Chapter ∞

THE INFLUENCE OF EMBRYO CULTURE MEDIUM ON NEONATAL BIRTHWEIGHT AFTER SINGLE EMBRYO TRANSFER IN IVF

C.G. Vergouw
E.H. Kostelijk
E. Doejaaren
P.G.A. Hompes
C.B. Lambalk
R. Schats

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ABSTRACT

Study question: Does the type of medium used to culture fresh and frozen-thawed embryos influence neonatal birthweight after single embryo transfer (SET) in IVF?

Summary answer: A comparison of two commercially available culture media showed no significant influence on mean birthweight and mean birthweight adjusted for gestational age, gender and parity (z-scores) of singletons born after a fresh or frozen-thawed SET. Furthermore, we show that embryo freezing and thawing may lead to a significantly higher mean birthweight.

What is known and what this paper adds: Animal studies have shown that culture media constituents are responsible for changes in birthweight of offspring. In human IVF, there is still little knowledge of the effect of medium type on birthweight. Until now, only a small number of commercially available culture media have been investigated (Vitrolife, Cook® Medical and IVF online medium). Our study adds new information: it has a larger population of singleton births compared to the previously published studies, it includes outcomes of other media types (HTF and Sage®), not previously analysed, and it includes data on frozen-thawed SETs.

Design: This study was a retrospective analysis of birthweights of singleton newborns after fresh (day 3) or frozen-thawed (day 5) SET cycles, using embryos cultured in either of two different types of commercially available culture media, between 2008 and 2011.

Participants and setting: Before January 2009, a single-step culture medium was used: human tubal fluid (HTF) with 4 mg/ml human serum albumin (HSA). From January 2009 onwards, a commercially available sequential medium was introduced: Sage®, Quinn's advantage protein plus medium. Singletons born after a fresh SET (99 embryos cultured in HTF and 259 in Sage®) and singletons born after a frozen-thawed SET (32 embryos cultured in HTF only, 41 in HTF and Sage® and 86 in Sage® only) were analysed. Only patients using autologous gametes without the use of a gestational carrier were considered. Also excluded were: (vanishing) twins, triplets, babies with congenital or chromosomal abnormalities and babies born before 22 weeks of gestation.

Main results and the role of chance: Analysis of 358 singletons born after a fresh SET and 159 singletons born after a frozen–thawed SET showed no significant difference between the HTF and Sage® groups in terms of birthweight. Gestational age, parity and gender of the baby were significantly related to birthweight in multiple linear regression analyses, and other possible confounding factors included maternal age, BMI and smoking, the number of blastomeres of the transferred embryo and the type of culture medium. Maternal age, BMI and smoking, gestational age at birth, gender of

the baby and the percentage of firstborns did not differ significantly between the HTF and Sage® groups, however, among the fresh embryos, those cultured in Sage® had significantly more blastomeres at the time of embryo transfer compared to the embryos cultured in HTF. Birthweights adjusted for gestational age and gender or gestational age and parity (z-scores) were not significantly different between the HTF and Sage® groups for fresh or frozen–thawed SETs. Mean birthweight, as well as the mean birthweight among firstborns and the mean birthweights adjusted for gestational age and gender or parity (z-scores) were significantly higher in the cryopreservation group compared to the fresh embryo transfer group.

Bias, confounding and other reasons for caution: Our study is limited by its retrospective design and only two commercially available culture media were tested. More research is necessary to investigate the potential influence of culture media on gene expression.

Generalizability to other populations: Although our data do not indicate major influences of the HTF and Sage® culture media on birthweight, our results cannot be extrapolated to other culture media types. Furthermore, there remains a potential influence of embryo culture environment on epigenetic variation not represented by birthweight differences but by more subtle features.

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INTRODUCTION

Singletons born after assisted reproductive technologies (ARTs) are at increased risk of adverse neonatal outcome, such as low birthweight and preterm delivery[1-5]. The origin of adverse neonatal outcomes after ART may be related to the technical procedures themselves or to patient-related factors. Several studies suggest that adverse neonatal outcomes are associated with subfertility[1,6-8], while others declare no relation between subfertility and low birthweight and preterm delivery[1,9]. Other well-known factors that influence neonatal outcome are BMI of the mother, smoking and alcohol consumption[10-12]. Furthermore, increased birthweights have been linked to gender, with boys having on average higher birthweights than girls, and birthweight also increases as parity and gestational age increase[13]. Mean birthweight increases with an increasing average cell number in the embryo at day 3 as well[14].

Impacts of the IVF technology, such as ovarian stimulation (controlled ovarian hyperstimulation [COH]), on birthweights have also been studied. Pelinck et al.[15] found a trend towards lower birthweight after ovarian stimulation while eliminating the laboratory procedures as a cause. In addition, embryo freezing and thawing does not seem to have a negative influence on the incidence of preterm delivery and low birthweight[9,16,17].

Another important factor, the embryo culture medium used in IVF laboratories, may have an influence on neonatal outcome, including birthweight. Animal studies have shown that culture media constituents are responsible for changes in birthweight of offspring (reviewed by Young et al.[18]). In human IVF, there is still little knowledge of the effect of medium type on birthweight. Studies by Dumoulin et al.[19] and Nelissen et al.[20] showed that the type of embryo culture medium used has a significant effect on early embryonic development, fetal development and birthweight of the baby. In contrast, Eaton et al.[21] recently demonstrated no significant association between embryo culture medium and birthweight. In these studies, only a small number of the commercially available culture media were investigated: GI.3 and GI.5 medium (Vitrolife, Göteborg, Sweden); Cook® medium (Cook® Medical, Brisbane, Australia) and Global medium (IVF online, Toronto, Canada) and mainly children born after a fresh embryo transfer were included. To investigate further the influence of medium type on neonatal outcome, more commercially available media should be included in a study design. The aim of the current study was to compare birthweights of neonates after IVF treatment with a single embryo transfer (SET), where fresh and frozen-thawed embryos were cultured in one of two commercially available types of media.

MATERIALS AND METHODS

PATIENTS

The base population consisted of couples with an ongoing pregnancy after either a fresh IVF/ICSI cycle, or a frozen-thawed embryo transfer cycle at VU University Medical Center between 2008 and 2010. During this time, two different types of culture media were used, as a complete switch was instigated in January 2009. Before January 2009, a single-step commercially available culture medium was used: human tubal fluid (HTF; Lonza, Belgium) with 4 mg/ml human serum albumin (HSA; GPO, Sanquin, the Netherlands). From January 2009 onwards, a commercially available sequential medium was introduced: Sage®, Quinn's advantage protein plus medium (CooperSurgical, USA).

Embryos derived from a fresh IVF/ICSI cycle were cultured either in HTF (before January 2009) or in Sage® (after January 2009). Frozen-thawed embryos, however, could have three origins: cultured completely in HTF (freezing and thawing before January 2009, 'HTF group'); cultured in HTF before freezing and cultured in Sage® after thawing (freezing before and thawing after January 2009, 'HTF/Sage® group'); or cultured completely in Sage® (freezing and thawing after January 2009, 'Sage® group'). Except for the switch in the type of embryo culture medium, no other significant changes were made in the laboratory protocols.

STIMULATION PROTOCOL FRESH IVF/ICSI CYCLES

Women underwent COH in a long GnRH agonist (triptoreline [Decapeptyl®; Ferring, Denmark] protocol, starting the agonist in the last week of an oral contraceptive (Microgynon® 30; Shering, Germany). For women with a previous poor response, a short GnRH agonist protocol was applied. Ovarian stimulation was performed with recombinant FSH (Gonal-F®; Merck Serono, Germany or Puregon®; MSD, USA). Cycles were monitored by transvaginal ultrasounds and serum estradiol measurements. Human chorionic gonadotropin (hCG; [Pregnyl®; Organon, the Netherlands]) 10,000 IU s.c. was given 36 h before ultrasonographic-guided oocyte retrieval, when there was at least one follicle ≥18 mm and three or more follicles ≥16 mm. Luteal phase support (progesterone intravaginally; 3X200 mg daily; Utrogestan®; Besins Health care, Belgium) was started on the day of oocyte retrieval.

FROZEN-THAWED EMBRYO TRANSFER CYCLES

Transfer of frozen-thawed embryos was performed predominantly during a natural menstrual cycle. Follicle growth was monitored by vaginal ultrasonography and hCG was administered when a follicle reached the size of 18 mm or more in diameter and the two endometrial layers had reached at least a thickness of 6 mm or more. Embryo transfer was performed 6 days after hCG injection. In a minority of the patients, a hormonal substitution scheme was applied: estradiolvalerate (Progynova® 2 mg; Bayer, Germany) in increasing doses up to 6 mg per day. As at least a thickness of the two endometrial layers of 6 mm was reached, micronized progesterone was started intravaginally 3X100 mg per day. The dose of estradiolvalerate was decreased to 4 mg per day after starting the micronized progesterone.
The embryo transfer was scheduled on the 6th day following the start of the micronized progesterone.

LABORATORY PROTOCOLS BEFORE JANUARY 2009

On the day of oocyte retrieval (day 0), IVF and ICSI were performed according to the laboratory's routine insemination procedures. At day 1, 18-20 h after insemination, fertilization was checked. The embryos were cultured individually in 25-µl pre-equilibrated medium drops under oil in incubators at 37°C, under 5% CO_2 and atmospheric O_2 concentration. The culture medium was HTF (Lonza, Belgium) with a protein solution (GPO; Sanquin, the Netherlands) containing 4 mg/ml HSA. Embryonic development was recorded daily (25-27, 44-48 and 68-72 h after insemination).

LABORATORY PROTOCOLS FROM JANUARY 2009 ONWARDS

IVF oocytes were placed in fertilization medium (Sage®, Quinn's advantage protein plus fertilization medium) after oocyte retrieval. Insemination was performed 2-4 h after retrieval. During the fertilization check 18-20 h after insemination, the IVF zygotes were transferred into 25-µl pre-equilibrated medium drops of cleavage medium (Sage®, Quinn's advantage protein plus cleavage medium). ICSI oocytes were placed directly into 25-µl pre-equilibrated cleavage medium drops after injection. The embryos were cultured individually under oil in incubators at 37°C, under 5% CO_2 and atmospheric O_2 concentration. The embryos were morphologically assessed 25-27, 44-48 and 68-72 h after insemination. In the morning of day 3, the embryos were transferred into a new culture dish with blastocyst medium (Sage®, Quinn's advantage protein plus blastocyst medium).

EMBRYO SELECTION FOR TRANSFER

Prior to transfer on day 3 in the afternoon (73-75 h after insemination), embryo morphology was checked again by combining the number and regularity of blastomeres and the degree of fragmentation. The morphological choice of which embryo to transfer was based on the laboratory's routine procedures.

CRYOPRESERVATION AND THAWING PROCEDURES

Cryopreservation and thawing of supernumerary good quality embryos on day 4 was performed as previously described[22]. Briefly, a standard slow freezing protocol with dimethyl sulphoxide (DMSO; Sigma Alderich, Germany) as a cryoprotectant was used. Thawing was done in a series of decreasing DMSO media solutions. The embryos were assessed after thawing by routine morphological criteria and then cultured for 20-24 h in individual 25-µl media drops. Before January 2009, HTF plus 4 mg/ml HSA was used to culture the embryos overnight; after January 2009, Sage®, Quinn's advantage protein plus blastocyst medium was used for the overnight culture. Prior to embryo transfer, the embryos were assessed again by routine morphological criteria and the embryo with the best morphology (preferably a blastocyst) was selected for transfer. No assisted hatching was performed.

DATA COLLECTION

After establishing an ongoing pregnancy at 10 weeks following ovum pickup or ovulation, a survey was carried out on all the pregnant patients. This survey contained questions about the course of pregnancy, duration of pregnancy, mode of delivery, birthweight, sex and Apgar scores. The majority of patients sent the questionnaire back after giving birth. When a patient delivered in our hospital, data was collected from the hospital's database.

The study included all singleton babies born after a gestation of at least 22 weeks. Those excluded were singletons born after a double embryo transfer (DET), vanishing twins (women who gave birth to a singleton, but had a twin pregnancy recorded in the first ultrasound at 7 weeks of gestational age), twins, triplets and newborns with congenital or chromosomal abnormalities. Only patients using autologous gametes without the use of a gestational carrier were considered. Day 2 embryo transfers were excluded as well.

To compare children of different gestational age and gender or parity, a z-score ([weight of individual – medium weight of reference population] / 1 SD in reference population) was calculated for each newborn[13].

STATISTICAL ANALYSIS

Student's t-tests (continuous variables) and chi-square tests (binary and categorical variables) were used to compare the mean values of patient baseline characteristics between the two types of culture media after fresh embryo transfers. One-way analysis of variance tests were used to compare the mean values of patient baseline characteristics between the three culture groups after frozen-thawed embryo transfers. When a significant difference was found, a Bonferroni multiple comparison test was performed to test which groups were significantly different from each other. For the fresh embryo transfers, possible confounding factors, including maternal age, BMI, smoking, parity, gestational age and gender of the baby and number of blastomeres of the transferred embryo were entered into multiple linear regression analyses together with type of culture media, to analyse their association with birthweight. In a sub-analysis, Student's t-tests (continuous variables) and chi-square tests (binary and categorical variables) were used to compare mean values of patient baseline characteristics between all fresh and frozen-thawed embryo transfers. Data were analysed using SPSS 18.0 (Statistical package for the Social Sciences: SPSS Inc., Chicago, IL, USA). A two-sided P-value of 0.05 or less was considered statistically significant.

RESULTS

A total of 814 patients with an ongoing pregnancy were identified as inclusive study candidates, and 779 patients (96%) sent the survey back or delivered in our hospital. Of this total, 262 cases were excluded: 143 singletons resulted from a DET, 11 singletons from a fresh day 2 embryo transfer, 24 vanishing twins, 61 twins, 1 triplet, 16 singletons where donor gametes or a gestational carrier were used, 2 singletons with major congenital defects (both after a frozen-thawed SET, one in the HTF/Sage® group and one in de Sage® group) and 4 singletons with a trisomy 21 (two in the fresh SET Sage® group and 2 in the frozen-thawed SET Sage® group). This resulted in a final study population of 517 women who gave birth to a singleton after SET: 358 after a fresh embryo transfer and 159 after a frozen-thawed embryo transfer (Figure 1).

FRESH SET

Of the 358 singletons from fresh embryo transfers, 99 were cultured in HTF and 259 in Sage® as an embryo. The maternal characteristics and the birth outcomes are shown in Table 1. The analysis of 358 singletons born after SET showed no significant difference between the HTF and Sage® groups in birthweight. Gestational age, parity and gender

of the baby were significantly related to birthweight in multiple linear regression analyses, and other possible confounding factors were maternal age, BMI and smoking, the number of blastomeres of the transferred embryo and type of culture medium (R^2=0.46).

The percentage of small-for-gestational-age (SGA) babies, percentage of large-for-gestational-age (LGA) babies, maternal age, BMI and smoking, gestational age at birth, gender of the baby and the percentage of firstborns did not differ significantly between the HTF and Sage® groups. However, the embryos cultured in Sage® had significantly more blastomeres at the time of embryo transfer compared to the embryos cultured in HTF. Birthweights adjusted for gestational age and gender or gestational age and parity (z-scores) were not significantly different between the HTF and Sage® groups.

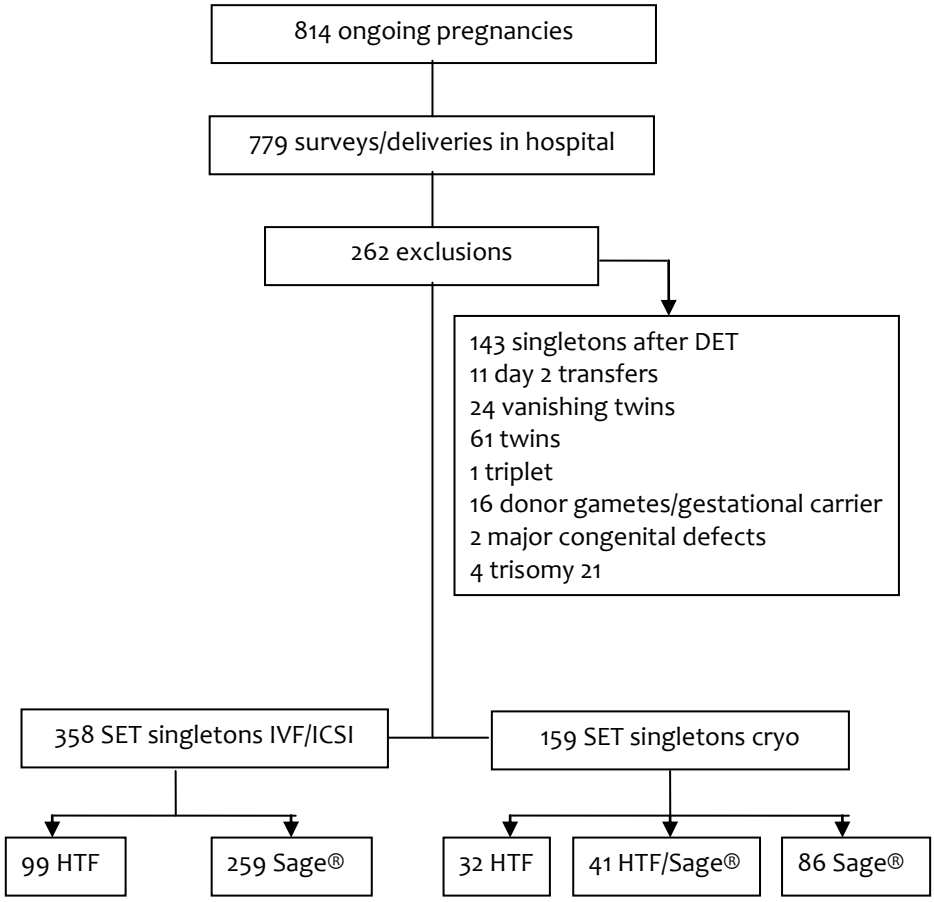

Figure 1. Breakdown of the size of different groups.

Table 1. Maternal characteristics and neonatal outcome of 358 fresh embryo transfers.

	HTF n= 99	Sage® n= 259	P-value
Maternal age (years)	34.0 ± 4.1	34.2 ± 4.0	0.75
Maternal BMI	23.9 ± 4.0 n=89	24.1 ± 3.9 n=241	0.79
Maternal smokers	14 (15.4) n=91	42 (17.9) n=234	0.59
Birthweight (grams)	3446 ± 508	3341 ± 575	0.11
Small for gestational age (< 10th percentile)	6 (6.1)	28 (10.8)	0.17
Large for gestational age (>90th percentile)	8 (8.1)	22 (8.5)	0.90
Z-score corrected for gestational age and gender	0.04 ± 0.87	0.09 ± 0.99	0.67
Z-score corrected for gestational age and parity	-0.02 ± 0.90	0.08 ± 0.99	0.38
Gestational age at birth (days)	278 ± 11	275 ± 15	0.06
Pregnancy duration (weeks)			
Term birth (≥37 weeks) Preterm birth (<37 weeks) Very preterm birth (<32 weeks)	98 (99.0) 1 (1.0) 0 (0.0)	255 (98.5) 2 (0.8) 2 (0.8)	0.67
Female infant	45 (45.5)	127 (49.0)	0.67
Firstborn children	74 (74.7)	196 (75.7)	0.86
Birthweight among firstborns (grams)	3388 ± 448 n=74	3292 ± 579 n=196	0.20
Number of cells per transferred embryo	7.7 ± 0.7	8.0 ± 1.3	0.01

Data are mean ± SD or n (%).

Table 2. Maternal characteristics and neonatal outcome of 159 frozen-thawed embryo transfers.

	HTF n= 32	HTF/Sage® n= 41	Sage® n= 86	P-value
Maternal age (years)	34.8 ± 4.4	35.2 ± 3.4	34.1 ± 3.8	0.59
Maternal BMI	23.3 ± 3.8 n=32	23.4 ± 4.0 n=37	24.1 ± 3.6 n=83	0.33
Maternal smokers	5 (15.6)	4 (10.8) n=37	14 (17.9) n=78	0.74
Birthweight (grams)	3547 ± 513	3700 ± 543	3550 ± 571	0.46
Small for gestational age (< 10th percentile)	2 (6.3)[a]	2 (4.9)[a]	2 (2.3)	<0.05*
Large for gestational age (>90th percentile)	2 (6.3)	12 (29.3)	13 (15.1)	0.09
Z-score corrected for gestational age and gender	0.27 ± 0.91	0.49 ± 1.08	0.31 ± 1.01	0.65
Z-score corrected for gestational age and parity	0.26 ± 0.91	0.47 ± 1.06	0.38 ± 1.00	0.66
Gestational age at birth (days)	278 ± 11	279 ± 8	277 ± 13	0.37
Pregnancy duration (weeks)				
Term birth (≥37 weeks)	30 (93.8)	40 (97.6)	80 (93.0)	
Preterm birth (<37 weeks)	2 (6.3)	1 (2.4)	5 (5.8)	0.40
Very preterm birth (<32 weeks)	0 (0)	0 (0)	1 (1.2)	
Female infant	16 (50.0)	18 (43.9)	48 (55.8)	0.90
Firstborn children	14 (43.8)[a]	9 (22.0)[b]	64 (74.4)[a, b]	<0.001*
Birthweight among firstborns (grams)	3512 ± 495 n=14	3361 ± 509 n=9	3470 ± 584 n=64	0.37

Data are mean ± SD or n (%).

*Pairs of means that are significantly different from each other in a Bonferroni multiple comparison test are marked ([a, b]).

FROZEN-THAWED SET

The maternal characteristics and birth outcomes of the frozen–thawed embryo transfers are shown in Table 2. Of the 159 singletons, 32 were cultured in HTF only, 41 were cultured in HTF and Sage® and 86 were cultured in Sage® only. The women whose embryos were cultured in Sage® only (Sage® group) were significantly more likely to be nulliparous compared to the women with embryos from the HTF and HTF/Sage® groups. The women in the HTF group were significantly more likely to be nulliparous compared to the HTF/Sage® group as well. The babies born in the HTF/Sage® group were significantly less likely to be small for gestational age compared to the babies born in the HTF group. However, the percentage SGA and LGA babies between the Sage® group and the HTF or HTF/Sage® groups did not differ significantly. There were no significant differences between the HTF, HTF/Sage® and Sage® groups in terms of maternal age, BMI and smoking, gestational age at birth and gender of the babies. The birthweights, birthweights among firstborns and the birthweights adjusted for gestational age and gender or gestational age and parity (z-scores) were not significantly different between the HTF, HTF/Sage® and Sage® groups.

FRESH VERSUS FROZEN-THAWED SET

The maternal characteristics and birth outcomes for fresh and frozen–thawed SETs are shown in Table 3. The women who had a frozen–thawed embryo transfer were significantly older and less likely to be nulliparous than the women who had a fresh embryo transfer. The mean birthweights, as well as the mean birthweights among firstborns and the mean birthweights adjusted for gestational age and gender or gestational age and parity (z-scores), were significantly higher in the frozen-thawed embryo transfer group compared to the fresh embryo transfer group. There were significantly more LGA babies and significantly less SGA babies in the frozen–thawed embryo transfer group compared to the fresh embryo transfer group. Gestational age at birth, gender of the baby and maternal BMI and smoking were not significantly different between the frozen–thawed and the fresh embryo transfer groups.

DISCUSSION

This study shows that, for two commercially available types of culture media, the choice of medium does not significantly influence the mean birthweight as well as the mean birthweight adjusted for gestational age and gender or gestational age and parity (z-scores) of singletons born after a fresh or frozen–thawed SET. Furthermore,

Table 3. Maternal characteristics and neonatal outcome of 358 fresh and 159 frozen-thawed embryo transfers.

	Fresh SET n=358	Frozen-thawed SET n=159	P-value
Maternal age (years)	33.0 ± 3.8	34.5 ± 3.8	<0.0001
Maternal BMI	24.1 ± 4.1 n=330	23.7 ± 3.6 n=209	0.32
Maternal smokers	56 (17.2) n=325	23 (15.6) n=147	0.67
Birthweight (grams)	3370 ± 558	3588 ± 553	<0.0001
Small for gestational age (< 10th percentile)	34 (9.5)	6 (3.8)	0.03
Large for gestational age (>90th percentile)	30 (8.4)	27 (17.0)	0.004
Z-score corrected for gestational age and gender	-0.03 ± 0.94	0.35 ± 1.00	<0.0001
Z-score corrected for gestational age and parity	0.03 ± 0.94	0.38 ± 0.99	<0.0001
Gestational age at birth (days)	276 ± 14	278 ± 11	0.14
Pregnancy duration (weeks)			
Term birth (≥37 weeks)	337 (94.1)	150 (94.3)	
Preterm birth (<37 weeks)	16 (4.5)	8 (5.0)	0.73
Very preterm birth (<32 weeks)	5 (1.4)	1 (0.6)	
Female infant	172 (48.0)	82 (51.6)	0.46
Firstborn children	270 (75.4)	87 (54.7)	<0.0001
Birthweight among firstborns (grams)	3319 ± 547	3466 ± 559	0.03

Data are mean ± SD or n (%).

we have shown that embryo freezing and thawing may lead to a significantly higher mean birthweight.

Our retrospective study is limited in design: a randomized controlled trial would have been more robust. The immediate switch in our laboratory culture system allowed us to analyse the birthweights of the babies who were cultured as an embryo in two different types of commercially available culture media within a relatively short period of time. Although only two commercially available types of culture media were tested, the data can be combined with other studies that review other commercially available

culture media, increasing the knowledge base of the effect of several media types on neonatal outcome.

Until now, only two groups have analysed the effect of medium type on neonatal outcome, with conflicting results. Dumoulin et al.[19] and Nelissen et al.[20] showed that embryos cultured in Vitrolife sequential medium (GI.3) resulted in significantly heavier infants than infants from embryos cultured in Cook® sequential medium. This result might be due to the higher number of blastomeres of the transferred embryos in the Vitrolife group. In addition, the mothers in the Vitrolife group were significantly taller and heavier, which may have influenced the newborn's birthweights. Another study, comparing births from embryos cultured in Global medium or GI.3 medium (Vitrolife) or GI.5 medium (Vitrolife) found no significant differences in the birthweights of neonates[21]. Embryos in the study of Eaton et al.[21] were cultured in Ham's F10 medium (InVitroCare) until the zygote stage, which made the culture system sequential. The current study incorporates a larger population of singletons compared to the three previously published studies and includes outcomes of other, not previously analysed, media types (HTF and Sage®) from both fresh and frozen–thawed SETs. Furthermore, we compared two different culture systems: a simple, one medium culture system (HTF with 4 mg/ml HSA) with a more complex sequential culture system (Sage®).

The major differences between the two types of culture media are the protein source and the addition of amino acids. HSA was used as a protein source in HTF (4 mg/ml) and in the fertilization media of Sage® (3 mg/ml). However, the cleavage and blastocyst media of Sage® contain Serum Protein Substitute (5 mg/ml), a synthetic protein source. Furthermore, several essential (arginine, I-cystine dihydrochloride, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine) and nonessential (alanyl-glutamine, asparagine, aspartic acid, glycine, proline, serine) amino acids are added to Sage® that are not present in HTF. In animal studies, it has been shown that the amount of proteins in culture media is, at least in part, responsible for the existence of large offspring syndrome in cattle and sheep[18,23]. High levels of proteins proved to alter embryo development kinetics, the expression of important genes for fetal development and subsequent weight of the offspring[24].

Both HTF and Sage® contain sodium chloride, potassium chloride, magnesium sulphate heptahydrate, phenol red, sodium bicarbonate, sodium pyruvate, glucose, lactate, calcium and gentamicin. In addition, Sage® cleavage is the only medium that does not contain potassium phosphate, while only Sage® fertilization and Sage® cleavage medium contain taurine, citric acid and EDTA. Finally, several vitamins are added to Sage® blastocyst medium, such as thiamine, ribovlavin, folic acid, D-calcium pantothenate, choline cloride, i-inositol, nicotinamide and pyridoxine. Despite the large

differences in components between HTF and Sage®, the birthweight of babies who were cultured as an embryo in one of the two medium types did not differ. Because the amount of protein added to culture medium caused differences in birthweight in animal studies[18,23], we speculate that the lack of difference in birthweight between HTF and Sage® babies might be caused by the almost similar amount of protein that is contained in both culture media.

Birthweight in humans is associated with short- and long-term morbidity and mortality, and is therefore commonly used for the assessment of neonatal outcome[25]. Although our study showed no significant differences in birthweight of infants cultured as an embryo in two different types of culture media, animal studies have shown that different culture conditions may lead to epigenetic changes through altered methylation of genes[24,26,27]. To this end, more research remains necessary to truly determine whether different culture media have an influence on gene expression.

Our multiple regression analyses showed that gestational age, parity and gender of the baby were significantly related to birthweight. This is a confirmation of previous data from Oken et al.[13], who found that gestational age, parity of the mother and gender of the infant were the most influencing factors of birthweight in singleton deliveries. The preferred method of comparing birthweights in a uniform way is by the z-score, a value customized for these highly correlated variables[25]. To compare children of different gender and gestational age, we calculated z-scores for birthweights, adjusting for gestational age and either gender of the baby or parity. These z-scores were not significantly different between the two analysed types of culture media, confirming the lack of association found in the unadjusted birthweights.

It has been shown that singletons born after DET have significantly lower birthweights and a higher incidence of preterm births and low birthweight compared to singletons born after SET. This was probably caused by the relatively high frequency of vanishing twins[28]. Survivors of vanishing twins have lower birthweights than singletons born after SET[29]. In this study, only singletons born after SET were included in order to analyse a more homologous group and to avoid possible confounding influences of patients with a vanishing twin.

Lieberman[14] has shown a higher mean birthweight with increasing blastomere cell number at embryo transfer. Because the number of blastomeres of the transferred embryo was significantly higher in the Sage® group, we may have expected a higher mean birthweight in the Sage® group.

The data from the current study confirm results of previous studies[9,16,17] which have concluded that embryo freezing and thawing lead to a significantly higher mean birthweight. This might be explained by a positive selection of women and embryos,

but also by the more natural uterine environment in a frozen embryo transfer cycle[9,16]. Another possible explanation might be the interaction of the used cryoprotectants with the main enzyme involved in epigenetic reprogramming, resulting in a normalization of the imprinting process[30]. Additionally, the day of embryo transfer might possibly have contributed to the difference in mean birthweight of babies born in the fresh and frozen–thawed embryo transfer group. All fresh embryo transfers were day 3 transfers and all frozen–thawed embryo transfers were day 5 transfers. Some previous studies, however, have shown that day of fresh embryo transfer has no effect on mean birthweight[31,32]. Unfortunately, in these studies, SET was not exclusively used. We also observed significantly more LGA babies and less SGA babies in the frozen–thawed embryo transfer group compared to the fresh embryo transfer group. Previous studies comparing fresh cleavage stage and blastocyst stage embryo transfers showed no significant differences in the percentage of LGA and/or SGA babies[33,34]. Therefore, we believe that the significant difference in LGA and SGA babies in the current study is not a result of the difference in day of embryo transfer between fresh and frozen-thawed embryos, but that this might be caused by the embryo freezing and thawing. The significant difference in percentage of SGA babies between the HTF/Sage® group and the HTF group for cryopreserved embryos is most likely caused by the small number of patients in both groups. The women in the cryopreservation group were significantly older and less likely to be nulliparous compared to the women in the fresh embryo transfer groups. This was expected, because more women gave birth after a fresh embryo transfer cycle and returned later for another pregnancy from their cryopreserved embryos.

The percentage of smokers, a factor well known to influence neonatal outcome[10,12], was not significantly different between all groups of patients compared.

In conclusion, within the limitation of evaluating solely two commercially available types of culture media, the choice of medium does not significantly influence the mean birthweight, nor the mean birthweight adjusted for gestational age and gender or gestational age and parity (z-scores), of singletons born after a fresh or frozen–thawed SET. In addition, embryo freezing and thawing may lead to a significantly higher mean birthweight. Although our data do not indicate a major influence of the tested types of culture media on birthweight, there may be a potential influence of embryo culture environment on epigenetic variation. These variations may not be represented by birthweight differences but by more subtle features that may surface after prolonged follow-up in adult tissues[35] and even in future generations[36]. It is, therefore, of great importance to continue research that evaluates effects of embryo culture systems on the health of ART children and further on the life of the adults and their children.

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