recipient age, oocyte donor age, recipient BMI, endometrial thickness at transfer and day of embryo development at

transfer, the odds of preterm delivery (OR 0.99; 95% CI 0.4 to 2.3), LBW (OR 0.44; 95% CI 0.1 to 1.6) and macrosomia (OR 1.0; 95% CI 0.99 to 1.0)] were similar among the fresh and vitrified–warmed cohorts.

The effect of duration of cryostorage on reproductive competence and perinatal outcomes after the transfer of vitrified and warmed blastocysts

Patients (n = 1297) underwent 1760 cycles involving the transfer of single, vitrified–warmed, euploid blastocysts derived from autologous oocytes. Of the 1804 blastocysts warmed, 97.6% (n = 1760) survived and were transferred. The average duration of cryostorage was about 4 months, with a maximum duration of up to 4.6 years (mean 122.5 ± 194.1 days; range 21–1671 days). A 73.0% (n = 1284/1760) positive pregnancy test rate was achieved after FET. The live birth rate was 47.8% (n = 842/1760). Of all single FET, 60.3% (n = 1061/1760) resulted in implantation and 55.9% (n = 984/1760) resulted in clinical pregnancy. The pregnancy loss rate was 34.4% (n = 442/1284). The average gestational age at delivery was 38.2 ± 2.0 weeks with an average infant birth weight of 3331.8 ± 591.5 g. The overall rate of

Table 2 – IVF and embryo transfer outcomes in donor oocyte recipients using preimplantation genetic screening: fresh versus vitrified–warmed single embryo transfers.

	Fresh	Vitrified–warmed
Patients (n)	17	57
Cycles (n)	17	82
Positive Beta-HCG test, %, n	82.4 (14/17)	73.2 (60/82)
Implantation rate, %, n	76.5 (13/17)	73.2 (60/82)
Clinical pregnancy rate, %, n	76.5 (13/17)	56.1 (46/82)
Live birth rate, %, n	58.8 (10/17)	40.2 (33/82)
Pregnancy loss rate, %, n	28.6 (4/14)	45.0 (27/60)

^a No statistically significant differences between the two groups.

Table 3 – Donor oocyte recipients' first fresh versus vitrified–warmed single embryo transfers: patient demographic data, embryo transfer cycle characteristics and clinical outcomes.

	Fresh	Vitrified–warmed	P-value
Donor oocyte recipients' first single-embryo transfer cycle	194	230	
Recipient age (years) ^a	43.6 ± 4.1	44.7 ± 3.6	NS
Oocyte donor age (years) ^a	27.0 ± 3.1	27.3 ± 3.4	NS
Recipient BMI (kg/m ²) at transfer ^a	24.5 ± 5.0	24.6 ± 5.2	NS
Endometrial thickness at transfer (mm) ^a	9.3 ± 2.2	8.9 ± 2.2	NS
Peak serum estradiol (pg/ml) ^a	411.8 ± 233.82	453.5 ± 192.2	NS
Blastocysts transferred on day-6 of development, % (n)	10.3 (20/194)	55.2 (127/230)	<0.0001
Blastocysts with morphology grade ≥4BC transferred, % (n)	90.2 (175/194)	92.6 (213/230)	NS
Proportion of blastocysts that underwent trophectoderm biopsy for PGS, % (n)	8.8 (17/194)	24.8 (57/230)	<0.0001
Positive beta-HCG test, % (n)	71.6 (139/194)	67.8 (156/230)	NS
Implantation rate, % (n)	61.3 (119/194)	57.4 (132/230)	NS
Clinical pregnancy rate, % (n)	60.3 (117/194)	54.3 (125/230)	NS
Live birth rate, % (n)	51.5 (100/194)	44.3 (102/230)	NS
Pregnancy loss rate, % (n)	28.1 (39/139)	34.6 (54/156)	NS

^a Results are expressed as mean ± SD with 95% CI.
BMI, body mass index; NS, not statistically significant.

Table 4 – Donor oocyte recipient cycles resulting in singleton live birth: patient demographic data and embryo transfer cycle characteristics.

	Fresh	Vitrified-warmed
Donor oocyte recipients achieving singleton live birth from SET (n)	100	102
Recipient age (years) ^b	43.1 ± 4.5	44.1 ± 3.9
Oocyte donor age (years) ^b	27.0 ± 3.1	27.3 ± 3.4
Recipient BMI (kg/m ²) at transfer ^b	24.4 ± 5.0	24.9 ± 5.3
Endometrial thickness at transfer (mm) ^b	9.7 ± 2.3	9.1 ± 2.1
Peak serum estradiol (pg/ml) ^b	481.8 ± 513.8	333.5 ± 188.9
Embryos transferred on day 6 of development, %, n	10.0 (10/100)	55.9 (57/102)
Blastocysts with morphology grade ≥4BC transferred, %, n	91.0 (91/100)	93.1 (95/102)
Proportion of blastocysts that underwent trophectoderm biopsy for PGS, %, n	9.0 (9/100)	14.7 (15/102)

^a Results are expressed as mean ± SD with 95% CI.
^b P < 0.0001.
 BMI, body mass index; PGS, preimplantation genetic screening; SET, single embryo transfer.

preterm delivery was 17.8% (n = 150/842) and the rate of LBW was 6.8% (n = 57/842).

Univariate logistic regression analyses within a generalized estimating equations model were used to assess whether baseline demographic characteristics (age at time of embryo transfer, age at time of oocyte retrieval, BMI, anti-Müllerian hormone, day-3 FSH) or cycle data (peak serum oestradiol level, endometrial thickness at transfer, developmental day and stage of embryo transferred) had significant effects on transfer outcome. The odds of implantation were significantly decreased with advancing patient age at time of embryo transfer (OR 0.97; 95% CI 0.95 to 0.997; P = 0.03). The odds of pregnancy loss were significantly decreased with increasing endometrial thickness at the time of embryo transfer (OR 0.9; 95% CI 0.82 to 0.99; P = 0.047).

After controlling for patients who underwent multiple transfers, patient age at time of IVF and embryo transfer, day of trophectoderm biopsy, BMI and endometrial thickness at transfer, the duration of cryostorage did not significantly affect the odds of implantation (OR

1.002; 95% CI 0.9995 to 1.004), clinical pregnancy (OR 1.0015; 95% CI 0.9993 to 1.004), pregnancy loss (OR 0.9991; 95% CI 0.9967 to 1.002), or live birth (OR 1.001; 95% CI 0.9988 to 1.0033). The duration of cryostorage was not a significant predictor of birth weight ($\beta = -15.7$) or gestational age at delivery ($\beta = -0.996$) and did not modify the odds of preterm delivery (OR 1.0005; 95% CI 0.989 to 1.012). The odds of LBW could not be calculated owing to a low number of occurrences (n = 57).

Discussion

Increased use of elective SET and the proposed clinical benefits of transferring embryos to a uterine cavity with a physiologic hormonal milieu, has led to embryo vitrification and warming to become an integral, intermediate step between IVF and embryo transfer in modern-day assisted reproduction. As cryopreservation technology has evolved to the routine use of vitrification, a careful assessment of its potential effects on IVF and embryo, and perinatal outcome is warranted. In this single-centre, cohort study, we have demonstrated that live birth rate, birth weight and timing of delivery are not affected by blastocyst vitrification, cryostorage and warming.

The donor oocyte recipient model was used to compare fresh and vitrified-warmed embryo transfers, and allowed for an assessment of whether embryo cryopreservation affects reproductive potential, independent of the effects of ovarian stimulation on the endometrial environment. After controlling for the number of blastocysts transferred, oocyte donor age, recipient age, BMI and the day of embryo development the odds of implantation, clinical pregnancy and pregnancy loss were not affected by whether a fresh or a vitrified-warmed blastocyst was transferred. Furthermore, transfer of single vitrified-warmed blastocysts did not increase the odds of preterm delivery, low birth weight or macrosomia. In a large population of patients undergoing the transfer of single, vitrified, euploid blastocysts derived from patients' autologous oocytes, the duration of blastocyst storage in liquid nitrogen (≤ 4 years) was not found to affect embryo transfer success, birth weight and timing of delivery.

The results of early studies that evaluated the isolated effect of embryo cryopreservation on IVF and embryo transfer outcome, using the donor oocyte study model, questioned the efficacy of cryopreservation by the slow-freezing method, and emphasized the need to continue synchronizing donor and recipient cycles in order to facilitate fresh transfer (Check et al., 1995, 2001; Selick et al., 1995). Check et al. (1995) compared fresh and slow-frozen zygote and cleavage-stage embryo transfers in a cohort of donor oocyte recipients and reported a significantly higher implantation rate after fresh transfer (15.6% versus 7.1%; P < 0.05), suggesting that cryopreserved embryos had compromised quality. A subsequent study by the same investigators (Check et al., 2001) confirmed that donor oocyte recipients who underwent transfer of fresh zygote and cleavage-stage embryos had significantly higher clinical pregnancy rates (63.4% versus 43.6%; P < 0.05) compared with those who received slow-frozen embryos. In a comparative analysis of donor oocyte recipient cycles involving the transfer of fresh and slow-frozen zygotes, Selick et al. (1995) reported that slow-freezing and thawing led to the loss of 23% of embryos and an overall decline in post-thaw morphological grade. Interestingly, the latter finding did not translate into significantly different implantation rates per transfer between the fresh and vitrified-warmed cohorts.

Table 5 – Perinatal outcome after fresh versus vitrified-warmed single embryo transfers in donor oocyte recipients achieving singleton live birth at 24 weeks or over.

	Fresh	Vitrified-warmed
Patients achieving singleton live births from SET (n)	100	102
Gestational age at delivery (weeks) ^a	37.9 ± 1.8	37.7 ± 1.5
Preterm delivery, %, n	25.3 (25/99)	24.0 (24/100)
Height (inches) ^a	20.1 ± 1.1	20.2 ± 1.2
Birth weight (g) ^a	3326.8 ± 638.1	3285.7 ± 599.0
Low birth weight (<2500 g) %, n	3.1 (3/97)	8.1 (8/99)
Normal birth weight (2500–4200 g), %, n	89.7 (87/97)	91.9 (91/99)
Macrosomia (>4500 g), %, n	2.1 (2/97)	0 (0/99)

^a Results are expressed as mean ± SD with 95% CI. There were no statistically significant differences between the two groups.
 SET, single embryo transfer.

Previously published data obtained with slow-freezing and transfer at the zygote or cleavage stage is not applicable to the current treatment paradigm, which involves blastocyst vitrification, warming and SET. Few studies have isolated and examined the effects of vitrification and warming on blastocyst competence. Taylor et al. (2014) compared the implantation and pregnancy rates resulting from previously vitrified blastocysts that underwent warming, trophectoderm biopsy and re-vitrification with blastocysts that were biopsied and vitrified-warmed only once before transfer (Taylor et al., 2014). Twice-vitrified and warmed blastocysts had a lower warming survival rate than once-vitrified and warmed blastocysts (87.5% versus 98.3%; $P < 0.05$); however, no effect was observed on implantation, pregnancy and live birth rates. The present study is the first to use a donor oocyte recipient model to assess the effects of blastocyst vitrification and warming on transfer outcome.

Multivariate regression model demonstrated that vitrification and warming did not significantly affect outcome; however, the pregnancy loss rate was 45% in the cohort of donor oocyte recipients who underwent single euploid FET, compared with 28.6% after fresh single euploid embryo transfer. The reasons for this high loss rate are unknown at present but it is not easy to draw conclusions in this cohort owing to the small sample size. When a larger cohort of all fresh ($n = 194$) and vitrified-warmed ($n = 230$) SET in donor oocyte recipients (including both PGT and non PGT cycles) was analysed, biochemical and clinical pregnancy loss after FET (34.6%) was more consistent with that seen in clinical practice and in the study cohort of autologous cycle, single, euploid FET (34.4%). Forman et al. (2012) demonstrated a similarly high pregnancy loss rate (29.6%, $n = 32/108$) in patients who underwent SET of unselected embryos (Forman et al., 2012). Nonetheless, these high loss rates remain to be investigated further.

This single-centre study is the first to use a donor oocyte recipient model to assess the effects of blastocyst vitrification and warming on perinatal outcomes. The transfer of single, vitrified-warmed blastocysts did not significantly influence infant birth weight, height or gestational age at delivery. These findings agree with multicentre (Galliano et al., 2015) and registry-based (Kalra et al., 2011) studies, which compared perinatal outcomes after the transfer of fresh and cryopreserved donor-oocyte derived embryos. On the basis of national live birth data from the Society for Assisted Reproductive Technology database, Kalra et al. (2011) reported no difference in the rate of low birth weight infants after fresh compared with cryopreserved embryo transfers in donor oocyte recipients. This study, however, did not specify the stage of embryo development at cryopreservation and transfer, or the cryopreservation technology used (Kalra et al., 2011). In a retrospective analysis of data pooled from multiple IVF centres, Galliano et al. (2015) reported no differences in gestational age at delivery or birth weights of infants conceived from the transfer of fresh and cryopreserved embryos derived from sibling donor oocytes. This study included a combination of cleavage and blastocyst stage embryos that were cryopreserved using either slow freeze or vitrification. The investigators, however, conducted a sub-analysis demonstrating no effect on perinatal outcome as a function of method of cryopreservation or stage of the embryo at transfer (Galliano et al., 2015).

Concerns have been raised about the effects of cryopreservation on placentation and perinatal outcome. A large, national, register-based cohort study that compared the outcomes of spontaneously conceived pregnancies with those derived from the transfer of slow-frozen embryos reported significantly higher rates of preterm birth

and low birth weight after the transfer of embryos slow-frozen from the zygote to the blastocyst stage (Pelkonen et al., 2010). Conversely, a more recent Danish, register-based cohort study (Pinborg et al., 2014) reported a significantly increased risk of large-for-gestational age infants and macrosomia in offspring from FET of slow-frozen cleavage stage embryos compared with those resulting from natural conceptions. It is possible that in-vitro culture of embryos promotes intrauterine overgrowth and that this effect might be masked after fresh embryo transfers by the deleterious effects of ovarian stimulation on endometrial receptivity, placentation and fetal growth. This study's findings refute the hypothesis that embryo cryopreservation and warming may contribute to alterations in fetal growth. Differences between our results and previous findings assessing pregnancies conceived from FET and spontaneous conception might be attributable to differences in laboratory methodology, i.e., current use of vitrification and transfer of blastocysts rather than slow freezing and transfer of cleavage stage embryos, or to other non-cryopreservation laboratory-related exposures such as differences in-vitro culture. By only including fresh and vitrified-warmed embryos cultured to the blastocyst stage and transferred into a synthetically prepared endometrial cavity, our study design controlled for these potential confounders.

An assessment of blastocyst vitrification and warming and its effect on clinical outcomes requires evaluation for independent effects of cryostorage duration. As vitrification involves the rapid solidification of fluid into a disorganized, unstable state, possibly leading to dynamic, structural changes over time, it is conceivable that the interval of time in cryostorage could affect the stability of embryos (Wirleitner et al., 2013). Early studies that examined the effect of cryostorage duration on slow-frozen embryos reported a decline in survival rate after only 6–15 months of storage (Testart et al., 1987) as well as a tendency towards lower pregnancy rates after prolonged storage (Machtinger et al., 2002; Schalkoff et al., 1993). The findings of more recent animal (Eum et al., 2009; Lavara et al., 2011; Sanchez-Osorio et al., 2010) and human studies (Afatoonian et al., 2013; Riggs et al., 2010; Wirleitner et al., 2013), however, have been reassuring and reported a lack of effect of cryostorage duration on post-warming embryo quality, survival, implantation and live birth potential. Most recently, Li et al. (2017) reported that cryostorage duration did not affect clinical pregnancy and live birth rates after 786 FET cycles involving the transfer of vitrified-warmed cleavage stage embryos (Li et al., 2017). This study, however, was limited as it did not include blastocyst transfers and failed to control for patients who underwent multiple cycles and the transfer of multiple embryos in a given cycle. The only published study to evaluate the effect of cryostorage duration on the reproductive potential of vitrified blastocysts included 603 embryos stored for up to 6 years. When comparing cohorts of patients whose embryos were cryostored for different periods of time, no differences were found in implantation, clinical pregnancy, pregnancy loss or live birth rates (Wirleitner et al., 2013). Other studies have demonstrated significant benefits of routine blastocyst biopsy, vitrification and warming to their success rates across autologous IVF patients of varying age groups (Whitney et al., 2016); however, this is the first study to evaluate whether the duration of storage of vitrified-warmed PGT-screened blastocysts affects blastocyst competence when controlling for ploidy. Additionally, this study is the first to explore whether the total period of time that blastocysts remain vitrified has an effect on perinatal outcome. Reassuringly, the study's results demonstrated that the storage of vitrified-warmed blastocysts for up to a 4-year duration did not affect the likelihood of implantation,

clinical pregnancy, live birth or pregnancy loss. Moreover, storage duration did not influence infant birth weight or gestational age at delivery. These results provide reassurance that blastocyst vitrification followed by prolonged cryostorage, before warming and transfer, is a safe and effective practice.

To the best of our knowledge, this is the largest, single-centre study to evaluate the effects of blastocyst vitrification and warming on blastocyst competence and perinatal outcomes. It is the first study to assess the effects of cryopreservation on implantation potential while controlling for ploidy, by only including patients that used PGT and underwent exclusive transfer of euploid embryos. The lack of control for embryonic ploidy in previously published studies represents a significant limitation and potential source of confounding bias, even in cases in which the donor oocyte model was used. Blastocysts derived even from young donor oocytes have been demonstrated to have clinically significant rates of aneuploidy (Sekhon et al., 2016; Sills et al., 2014; Yang et al., 2012). By focusing on the donor oocyte model, our study alleviates many confounding variables that have not been addressed to date, such as the effect of ovarian stimulation on the endometrium. In contrast to previously published studies based on pooled data from registries or multiple programmes, the present study included patients treated at a single centre, thus ensuring homogeneity of IVF and embryo transfer, and laboratory practices.

The findings of this study are limited owing to its retrospective, non-randomized design. Although use of the donor–oocyte model allows for isolation of effect of vitrification on clinical outcome, the results seen in donor oocyte recipients may not be generalizable to the general infertile population. Despite having adequate power to evaluate clinical outcomes, certain study cohorts, such as recipients of donor oocyte derived blastocysts that underwent PGT, had a limited sample size. The clinical utilization of PGT by donor oocyte recipients to date has been low because of the perception of a minimal baseline risk of aneuploidy in embryos derived from young donor oocytes. We chose to narrow the inclusion criteria to donor oocyte recipients that used PGT, despite the limitations it imposed on our study's sample size, based on the premise that controlling for ploidy status is essential to rule out the confounding effect of chromosome constitution on implantation potential. Because the oocytes used by recipients of fresh and vitrified–warmed embryos were derived from a variety of oocyte donors, we cannot exclude potential confounding effects of differing oocyte source, as maternal drivers of gene expression could influence the quality of fertilization, implantation and placentation. The ideal future study design would involve randomization of oocyte donor recipients, using sibling oocytes from the same donor, to the transfer of fresh as compared with vitrified, single, euploid blastocysts.

Additionally, the study did not have the ability to track and report perinatal outcomes beyond birth weight and gestational age at delivery, i.e., gestational hypertension, gestational diabetes, preeclampsia and placental disease, as patients were discharged from the IVF practice at 9 weeks' gestation. As patients were discharged to various obstetricians, variations in clinical antepartum management that could have contributed to differences in perinatal outcome could not be controlled for in the analysis. Previously published studies have reported an association between morbidly adherent placentation and FET of slow–frozen (Kaser et al., 2015) and vitrified (Ishihara et al., 2014) embryos. Others have reported a higher rate of postpartum haemorrhage after transfer of vitrified versus fresh blastocysts (Wikland et al., 2010). The underlying mechanism(s) behind these associations are not clear. It has been hypothesized that the vitrification process modifies trophectoderm cells in a way that might nega-

tively affect placentation (Wikland et al., 2010). Future studies should include detailed perinatal outcomes reflective of the quality of placentation, beyond birth weight and gestational age at delivery.

To ensure the safety of routine blastocyst vitrification and warming, future studies should evaluate the long-term health of infants after blastocyst vitrification and long-term cryostorage. It is possible that the environmental changes and laboratory handling during vitrification and warming induce epigenetic changes too subtle to demonstrate an effect on IVF and embryo transfer or perinatal outcomes but could have possible effects on the development and health of resulting offspring. Defects in methylation have been correlated with impaired fetal growth (Lefebvre et al., 1998). Human studies investigating the epigenetic effects of embryo cryopreservation are lacking. The collection of placentae and cord blood at birth could be useful to study whether any major transcriptional, epigenetic changes, or both, are associated with blastocyst vitrification and warming.

In conclusion, the ability to reliably vitrify, cryostore, warm and select a single euploid embryo for transfer has improved reproductive outcome by decreasing cycle failure rates and the incidence of multiple gestations (Forman et al., 2012). The similar live birth rates, birth weight and timing of delivery after fresh and vitrified–warmed embryo transfer in donor oocyte recipients implicates embryo–endometrial asynchrony, owing to a supraphysiologic hormonal environment, as the cause for reduced implantation and birth weight often reported when autologous IVF with fresh embryo transfer are compared with FET cycles. The finding that blastocyst vitrification, cryostorage and warming do not affect embryonic implantation potential or perinatal outcome is reassuring and demonstrates that a routine strategy of vitrifying, warming and selecting embryos for transfer to a synthetically prepared endometrium can maximize treatment efficacy, efficiency and safety.

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