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ARTICLE

Vitrification does not affect birth weight: lessons from the oocyte donation model

BIOGRAPHY

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KEY MESSAGE

Evidence from the present study suggests that embryo vitrification–warming procedures have no effect on birth weight of children born to donor oocyte recipients. The data are reassuring and confirm previously published data that exclude vitrification as a contributing factor to adverse neonatal outcomes.

ABSTRACT

Research question: Is embryo cryopreservation a cause of high birth weight and large for gestational age (LGA) in singletons resulting from vitrified–warmed embryo transfer?

Design: Retrospective cohort study evaluating 670 oocyte recipients who underwent fresh (367 cycles) or vitrified–warmed embryo transfer (303 cycles) at Instituto Bernabeu between July 2017 and March 2019. All single blastocyst transfers carried out in an artificial cycle that resulted in a singleton live birth were included.

Results: Maternal age (42.21 ± 4.45; 42.79 ± 3.83; $P = 0.519$), body mass index (23.34 ± 3.69; 23.80 ± 3.78; $P = 0.075$), gestational age (38.96 ± 1.97; 38.77 ± 2.15; $P = 0.207$), maternal smoking (10.8%; 13.0%; $P = 0.475$), gestational diabetes (4.9%; 4.3% $P = 0.854$), preeclampsia (2.7%; 5.6%; $P = 0.074$), hypertensive disorders (3.3%; 2.3%; $P = 0.494$), maternal parity (multiparous 18.5%; 14.5%; $P = 0.177$) and liveborn gender (female 44.5%; 48.8%; $P = 0.276$) were not significantly different between fresh or vitrified–warmed groups. Endometrial thickness was significantly higher in the fresh versus vitrified–warmed group (8.83 ± 1.73 versus 8.57 ± 1.59; $P = 0.035$, respectively). Oocyte donor height was similar between the fresh versus vitrified–warmed group (163.22 ± 5.88 versus 164.27 ± 6.66 cm; $P = 0.057$, respectively). Mean birth weight was not significantly different (3239.21 ± 550.43; 3224.56 ± 570.83; adjusted $P = 0.058$). No differences were observed in macrosomia (7.1%; 6.3%; adjusted OR 0.857, 95% CI 0.314 to 2.340, $P = 0.764$), LGA (6.0%; 6.7%; adjusted OR 0.450, 95% CI 0.176 to 1.149, $P = 0.095$), pre-term birth (10.9%; 9.0% adjusted $P = 0.997$), very pre-term birth (0.8%; 1.3%; adjusted $P = 1.000$), extremely pre-term birth (0%; 1.0%; adjusted $P = 0.998$); underweight (10.0%; 7.0%; adjusted $P = 0.050$); very low weight (0.6; 1.1%; adjusted $P = 1.000$) and small for gestational age (1.9%; 0.7%; adjusted $P = 0.974$) between fresh or vitrified–warmed groups.

Conclusion: This study eliminates potential confounders that might influence fetal growth and demonstrates that embryo vitrification and warming procedures do not affect birth weight.

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KEYWORDS
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Large for gestational age
Vitrified–warmed embryo transfer

INTRODUCTION

In recent years, the field of assisted reproductive technology (ART) has expanded considerably, and the number of IVF cycles has increased. In Europe, one in 50 children are born as a result of ART treatments (nearly 1.5 million infants) as shown in the most recent report from the European Society of Human Reproduction and Embryology (*Calhaz-Jorge et al., 2020*). Current efforts mainly focus on reducing complications in the mother and newborn.

IVF constitutes a unique situation in which fertilization and initial embryo development take place in an artificial environment. Legitimate concerns about potential perinatal risks and complications were raised in the early days of IVF treatment. Current medical evidence shows poorer perinatal outcomes in pregnancies after ART even when singleton pregnancies conceived naturally and by ART in the same woman are compared (*Pinborg et al., 2013*). Concerns remain about whether laboratory procedures carried out during the early stages of a human embryo may later affect fetal growth, leading to disease in adulthood (Barker hypothesis) (*Barker et al., 2007*).

Of all the laboratory procedures, cryopreservation constitutes one of the processes that potentially affects the health of the newborn. Data from register-based studies (*Pelkonen et al., 2010*; *Nakashima et al., 2013*; *Wennerholm et al., 2013*; *Schwarze et al., 2015*; *Luke et al., 2017*), randomized controlled trials (RCTs) (*Shi et al., 2018*; *Vuong et al., 2018*; *Zhang et al., 2018*) and meta-analyses (*Maheshwari et al., 2018*) have found an increase in birth weight with a higher incidence of large for gestational age (LGA) in newborns derived from frozen embryo transfers (FET), but the precise underlying mechanism remains unclear.

Many potential variables may influence these weight-related perinatal outcomes. Endometrial factors, and more specifically those related to ovarian stimulation and supraphysiological hormone levels in fresh transfers, may justify the differences in the perinatal prognosis compared with FETs (*Pereira et al., 2015*). Nonetheless, it is difficult to disentangle the influence of the cryopreservation process and the epigenetic alterations concomitantly suggested to explain the differences (*Grace and Sinclair, 2009*; *Pinborg et al., 2014*; *Hiura et al., 2017*).

In daily practice, improvements in cryopreservation techniques have led to the generalization of single embryo transfers and the widespread use of the 'freeze-all' approach, with FET outnumbering fresh transfers. Therefore, ensuring the safety of cryopreservation techniques is of paramount importance.

Oocyte donation represents an ideal model to study the effect of embryo cryopreservation on perinatal prognosis with the advantage of eliminating the influence of ovarian stimulation as a potential confounder. Additionally, recipients from oocyte donation treatments usually undergo similar endometrial preparation for both fresh and FET cycles.

In view of the uncertainty surrounding the potential effect of the vitrification–warming process on perinatal outcomes, a single centre cohort study in women receiving embryos from oocyte donation was conducted. Confounding factors relating to birth weight were corrected for. The aim was to determine whether FET is related to higher birth weight.

MATERIALS AND METHODS

Study design and setting

In this retrospective cohort study, oocyte recipients who underwent fresh embryo transfer (367 cycles) or vitrified–warmed embryo transfer (303 cycles) were evaluated at Instituto Bernabeu between July 2017 and March 2019. All included cycles were single embryo transfers carried out at blastocyst stage, which resulted in a singleton live birth of at least 25 weeks (FIGURE 1). Overall, 388 different donors provided oocytes for the 670 recipients. Recipients participated only once in the study.

Included maternal characteristics were age, ethnicity, pregnancy and parity, body mass index and smoking. Included oocyte donor characteristics were height and ethnicity.

Embryo transfer data included the type of oocyte (fresh n = 586; 271 fresh embryo transfer and 315 FET) or vitrified–warmed (n = 84; 52 fresh embryo transfer and 32 FET), type of embryo transfer (fresh or vitrified–warmed), quality of the blastocysts, if the transferred embryo had been biopsied and the blastocyst stage (day 5/6).

Cycle and pregnancy data included endometrial thickness, report of obstetric complications (preeclampsia, hypertension or gestational diabetes), date of delivery and weeks of gestation and type of delivery (eutocic or caesarean section). Characteristics of the newborns included gender, weight and weight category (small for gestational age [SGA] and LGA). Data on ART, including baseline clinical and laboratory variables, were exported from our database. Data on obstetric outcomes were obtained through self-reports from patients (questionnaires or were contacted by cohort staff).

The study was approved by the Ethics Institutional Committee of Instituto Bernabeu on 14 January 2020 (reference BR17).

IVF procedures

The vitrification–warming process in those cases in which vitrified oocytes were used was carried out using the Kitazato Vit Kit® - Freeze and Kitazato Vit Kit® - Thaw. Fertilization was assessed 16–18 h after insemination or microinjection (day 1). Oocytes were considered fertilized when they contained two pronuclei.

Zygotes were cultured in single-step medium (Global®Total®) (CooperSurgical, Målov, Denmark) individually in 20-µl drops, with a maximum of six per dish, covered with 3 ml of mineral oil at 37°C in an atmosphere of 6% CO_2 and 5% O_2. Embryo morphology was evaluated under an inverted microscope on day 3 of development and at the blastocyst stage (day 5/6).

Embryo quality was classified into four categories (A–D) according to The Association for Reproductive Biology Research (ASEBIR) (2008). Type A and B embryos were considered good-quality embryos; if possible, these were transferred to the uterus on day 5 or 6 using ultrasound guidance and the Rocket catheter (Medical, Washington, USA). After transfer, the remaining good-quality embryos (types A and B) were cryopreserved using the Irvine vitrification kit (Vit Kit®-Freeze) (FujiFilm Irvine, Santa Ana, CA, USA).

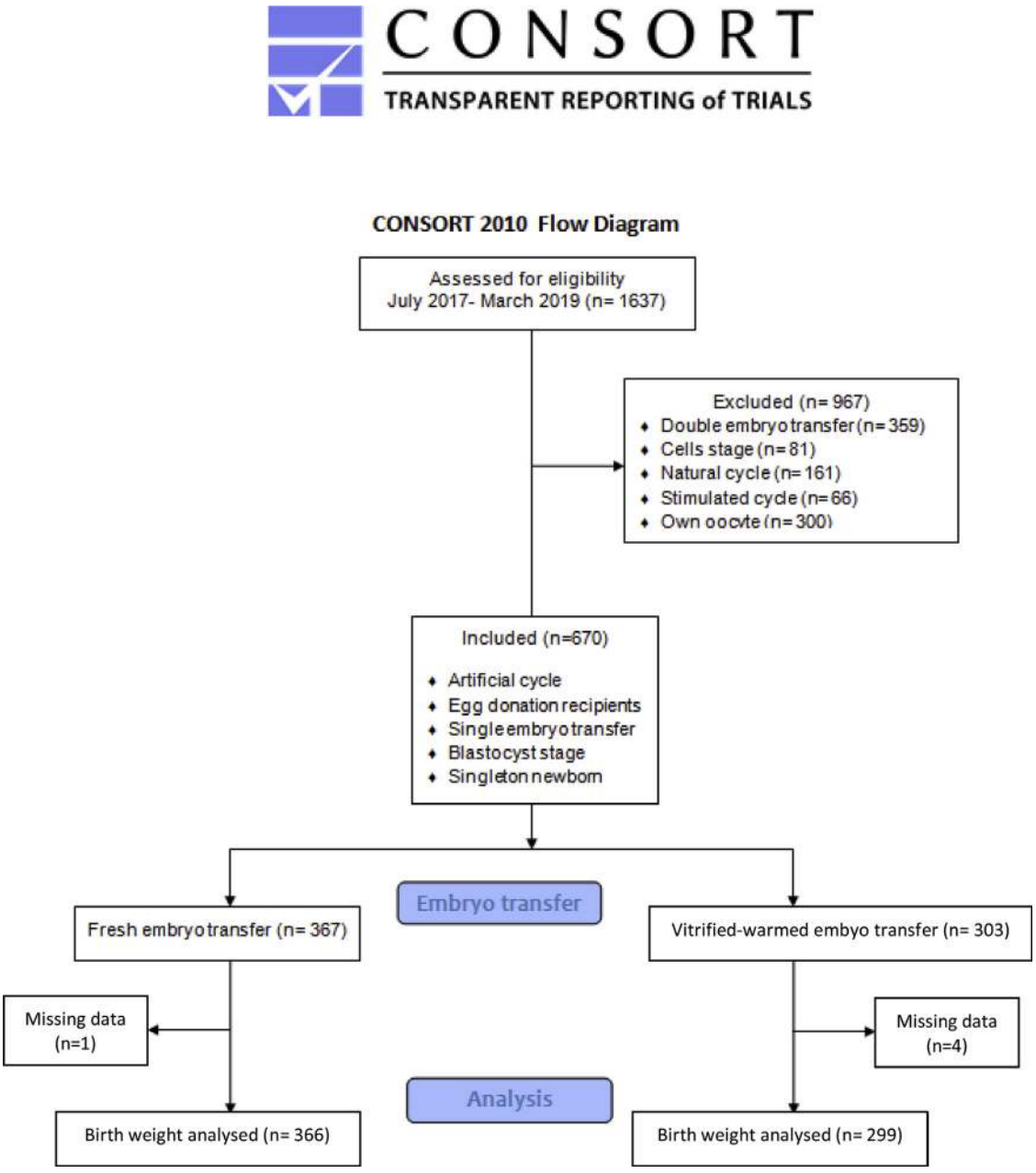

FIGURE 1 The selection of oocyte recipients with a singleton live birth for inclusion in the study.

In the embryo warming cycles, the process was carried out using the Vit Kit®-Thaw (FujiFilm Irvine, Santa Ana, CA USA). The vitrification and warming protocol has previously been described in detail (VerMilyea and Brewer, 2017).

In cases in which preimplantation genetic testing (PGT) was carried out (10 PGT cycles in the fresh embryo transfer group and 108 in the FET group), the embryos were biopsied at the blastocyst stage using an RI Saturn Active laser (CooperSurgical, Målov, Denmark).

Endometrial preparation

All patients received hormone replacement therapy for endometrial preparation, and all those showing ovarian function were downregulated with a depot gonadotrophin releasing hormone agonist (Ginecrin® depot 3.75 mg, administered intramuscularly as a single dose) (Abbott Laboratories, Madrid, Spain) given in the midluteal phase (approximately day 21) of the previous cycle (El-Toukhy *et al.*, 2004). On day 1 of subsequent menstruation (or at any point in recipients without ovarian function), oestrogen treatment was started using either daily oral oestradiol valerate (Progynova®) (Schering Spain, Madrid, Spain) or transdermal oestradiol every 2 days (Progynova® transdermal patch) (Schering Spain, Madrid, Spain). The dosage was 4 mg (or 100 µg of transdermal oestradiol) during the first 7 days and 6 mg (or 150 µg of transdermal oestradiol) from day 8 onwards. In the fresh embryo transfer group, starting in the evening of oocyte retrieval, 400 mg twice daily of micronized progesterone pessaries (Utrogestan®) (Seid Laboratories, Barcelona, Spain)

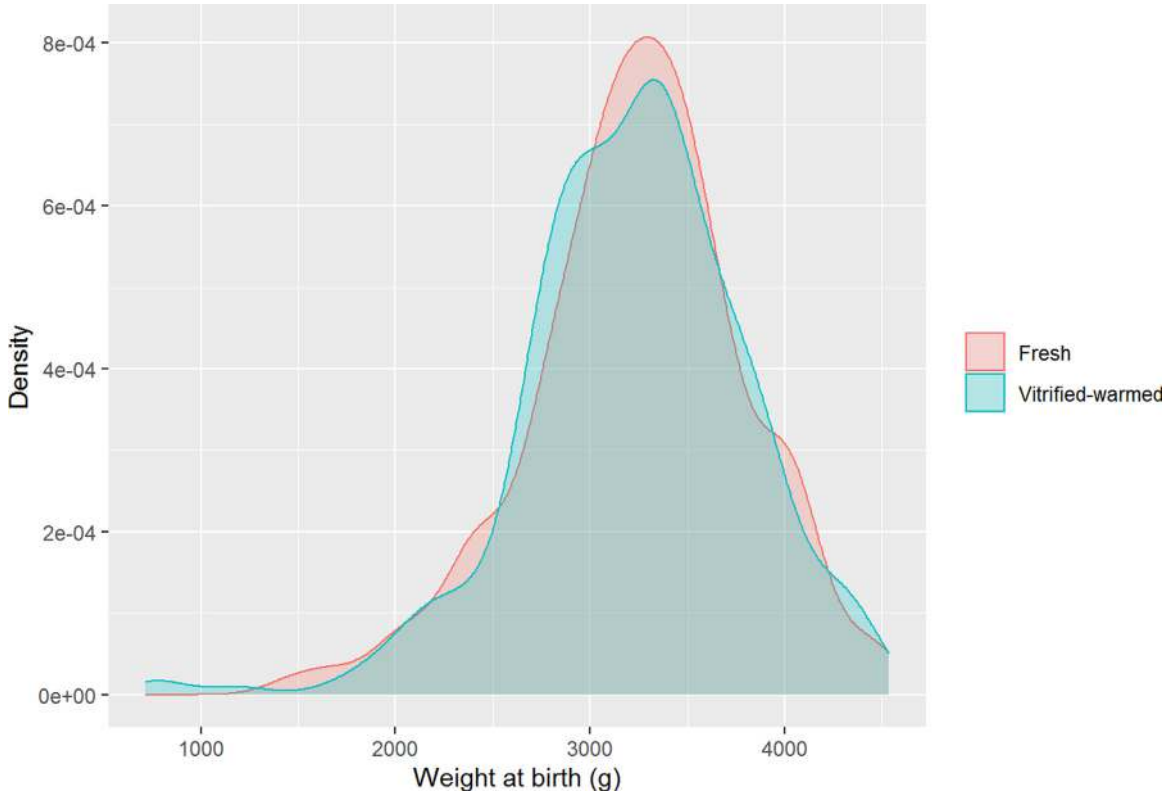

FIGURE 2 Birth weight distribution between groups fresh versus vitrified–warmed embryo transfer. Y-axis shows the probability of each birth weight value.

were added. In the FET group, a similar dose of micronized progesterone was started 5 days before the day of embryo transfer. Hormone replacement therapy was maintained until the end of the 12th gestational week.

Outcome measures

Primary outcome was birth weight. Secondary data were also obtained for gestational age at delivery, macrosomy (birth weight above 4500 g), underweight (birth weight below 2500 g), very low weight (birth weight below 1500 g), SGA (birth weight below the 10th percentile), LGA (above the 90th percentile), pre-term birth, very pre-term birth (before 32 weeks of gestation) and extremely pre-term birth (before 28 weeks of gestation). Population-specific birth weight charts according to parity, gender and type of delivery were used to establish percentile case by case (*Terán et al., 2017*).

Statistical analysis

The sample size was calculated from data previously published by *Ainsworth et al. (2019)*. On the basis of an alpha risk of 5% and a beta risk of 20%, a sample size of 266 patients (133 per group) is required to detect a minimum mean difference of 275 g with a SD of 800 g. Continuous variables were presented as number of cases, mean and SD. The Kolmogorov–Smirnov and Shapiro–Wilk tests were used to assess whether the continuous variables were normally distributed. $P < 0.05$ was considered statistically significant after a Mann–Whitney U Test (univariate) and linear regression (multivariate for confounding factors) were carried out. Categorical variables were presented as number of cases and percentage. Pearson's Chi-squared test (univariate) and binary logistic regression (multivariate for confounding factors) were used to analyse the association between variables. The primary outcome measure, i.e. the difference in the mean neonatal weight between cohorts, was corrected for the following confounders: endometrial thickness, smoking status, number of deliveries, preeclampsia, hypertensive disorders during pregnancy, gestational diabetes, weeks of gestation, gender of the newborn, oocyte donor height, preimplantation genetic testing for aneuploidy (PGT-A) and fresh versus vitrified–warmed oocytes. The distribution of the newborn weight variable is represented by a density plot (FIGURE 2), which is a smoothed version of a histogram. The result of the density plot allows for smoother distributions than the histogram by removing noise. The x-axis shows the values of a numerical variable (newborn weight) and the y-axis shows the probability that this variable takes a certain value.

R Statistical Software version 4.0.3 (The R Foundation) and Statistical Package for the Social Sciences (SPSS) software (version 20.0, SPSS, Inc., Chicago, IL, USA) were used for statistical analysis.

RESULTS

The analysis included a total of 670 singleton livebirths, derived from recipients who received a single embryo at the blastocyst stage between July 2017 and March 2019. Of these, 367 were fresh transfers and 303 were FET. In the FET group, 232 out of 303 (76.6%) of the cycles were part of a freeze-all strategy. In the remaining 23.4% (71/303), the index cycle was preceded by a previous unsuccessful fresh embryo transfer.

TABLE 1 shows maternal age (42.21 ± 4.45; 42.79 ± 3.83; $P = 0.519$), BMI

TABLE 1 BASELINE CHARACTERISTICS, PREGNANCY COMPLICATIONS AND OBSTETRIC OUTCOMES FOR OOCYTE RECIPIENTS AND DONORS' HEIGHT IN FRESH VERSUS VITRIFIED–WARMED EMBRYO TRANSFERS

Parameters	Type of embryo transferred		*P*-value
	Fresh (*n* = 367)	Vitrified-warmed (*n* = 303)	
Maternal (recipients)			
Baseline characteristics			
Age, years	42.21 ± 4.45	42.79 ± 3.83	0.519[a]
Body mass index, kg/m^2	23.34 ± 3.69	23.80 ± 3.78	0.075[a]
Endometrial thickness, mm	8.83 ± 1.73	8.57 ± 1.59	0.035[a]
Parity			
Multiparous, *n* (%)	68 (18.53)	44 (14.52)	0.177[b]
Smoking, *n* (%)	40 (10.90)	40 (13.20)	0.475[b]
Pregnancy complications			
Gestational diabetes, *n* (%)	18 (4.90)	13 (4.29)	0.854[b]
Preeclampsia, *n* (%)	10 (2.72)	17 (5.61)	0.074[b]
Hypertensive disorders, *n* (%)	12 (3.27)	7 (2.31)	0.494[b]
Obstetric outcome			
Gestational age at birth, weeks	38.96 ± 1.97	38.77 ± 2.15	0.207[a]
Liveborn gender			
Female, *n* (%)	163 (44.41)	148 (48.84)	0.276[b]
Type of delivery			
Eutocic, *n* (%)	132 (35.97)	106 (34.98)	0.854[b]
Oocyte donors			
Height, cm	163.22 ± 5.88	164.27 ±6.66	0.057[a]
Number of oocyte donors	191	197	

Data presented as *n* (%) or mean ± SD.

[a] Mann–Whitney U Test.

[b] Fisher's Exact Test.

(23.34 ± 3.69; 23.80 ± 3.78; $P = 0.075$), gestational age (38.96 ± 1.97; 38.77 ± 2.15; $P = 0.207$), maternal smoking (10.8%; 13.0%; $P = 0.475$), gestational diabetes (4.9%; 4.3%; $P = 0.854$), preeclampsia (2.7%; 5.6%; $P = 0.074$), hypertensive disorders (3.3%; 2.3%; $P = 0.494$), maternal parity (multiparous 18.5%; 14.5%; $P = 0.177$), and liveborn gender (female 44.5%; 48.8%; $P = 0.276$); no statistically significant differences were found between fresh or vitrified–warmed groups. Endometrial thickness, however, was significantly higher in the fresh versus vitrified–warmed group (8.83% ± 1.73 versus 8.57 ± 1.59; $P = 0.035$), respectively. A total of 99% of the oocyte donors and recipients were white, and the mean height of the donors were 163.22 ± 5.88 versus 164.27 ± 6.66 cm in the fresh versus FET groups, respectively ($P = 0.057$).

FIGURE 2 and TABLE 2 show that the mean birth weight was similar between fresh versus vitrified–warmed cycles (3239.21 ± 550.43 versus 3224.56 ± 570.83, respectively; B = 7.96, 95% CI –105.15 to 121.07; $P = 0.710$) in the crude analysis and after adjusting for the confounders endometrial thickness, smoking status, number of deliveries, preeclampsia, hypertensive disorders during pregnancy, gestational diabetes, weeks of gestation, gender of the newborn, oocyte donor height, PGT-A and fresh versus vitrified–warmed oocytes (B = 99.26, 95% CI –3.42 to 201.93; $P = 0.058$).

TABLE 3 shows no differences in the following additional outcomes: macrosomia (7.1% fresh; 6.3% vitrified–warmed; adjusted OR 0.857, 95% CI 0.314 to 2.340; $P = 0.764$), LGA (6.0% fresh; 6.7% vitrified–warmed; adjusted OR 0.450, 95% CI 0.176 to 1.149; $P = 0.095$), pre-term birth (10.9% versus 9.0%; adjusted $P = 0.997$), very pre-term birth (0.8% versus 1.3%; adjusted $P = 1.000$), extremely pre-term birth (0% versus 1.0%; adjusted $P = 0.998$), underweight (10.0%; 7.0%; adjusted $P = 0.050$); very low weight (0.6; 1.1%; adjusted $P = 1.000$) and SGA (1.9%; 0.7% adjusted $P = 0.974$) between fresh versus vitrified–warmed groups.

Finally, the general analysis showed that birth weight did not vary according to the origin of the embryo (fresh or vitrified–warmed) in weeks of gestation (FIGURE 3).

DISCUSSION

To the best of our knowledge, this is the first study assessing the effect of fresh versus vitrified–warmed embryo transfer on birth weight using the oocyte donation model controlling for significant confounders. To provide robust evidence-based guidance, only singleton livebirths resulting from cycles under artificial hormonal replacement endometrial preparation in fresh versus vitrified–warmed single blastocyst stage transfer cycles were included in the analysis. No significant difference was found in the birth weight of singletons resulting from fresh versus vitrified–warmed embryo transfers.

In contrast to our findings, the body of available evidence shows an increased risk of LGA linked to FET compared with fresh embryo transfer (*Luke et al., 2017*; *Maheshwari et al., 2018*). In rare reports using the oocyte donation model, no differences were found in birth weight when fresh and cryopreserved embryo transfers were compared, in accordance with the results of the present study (*Galliano et al., 2015*; *Vidal et al., 2017*). One notable exception is the study by *Roeca et al. (2020)*. This was a large registry study (SART-CORS data registry) in which birth outcomes were reported to be superior after transfer of fresh versus cryopreserved embryos for donor oocyte recipients. Although this study includes the analysis of many cycles, some important differences with our trial should be addressed. First, the primary outcome in this study was 'good obstetric outcome', defined as a singleton birth at 37 weeks gestational age or greater with a birth weight 2500–3999 g, whereas birth weight and gestational age-adjusted weight were included as secondary outcomes. Second, according to *Luke et al. (2012)* because of 'extreme outliers and improbable values representing potential data entry errors', variable cleaning of data extracted from the

TABLE 2 LINEAR REGRESSION ANALYSES OF BIRTH WEIGHT VERSUS TYPE OF EMBRYO TRANSFERRED

Birth weight	Type of embryo transferred		Linear regression analyses			
	Fresh (*n* = 366)	Vitrified–warmed (*n* = 299)	Unadjusted (univariate)		Adjusted (multivariate)	
			B[a] (95% CI)	*P*-value	B[b] (95% CI)	*P*-value
Birth weight, g	3239.21 ± 550.43	3224.56 ± 570.83	7.96 (–105.15 to 121.07)	0.710	99.26 (–3.42 to 201.93)	0.058

Data presented as mean ± SD.

[a] Coefficient of the univariate linear regression.

[b] Coefficient of the multivariate linear regression adjusted for endometrial thickness, smoking status, number of deliveries, preeclampsia, hypertensive disorders during pregnancy, gestational diabetes, weeks of gestation, gender of the newborn, oocyte–donor height, preimplantation genetic testing for aneuploidy and fresh versus vitrified–warmed oocytes.

SART-CORS was undertaken (*Luke et al., 2012*). This indicates that specific additional statistical methodology should be used to mitigate these shortcomings. Therefore, additional sensitivity analyses are crucial for scrutinizing the validity of the findings. Third, the study extracted all donor oocyte IVF cycles reported to SART that resulted in a fresh or cryopreserved embryo transfer between 1 January 2013 and 31 December 2015. The above timeline may not be representative of the modern trend, especially the number of embryos recommended for transfer in recipients. In fact, in this study, about 50% of the patients received two embryos and about 3% received three or more. Moreover, the number of cycles receiving blastocyst-stage embryos in the fresh embryo transfer (49%) compared with cryopreserved embryo transfer (91.2%) was significantly significant. The above-mentioned differences may account for the diverging conclusions reached in the study by *Roeca et al. (2020)*, in the present study, and in other studies focused on oocyte donation cycles (*Galliano et al., 2015*; *Vidal et al., 2017*). It is worth noting, however, that, after adjustment, the primary outcome 'good obstetric outcome' was 27% more likely after a fresh rather than cryopreserved embryo transfer (adjusted risk ratio 1.27; 95% CI 1.21 to 1.35), in the sub-analysis including only singleton livebirths (*n* = 9154), and a significant difference was no longer observed for the birth weight between fresh and cryopreserved embryo transfer (mean difference 0.10; 95% CI 0.09 to 0.14), which is in line with the present results.

A notable strength of our study is the exclusion of possible confounding factors. Previous studies have shown that the birth weight of singletons after assisted reproduction is higher after single embryo transfer compared with double embryo transfer (*De Sutter et al., 2006*), after vitrification compared with slow freezing (*Liu et al., 2013*) and after blastocyst compared with cleavage stage embryo transfer (*Makinen et al., 2013*; *Zhu et al., 2014*). The exclusion of the aforementioned variables and the analysis, including known potential factors affecting fetal growth, e.g. parity, body mass index, smoking status, hypertensive disorders during pregnancy, gender of the newborn) differentiates the present study from similar previously published studies on the topic (*Galliano et al., 2015*; *Vidal et al., 2017*), and may explain why our findings contradict earlier reports in which a higher weight and higher incidence of LGA and macrosomy were described in children born as a result of FET compared with fresh embryo transfer (*Luke et al., 2017*; *Maheshwari et al., 2018*). In addition, a single-centre study design

TABLE 3 SECONDARY NEONATAL OUTCOMES

Neonatal outcomes	Type of embryo transferred					
	Fresh, *n* (%) (*n* = 366)	Vitrified–warmed, *n* (%) (*n* = 299)	Unadjusted (univariate)		Adjusted (multivariate)	
			OR (95% CI)[a]	*P*-value[b]	OR (95% CI)[a]	*P*-value[c]
Macrosomia	26 (7.1)	18 (6.0)	0.838 (0.450 to 1.559)	0.688	0.857 (0.314 to 2.340)	0.764
Underweight	35 (9.6)	20 (6.7)	0.678 (0.383 to 1.201)	0.232	4.055 (0.999 to 16.463)	0.05
Very low weight	2 (0.5)	3 (1.0)	1.845 (0.306 to 11.112	0.820	2.544 (–, –)	1.000
SGA	7 (1.9)	2 (0.7)	0.345 (0.071 to 1.675)	0.297	0,971 (0.162 to 5.806)	0.974
LGA	22 (6.0)	20 (6.7)	1.121 (0.600 to 2.096)	0.844	0.450 (0.176 to 1.149)	0.095
Pre-term birth	40 (10.9)	27 (9.0)	0.809 (0.484 to 1.353)	0.500	–	0.997
Very pre-term birth	3 (0.8)	4 (1.3)	1.641 (0.364 to 7.389)	0.788	0.070 (–, –)	1.000
Extremely pre-term birth	0 (0)	3 (1.0)	–	0.181	–	0.998

[a] Reference category: fresh embryos.

[b] Fisher's exact test for univariate analysis.

[c] Binary logistic regression adjusted for endometrial thickness, smoking status, number of deliveries, preeclampsia and hypertensive disorders during pregnancy, gestational diabetes, weeks of gestation, gender of the newborn, oocyte donor height, preimplantation genetic testing for aneuploidy and fresh versus vitrified–warmed oocytes.

LGA, large for gestational age; SGA, small for gestational age; –, Due to the low number of cases in these groups, it is not possible to calculate 95% confidential intervals for the OR.

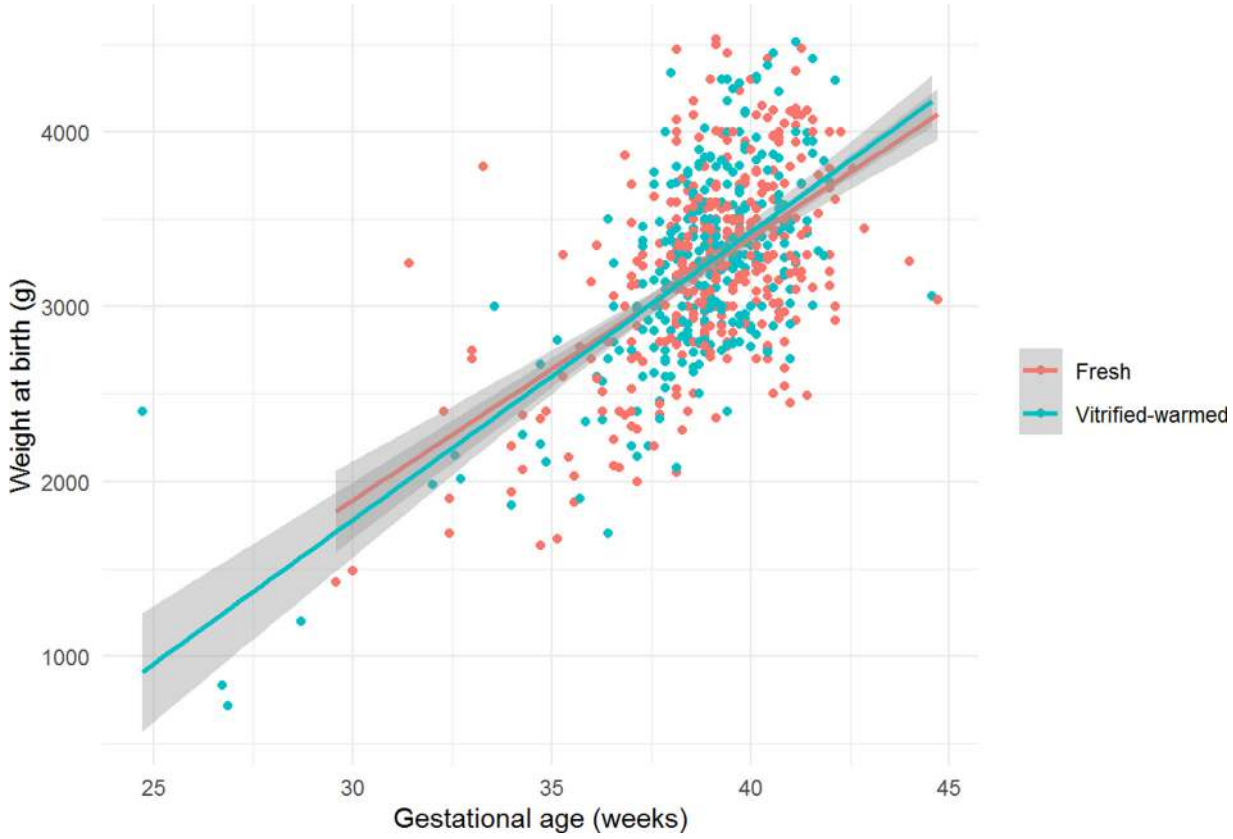

FIGURE 3 Birth weight versus gestational age for singleton birth in the two study groups (fresh versus vitrified–warmed). The grey shaded region represents the 95% confidence interval of each of the estimated lines.

permits access to medical records and treatment protocols, allowing for a statistical analysis adjusted for known confounding variables related to birth weight. Finally, the relatively short time of 3 consecutive years between 2017 and 2019 in the present study limits the heterogeneity of laboratory and clinical practice. Some publications relate the differences in birth weight to epigenetic changes in the preimplantation embryo, including demethylation and formation of new methylation patterns (reviewed in *Cedar and Bergman, 2012*), which could potentially be induced by the vitrification–warming procedures. These differences in methylation patterns have been clearly described only in the murine model to date (*Wang et al., 2010*). Our clinical findings challenge the notion of epigenetic factors induced by the cryopreservation technique influencing birth weight. Therefore, additional studies focused on the human embryo are warranted.

In the present study, the number of PGT cases was unevenly distributed, with 10 cases in the fresh embryo transfer compared with 108 in FET. Therefore, PGT was included as a confounder in the regression analysis of birth weight. When adjusting for the confounders endometrial thickness, smoking status, number of deliveries, preeclampsia, hypertensive disorders during pregnancy, gestational diabetes, weeks of gestation, gender of the newborn, oocyte donor height and fresh versus vitrified–warmed oocytes, but excluding PGT-A, the *P*-value was 0.211; when PGT-A was included, the *P*-value was 0.058). This finding is intriguing, especially in the light of available evidence suggesting that embryo biopsy does not seem to influence birth weight (*Sites et al., 2021*). The non-homogeneous distribution of PGT-A among groups might have contributed to this change in *P*-value; however, as the addition of PGT-A did not result in a statistically significant *P*-value, the core outcome was not affected.

As a hypothesis, if it is not the cryopreservation process, then, it could be the endometrium. The endometrial milieu constitutes a plausible independent factor correlated with later fetal growth after FET. In the animal model, *Weinerman et al. (2017)* assessed the perinatal outcomes after the transfer of mixed fresh and vitrified blastocysts to mice that underwent superovulation compared with mice that did not. The study reported that superovulation of the recipients led to significantly lower fetal weight at term, whereas blastocyst vitrification had no significant effect on fetal weight. In addition, Doppler ultrasound revealed increased median umbilical artery resistance in the placenta of mice exposed to superovulation compared with naturally mated mice, including a lower microvascular density (*Weinerman et al., 2017*). More recently, in IVF patients, *Wang et al. (2020)* found that singleton newborns conceived after an artificial FET cycle had a higher risk of being LGA. This study included a large cohort of cycles in pairwise comparisons between natural cycle FET, stimulated cycle FET and artificial cycle FET singletons. The authors concluded that the artificial priming of the endometrium is a possible factor for the increased newborn size in singleton pregnancies

after FET. In the same vein, the study by (*Zhou et al., 2022*) further supports a potential link between the absence of the corpus luteum and adverse perinatal outcomes, including a higher risk of LGA. The investigators found that the proportion of LGA infants significantly increased in the artificial cycle group (14.0%) compared with that in the natural cycle group (10.3%) and stimulated cycle group (7.6%), and the odds of very LGA was also higher in the artificial cycle group. Conversely, a recent post-hoc database analysis from de ANTARTICA randomized controlled trial (*Zaat et al., 2021*) found no differences in the weight of FET singleton newborns when the natural cycle was compared with the artificial cycle, fueling the controversy surrounding the topic. Although the study by *Zaat et al. (2021)* provides follow-up data analysis from a previously conducted RCT, the small sample size of 82 participants constitutes an evident statistical limitation; moreover, the main multi-centre RCT was conducted 6 to 11 years ago (2009–2014), which increases the risk for heterogeneity in the laboratory process, recall and selection bias. Clearly, the association between the endometrial preparation method for FET and birth weight still requires more evidence from prospective controlled trials.

Finally, the abnormal microRNA expression profiles found in term placentae derived from FET as described by *Hiura et al. (2017)* might be associated to the type of endometrial preparation rather than to the cryopreservation process *per se*, especially if we incorporate these findings to the conclusions from recent studies describing that artificial cycles for endometrial preparation are associated with a higher risk of preeclampsia compared with a natural cycle preparation for FET (*Saito et al., 2019*; *von Versen-Höynck, et al., 2019a*; *von Versen-Höynck et al., 2019b*). Future studies should address the potential effect on birth weight between embryos transferred in natural versus artificial endometrial preparations and the mechanism by which artificial cycle FET may affect birth weight.

We also acknowledge some potential limitations in our study. First, a thicker endometrium was found in the fresh embryo transfer group. The exact reason for this finding is unknown, but we can hypothesize that a longer endometrial preparation is needed to synchronize for a fresh embryo transfer cycle, and might be a possible explanation. Although we cannot completely exclude a possible influence in the results from this parameter, a relevant clinical effect of an average difference in endometrial thickness of a fraction of a millimeter (mean) seems highly unlikely. Second, self-reported obstetric outcomes may be prone to misclassification.

Additional information on secondary findings, i.e. macrosomia, underweight, very low weight, SGA, LGA, pre-term birth, pre-term birth, very pre-term birth and extremely pre-term birth between fresh and vitrified–warmed embryo transfer are presented in TABLE 3. Nonetheless, as the study was not powered to detect differences for these secondary outcomes, it is not possible to draw definite conclusions on these outcomes, and caution is warranted when interpreting these additional results. Overall, these additional descriptive data suggest that these clinical presentations do not seem to be influenced by the transfer of a vitrified–warmed embryo.

In conclusion, the present study of the oocyte donation model eliminates potential confounders that might influence fetal growth and demonstrates that embryo vitrification and warming procedures have no effect on birth weight. Given the widespread use of this modern cryopreservation technique in fertility centres, our findings on perinatal outcomes of vitrified embryos are reassuring. Prospective studies within this population are required to confirm our encouraging findings.

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