The freezing method of cleavage stage embryos has no impact on the weight of the newborns

N. Kaartinen¹ · K. Kananen² · H. Huhtala³ · S. Keränen⁴ · H. Tinkanen¹

Received: 6 October 2015 / Accepted: 21 December 2015 / Published online: 9 January 2016
© Springer Science+Business Media New York 2016

Abstract

Purpose The aim of this study was to study the effect of the embryo freezing method on the birth weight of newborns from frozen embryo transfer (FET) cycles, and the pregnancy results of cleavage stage embryos cryopreserved by slow freezing or vitrification.

Methods This is a retrospective cohort study undertaken in a University Hospital IVF unit using concurrently both the slow-freezing and the vitrification techniques. All frozen-thawed and vitrified-warmed day 2 and day 3 embryo transfers during the time period from 1 April 2009 to 31 November 2013 were included in the study.

Results There was no statistically significant weight difference between newborns from vitrified or slow-frozen embryos (3588 vs 3670 g). A higher post-thaw viability rate was achieved after cryopreservation by the vitrification technique compared to the slow-freezing protocol (83.4 vs 61.4 %). The miscarriage rate was lower in the vitrification group (15.7 vs

29.0 %). The live birth rates were similar (19.5 vs 19.1 %) in the slow-freezing and vitrification groups, respectively. Among vitrified embryos, 7.4 embryos needed to be thawed to produce one delivery; in the slow-freezing group, that number was 11.9.

Conclusions The freezing method has no impact on the weight of the newborn.

With lower post-thaw survival rates and higher miscarriage rates, the slow-freezing cryopreservation protocol is inferior to the vitrification technique.

Keywords Cryopreservation · Vitrification · Slow freezing · Frozen embryo transfer

Capsule The freezing method has no impact on the weight of the newborn. With lower post-thaw survival rates and higher miscarriage rates, the slow-freezing cryopreservation protocol is inferior to the vitrification technique.

✉ N. Kaartinen
noora.kaartinen@pshp.fi

¹ Department of Obstetrics and Gynaecology, Tampere University Hospital, University of Tampere, Teiskontie 35, 33521 Tampere, Finland

² Infertility Clinic Ovumia, Biokatu 12, 33520 Tampere, Finland

³ School of Health Sciences, Tampere University, T-Building, Medisiinarinkatu 3, 33520 Tampere, Finland

⁴ Central Finland Central Hospital, Keskussairaalantie 19, 40620 Jyväskylä, Finland

Introduction

Since the early days of the in vitro fertilization technology, a remarkable progress has been accomplished in the process of embryo culture while the implantation has remained the critical point limiting the pregnancy rate. Embryo freezing has revolutionized the entire process by enabling the use of the whole embryo cohort obtained from a single oocyte pick-up. The opportunity to transfer a single embryo at a time has diminished the risk of multiple pregnancies resulting in a better obstetrical outcome and increased cumulative delivery rate from the maximal utilization of embryos. The possibility to freeze all of the embryos in case of threatening ovarian hyperstimulation has reduced the complication rate associated with ovarian stimulation. The potential adverse effect of the superovulation on the endometrium receptivity may be avoided by cryopreserving all of the embryos and replacing them later in a more physiological milieu [1, 2].

Two cryopreservation techniques are mainly used in the IVF laboratories. In the slow-freezing protocol the temperature is decreased step by step first slowly to -30°C and then rapidly to -150°C , whereas in vitrification, the temperature is

reduced to -196°C by means of immediate exposure to liquid nitrogen [3]. The former technique requires costly equipment, while the latter one engages more labor force. The disadvantage of the slow freezing is the formation of ice crystals predisposing the embryo to cell damage during thawing. Cryoprotectants are used to cause expulsion of intracellular water, thus decreasing the ice crystal formation. In vitrification, higher concentrations of cryoprotectants are used for a short period of time before rapid cooling compared to slow freezing. The formation of ice crystals is inhibited as the solutes turn into a glass-like structure, i.e., the solutes vitrify [4].

Numerous studies have detected higher post-thaw survival rates in cleavage stage embryos cryopreserved by vitrification compared to embryos cryopreserved by slow freezing [5–12].

The efficiency of these two freezing methods has been studied in blastocyst stage embryos showing higher implantation and clinical pregnancy rates by vitrification compared to slow freezing (32 vs 20 % and 43 vs 28 %, respectively) [13]. A large population-based cohort study detected a significantly higher clinical pregnancy rate (adjusted relative risk ARR 1.47) and delivery rate (ARR 1.41) in benefit of vitrification [14]. No difference in the implantation rates of day 3 embryos was detected between the slow-freezing and vitrification techniques in two randomized, controlled studies (35.3 vs 35.4 % [15] and 21.4 vs 15.8 % [9]). A retrospective study found not only a significantly higher live birth rate after vitrification (35.3 %) compared to slow freezing (25.8 %) but also a higher miscarriage rate higher in the vitrification group (16.5 vs 7.7 %) [16]. In the only controlled, randomized study comparing the two cryopreservation methods at cleavage stage by Debrock et al. [17], a significant difference in live birth rate per embryo thawed/warmed for the benefit of vitrification was observed (16.1 vs 5.0 %). The use of vitrification at cleavage stage has raised interest in the possible epigenetic effects of the freezing method, the weight of the newborn as one expression.

The occurrence of increased birth weight after frozen-thawed embryo transfer compared to fresh embryo transfers has been observed in several studies [18–20]. However, the outcome of studies comparing the effect of the freezing method on birth weight is conflicting. Wang et al. [16] reported no statistically significant differences in birth weights after fresh, slowly frozen-thawed, or vitrified-warmed embryo transfers. Contradicting these results, Liu et al. [21] reported an increased birth weight after vitrified-warmed embryo transfers compared to slowly frozen thawed embryos, whereas Shi et al. [22] suggested a higher birth weight after vitrified-warmed day 3 embryos compared to fresh day 3 embryo.

Several IVF laboratories apply both cryopreservation techniques. The possible effect of the cryopreservation techniques on the birth weights could indicate epigenomic changes in the embryo, with further implications. We conducted a retrospective study to investigate the effect of the freezing method on the birth weight. The possible effect the cryopreservation

technique used had on the miscarriage rate and delivery rates was also studied.

Materials and methods

The study plan was approved by the Institutional Review Board of Tampere University Hospital. We analyzed all the frozen-thawed day 2–day 4 transfers from the time period from 1 April 2009 to 31 November 2013. The study material was collected from medical records. Only cycles with autologous oocytes were included in the study. The study included vitrified embryos and embryos cryopreserved by slow freezing. The difference between mean weights of the babies from frozen-thawed and vitrified-warmed embryo transfers was considered clinically significant when exceeding 100 g. The difference between the birth weight and the national gestational age- and gender-adjusted reference weight is expressed as the standard deviation score (SD-score). The SD-score is calculated using the formula $Z = (x - \mu) / \delta$, where x is the birth weight of a newborn, μ the mean weight of babies born at the same gestational age and gender, and δ is the standard deviation in the reference group. Only weights of newborns from single pregnancies were included.

The controlled ovarian hyperstimulation protocol

The controlled ovarian hyperstimulation was accomplished using either antagonist, long agonist, or short agonist protocol. A recombinant FSH was used to induce follicular growth, and the hCG was administered when the leading follicle reached 18 mm in the agonist protocol and 17 mm in the antagonist protocol. Thirty-six hours after hCG injection, the oocytes were collected using a transvaginal ultrasound-guided puncture. The luteal support consisted of vaginally administered progesterone and the embryo transfer was made under ultrasound monitoring.

Culture conditions

The culture media was supplied by Origio (Måløv, Denmark) or Vitrolife (Gothenburg, Sweden). Successful fertilization was confirmed 16 to 18 h after the insemination (hpi). The normally fertilized zygotes were cultured in sequential media using ISM1™ (Origio) until day 2 and BlastAssist™ (Origio) from day 2 to 3, or in G-1™ PLUS (Vitrolife) until day 3.

Embryo evaluation and selection

Cleavage stage embryos were evaluated on the day of the fresh embryo transfer on day 2 or 3 of the culture. The evaluation was performed as previously reported [23]. One or two of the best quality embryos were selected for fresh embryo

transfer and the remaining top or good quality embryos were selected for cryopreservation by either slow freezing or vitrification.

The cryopreservation and thawing protocols

The slow freezing was performed according to the instructions of the manufacturer using the Embryo Freezing Pack (Origio) and the standard slow-freezing method. The cooling rate was controlled by the Freeze Control (Cryologic Ltd., Mulgrave, Australia) liquid nitrogen freezing apparatus. Thawing was performed using the Embryo Thawing Pack (Origio). The cryoprotectants included propylene glycol and sucrose. Vitrification and warming were performed according to the instructions of the manufacturer (VitrifreezeES™, VitrithawES™, FertiPro, Beernem, Belgium). High security vitrification straws (Cryo BioSystems, L'aigle, France) were used as a vitrification device. In vitrification, the main cryoprotectants were dimethyl sulfoxide and ethylene glycol.

The embryos were thawed 1 day before the embryo transfer and the survived embryos (minimum of 50 % of blastomeres alive) were cultured overnight in BlastAssist. The embryos that had cleaved were evaluated and selected for embryo transfer. Embryo survival was defined as cleavage during an overnight culture.

The frozen-thawed embryo transfers

The frozen-thawed embryo transfers (FET) were performed in spontaneous or hormone-substituted cycles. In hormone-substituted cycles estradiol hemihydrate as tablets (2 mg three times a day) or transdermal patches (100–150 µg twice a week) from the beginning of the menstrual cycle was used, in addition to natural vaginal progesterone tablets (200 mg × 3 a day), starting when the endometrial thickness measured 7–8 mm. One or two embryos were transferred using ultrasound guidance.

A clinical pregnancy was assessed with a transvaginal ultrasound 5 weeks after the embryo transfer and defined as the presence of at least one gestational sac on the ultrasound scan. A spontaneous abortion was considered to be a pregnancy loss until week 22 of gestation after a previous confirmation of a clinical pregnancy.

Statistical analyses

The distribution of the birth weight of the babies was normal in all groups. A *t* test for independent samples was used when comparing the mean birth weights between the groups. When comparing the differences in median birth weight standard deviations calculated from the gender- and gestational age-adjusted population-based reference weight, a nonparametric test (Mann-Whitney) was used because of the nongaussian

distribution. A chi-square test was used to compare the delivery rates, clinical pregnancy rates, and miscarriage rates between the groups. All statistical analyses were performed using the IBM SPSS software (v19.0 Armonk, NY, USA).

Results

The study consisted of 871 IVF/ICSI cycles with 848 fresh embryo transfers resulting in 160 live births (18.9 %). There were 1346 days 2–3 frozen-thawed embryo transfers in the study, of which 663 were cryopreserved by slow freezing and 683 were vitrified. The frozen-thawed embryo transfers resulted altogether to 276 live births. The stimulation protocols, IVF/ICSI distribution, and the cause of infertility were comparable between the groups as well as the ages of the mothers (31.4 vs 32.1 years) (Table 1). The results of three patients were lost to follow up.

The mean weight of the babies born after fresh, slowly frozen, and vitrified embryo transfers were 3454.1, 3670.3, and 3588.7 g, respectively. Only babies from singleton deliveries were included in the analysis. We analyzed the weights

Table 1 The characteristics of the cycles of the two cryopreservation methods

	Slow freezing		Vitrification		<i>p</i> value
	<i>n</i>	%	<i>n</i>	%	
Number of cycles	418		453		
Age (years) mean (SD)	31.4 (4.0)		32.1 (4.2)		0.012
Stimulation protocol					0.032
Antagonist	190	45.5	237	52.3	
Long agonist	223	53.3	205	45.3	
Short agonist	5	1.2	11	2.4	
Insemination					0.961
IVF	206	49.3	228	50.3	
ICSI	211	50.5	224	49.4	
Shared	1	0.2	1	0.2	
Infertility					0.104
Primary	253	60.5	261	57.6	
Secondary	165	39.5	185	40.8	
Unknown			7		
Cause of infertility					0.094
Tubal	24	5.7	23	5.1	
Male factor	129	30.9	119	26.3	
Anovulation	54	12.9	50	11.0	
Unexplained	122	29.2	135	29.8	
Multiple reasons	35	8.4	41	9.1	
Endometriosis	52	12.4	73	16.1	
Poor ovarian reserve	2	0.5	12	2.6	

of the newborns using standard deviations from the national gestational age- and gender-adjusted population-based reference weight. The SD-scores in fresh, slow-freezing, and vitrification groups were -0.6 , -0.22 , and -0.27 , respectively (Table 2). Considering the two freezing methods, these SD-scores did not differ statistically ($p=0.893$). The difference between the mean weights of the babies born after slow-frozen and vitrified embryo transfers was 81.6 g, which is not statistically significant ($p=0.263$ with 95 % CI -61.5 – 224.7). The babies born after fresh embryo transfers were lighter than the babies born after frozen embryos transfers (3454.1 vs 3627.2 g, weight difference 173 g, $p=0.003$). The length of the gestation was similar in both FET groups, the mean delivery week being 39.8 in slow-freezing and 39.5 in vitrification group ($p=0.979$).

The percentages for SGA (small for gestational age) and LGA (large for gestational age) were 1.6 vs 3.6 % and 2.4 vs 5.0 % for slow freezing and vitrification, respectively.

The ratio of boys was 54.4 vs 50.7 %, respectively.

The slow-freezing group consisted of 359 (54.2 %) single and 304 (45.8 %) double embryo transfers, and the vitrification group consisted of 487 (70.9 %) and 196 (29.1 %) single and double embryo transfers, respectively. The viability rate of the embryos was 67.7 % in the slow-freezing group and 88.4 % in the vitrification group ($p<0.001$ with 95 % CI of 0.175 – 0.236).

The pregnancy results of the frozen embryo transfers are listed in Table 3. There was no statistical difference in the clinical pregnancy or live birth rates between the freezing methods. However, in pregnancies resulting from single embryo transfers, the miscarriage rate was significantly higher in the slow-frozen transfers compared to the vitrified embryo transfers (29 vs 15.7 %, $p=0.021$).

To produce one delivery, 7.4 vitrified embryos were needed to be thawed. In slow-freezing group, the corresponding number was 11.9.

Discussion

In this study, no statistically significant weight difference was observed in the newborns from slow-frozen or vitrified embryo transfers. Even though the freezing method did not have an influence on live birth rates, vitrification was shown to be more effective freezing method for cleavage stage embryos. The survival rate of thawed vitrified embryos was significantly higher and fewer frozen embryos were needed to produce a delivery.

In the present study, the clinical pregnancy and delivery rates were similar for both freezing methods. The miscarriage rate (the proportion of miscarriages in all clinical pregnancies) in the slow-freezing group was higher (29 vs 15.7 %), especially in the case of single embryo transfers. The miscarriage rate were higher than presented in previous studies in the case of day 3 FET cycles by Liu et al. (12.6 vs 12.1 %) or Rama Raju et al. (7.71 vs 9.24 %) [21, 24], but it did not differ from the results of a study on the miscarriage rate after fresh embryo transfers (17.4–22.2 %) in PCO and tubal infertility [25]. The most common reason for early pregnancy miscarriages is the chromosomal abnormalities irrespective the mode of fertilization (spontaneous or ART) [26–28]. Slow freezing and thawing has been detected to induce numerical chromosomal changes in human embryos, which could be explained by increased ice crystal formation [28]. In frozen-thawed blastocyst transfers, no difference has been detected in the miscarriage rates between the two cryopreservation methods [14]. In a study by Li et al. [29], the embryo DNA integrity index was higher in vitrified than in slow-frozen human blastocysts which could explain the lower miscarriage rate in vitrified blastocyst pregnancies compared to slow freezing.

The weight difference between newborns from fresh and frozen embryo transfers does not seem to be related to maternal factors, as the increased risk of LGA and macrosomia in singletons after FET has been detected in a sibling cohort as

Table 2 Neonatal characteristics of the live born singletons from frozen embryo transfer cycles

	Slow freezing <i>n</i> (%)	Vitrification <i>n</i> (%)	<i>p</i> value
Boys	68 (54.4)	71 (50.7)	
Gestational age at birth (weeks)	39.8	39.5	0.979
Preterm births (<37 weeks)	6 (4.8)	12 (8.6)	0.328
Very preterm births (<32 weeks)	1 (0.8)	1 (0.7)	1.000
Birth weight (g)	3670.3	3588.7	0.263
Low birth weight (<2500 g)	0	6 (4.3)	0.032
Low birth weight in term births (≥ 37 weeks)	2 (1.6)	3 (2.1)	1.000
Very low birth weight (<1500 g)	0	1 (0.7)	1.000
High birth weight (>4500 g)	5 (4.0)	10 (7.1)	0.3
Small for gestational age (< -2 SD)	2 (1.6)	5 (3.6)	0.452
Large for gestational age (> $+2$ SD)	3 (2.4)	7 (5.0)	0.342
Z score (mean)	-0.22	-0.27	0.893

Table 3 The pregnancy results after the transfer of slow-frozen/thawed and vitrified/warmed embryos, both single and double embryo transfers

	Slow freezing		Vitrification		<i>p</i> value
	<i>n</i>	%	<i>n</i>	%	
Single embryo transfers	359		487		
Clinical pregnancy	100	27.9	115	23.6	0.175
Miscarriage	29	29.0	18	15.7	0.021
Extrauterine pregnancy	0		3	2.6	
Induced abortion	0		1		
Still birth	1		0		
Live birth	70	19.5	93	19.1	0.87
Singletons	70		92		
Gemini	0		1		
Double embryo transfers	304		196		
Clinical pregnancy	94	30.9	68	34.7	0.381
Miscarriage	25	26.6	15	22.1	0.582
Extrauterine pregnancy	3	3.2	3	4.4	0.696
Induced abortion	1		1		
Still birth	1		0		
Live birth	64	21.1	49	25.0	0.325
Singletons	55		47		
Gemini	9		2		

well [30]. In a study by Wikland et al., no difference in birth weight was observed after the transfer of vitrified blastocysts or slow-frozen early cleavage stage embryos [31]. The blastocyst culture itself has been found to have an impact on the birth weight of newborns, complicating the comparison of two cryopreservation techniques using embryos from different stages [32, 33]. In Liu's study, the median birth weight of babies born from a vitrified cleavage stage embryo transfer was higher by a weight difference of 103 g, than the birth weight after the transfer of slow-frozen cleavage stage embryos. In their study, no weight difference existed between babies from fresh or slow-frozen cleavage stage embryos. This contradicts the study by Pelkonen et al. in which 134-g higher birth weight was detected among slow-frozen FET singletons compared to fresh singletons [19]. In Liu's study, the birth weights of the babies were obtained through patient questionnaires which may cause some uncertainty in the results. In our study, the precise length of the pregnancy of all of the newborns was known, and the weights were compared to the gender- and gestational age-adjusted population-based reference values, increasing the reliability of the results. The mean birth weight of the newborns was approximately 80 g lower in the vitrification group compared to slow-freezing group ($p=0.263$). We considered the weight difference more than 100 g clinically significant. The same tendency was seen in the study of Wang et al. of day 3 FET pregnancies; the 140-g difference in birth weights for the benefit of slow freezing

compared to vitrification was not statistically significant ($p=0.307$) [16]. The lack of significant difference in the weights of babies born after these two different cryopreservation techniques could indicate a somewhat similar effect on the epigenetic processes or similar embryo selection caused by the cryopreservation. On the other hand, it could also refer to minimal epigenetic effect of either one of the techniques.

The embryo survival has usually been defined as survival of at least 50 % of the pre-freeze blastomeres. A survival as high as 95 % has been associated with the cryopreservation of cleavage stage embryos by the vitrification technique and a higher survival rate after the vitrification of human cleavage stage embryos compared to the slow freezing has been detected in many studies [5, 6, 10, 11, 21]. This was also observed in our study (61.4 vs 83.4 %). Our viability rate refers to the percentage of embryos surviving the thawing and resuming cleavage after an overnight culture. Embryos with damaged cells after thawing have been shown to have a lower overnight developmental potential compared to intact cells both after slow freezing and vitrification [11]. Certainly, the decreased survival associated with slow freezing is caused by increased ice formation during freezing and thawing physically damaging cell membranes. Conversely, cryoprotectants and osmotic stress can have potential negative impact on the bioenergetic function of mitochondria [34].

It has been supposed that better endometrial receptivity could be one reason for the increased birth weight after FET compared to fresh embryo transfers. Another explanation could be the stress caused by the embryo freezing-thawing resulting in a selection of embryos with better growth potential [20]. Epigenetic mechanisms have also been proposed to explain the weight difference. The freezing and thawing during the delicate period of early embryo development, i.e., the activation of embryonic genome, may cause epigenetic alterations affecting the phenotype at birth.

The strength of the present study is the accurate birth weights compared to the population-based reference weight expressed as the SD-score from the reference weight taking into account the gestational age and the sex of the newborn. A shortcoming in this study was the retrospective design of the study. Furthermore, the maternal BMI and smoking were not included in the data collection. These factors are known to have impact on the birth weight. Especially, the smoking habits of the patients are poorly registered in the patient records.

In conclusion, no significant birth weight difference exists between the two cryopreservation techniques, although there seems to be a tendency toward a slightly lower mean birth weight after vitrification. Undoubtedly, the benefits of vitrification technology for preserving cellular integrity outweigh the trend toward a slightly lower live birth weight. Vitrification is a more efficient procedure, yielding higher rates of cell survival and viability which translates into greater

embryo utilization per IVF cycle and fewer pregnancy losses upon transfer.

Acknowledgments We thank Mrs. Elina Adams for language revision.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

- Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: a prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. *Fertil Steril*. 2011;96(2):344–8.
- Weinerman R, Mainigi M. Why we should transfer frozen instead of fresh embryos: the translational rationale. *Fertil Steril*. 2014;102(1):10–8.
- Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Hum Reprod Update*. 2012;18(5):536–54.
- Rall WF, Wood MJ, Kirby C, Whittingham DG. Development of mouse embryos cryopreserved by vitrification. *J Reprod Fertil*. 1987;80(2):499–504.
- Balaban B, Urman B, Ata B, Isiklar A, Larman MG, Hamilton R, et al. A randomized controlled study of human day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. *Hum Reprod*. 2008;9:1976–82.
- Levron J, Leibovitz O, Brengauz M, Gitman H, Yerushalmi GM, Katorza E, et al. Cryopreservation of day 2-3 embryos by vitrification yields better outcome than slow freezing. *Gynecol Endocrinol*. 2014;30(3):202–4.
- Lin T-K, Su J-T, Lee F-K, Lin Y-R, Lo H-C. Cryotop vitrification as compared to conventional slow freezing for human embryos at the cleavage stage: survival and outcomes. *Taiwan J Obst Gynecol*. 2010;49(3):272–8.
- Loutradi KE, Kolibianakis EM, Venetis CA, Papanikolaou EG, Pados G, Bontis I, et al. Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. *Fertil Steril*. 2008;90(1):186–93.
- Fasano G, Fontenelle N, Vannin A-S, Biramane J, Devreker F, Englert Y, et al. A randomized controlled trial comparing two vitrification methods versus slow-freezing for cryopreservation of human cleavage stage embryos. *J Assist Reprod Genet*. 2014;31(2):241–7.
- Rezazadeh Valojerdi M, Eftekhari-Yazdi P, Karimian L, Hassani F, Movaghar B. Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. *J Assist Reprod Genet*. 2009;26(6):347–54.
- Van Landuyt L, Van de Velde H, De Vos A, Haentjens P, Blockeel C, Tournaye H, et al. Influence of cell loss after vitrification or slow-freezing on further in vitro development and implantation of human day 3 embryos. *Hum Reprod*. 2013;28(11):2943–9.
- Kolibianakis EM, Venetis CA, Tarlatzis BC. Cryopreservation of human embryos by vitrification or slow freezing: which one is better? *Curr Opin Obstet Gynecol*. 2009;21(3):270–4.
- Pouget O, Scalici E, Hoa-Ferrieres A, Deutsch-Bringer S, Gala A, Dechaud H, et al. Comparison of frozen embryo transfer outcomes at blastocyst stage according to freezing method and type of endometrial preparation. *Gynécologie Obstétrique Fertil*. 2015;43(3):219–24.
- Li Z, Wang YA, Ledger W, Edgar DH, Sullivan EA. Clinical outcomes following cryopreservation of blastocysts by vitrification or slow freezing: a population-based cohort study. *Hum Reprod*. 2014;29(12):2794–801.
- Wilding MG, Capobianco C, Montanaro N, Kabili G, Di Matteo L, Fusco E, et al. Human cleavage-stage embryo vitrification is comparable to slow-rate cryopreservation in cycles of assisted reproduction. *J Assist Reprod Genet*. 2010;27(9-10):549–54.
- Wang X, Zhang X, Qin Y, Hao D, Shi H. Outcomes of day 3 embryo transfer with vitrification using Cryoleaf: a 3-year follow-up study. *J Assist Reprod Genet*. 2012;29(9):883–9.
- Debrock S, Peeraer K, Fernandez Gallardo E, De Neubourg D, Spiessens C, D'Hooghe TM. Vitrification of cleavage stage day 3 embryos results in higher live birth rates than conventional slow freezing: a RCT. *Hum Reprod*. 2015;30(8):1820–30.
- Ishihara O, Araki R, Kuwahara A, Itakura A, Saito H, Adamson GD. Impact of frozen-thawed single-blastocyst transfer on maternal and neonatal outcome: an analysis of 277,042 single-embryo transfer cycles from 2008 to 2010 in Japan. *Fertil Steril*. 2014;101(1):128–33.
- Pelkonen S, Koivunen R, Gissler M, Nuojua-Huttunen S, Suikkari A-M, Hydén-Granskog C, et al. Perinatal outcome of children born after frozen and fresh embryo transfer: the Finnish cohort study 1995–2006. *Hum Reprod*. 2010;25(4):914–23.
- Wennerholm U-B, Henningsen A-KA, Romundstad LB, Bergh C, Pinborg A, Skjaerven R, et al. Perinatal outcomes of children born after frozen-thawed embryo transfer: a Nordic cohort study from the CoNARTaS group. *Hum Reprod*. 2013;28(9):2545–53.
- Liu SY, Teng B, Fu J, Li X, Zheng Y, Sun XX. Obstetric and neonatal outcomes after transfer of vitrified early cleavage embryos. *Hum Reprod*. 2013;28(8):2093–100.
- Shi W, Xue X, Zhang S, Zhao W, Liu S, Zhou H, et al. Perinatal and neonatal outcomes of 494 babies delivered from 972 vitrified embryo transfers. *Fertil Steril*. 2012;97(6):1338–42.
- Kaartinen N, Das P, Kananen K, Huhtala H, Tinkanen H. Can repeated IVF-ICSI-cycles be avoided by using blastocysts developing from poor-quality cleavage stage embryos? *Reprod Biomed Online*. 2015;30(3):241–7.
- Rama Raju GA, Jaya Prakash G, Murali Krishna K, Madan K. Neonatal outcome after vitrified day 3 embryo transfers: a preliminary study. *Fertil Steril*. 2009;92(1):143–8.
- Han AR, Kim HO, Cha SW, Park CW, Kim JY, Yang KM, et al. Adverse pregnancy outcomes with assisted reproductive technology in non-obese women with polycystic ovary syndrome: a case-control study. *Clin Exp Reprod Med*. 2011;38(2):103–8.
- Bettio D, Venci A, Levi Setti PE. Chromosomal abnormalities in miscarriages after different assisted reproduction procedures. *Placenta*. 2008;29(Suppl B):126–12.
- Kim JW, Lee WS, Yoon TK, Seok HH, Cho JH, Kim YS, et al. Chromosomal abnormalities in spontaneous abortion after assisted reproductive treatment. *BMC Med Genet*. 2010;11:153.
- Martínez MC, Méndez C, Ferro J, Nicolás M, Serra V, Landeras J. Cytogenetic analysis of early nonviable pregnancies after assisted reproduction treatment. *Fertil Steril*. 2010;93(1):289–92.
- Li L, Zhang X, Zhao L, Xia X, Wang W. Comparison of DNA apoptosis in mouse and human blastocysts after vitrification and slow freezing. *Mol Reprod Dev*. 2012;79(3):229–36.
- Pinborg A, Henningsen AA, Loft A, Malchau SS, Forman J, Andersen AN. Large baby syndrome in singletons born after frozen embryo transfer (FET): is it due to maternal factors or the cryotechnique? *Hum Reprod*. 2014;29(3):618–27.

31. Wikland M, Hardarson T, Hillensjö T, Westin C, Westlander G, Wood M, et al. Obstetric outcomes after transfer of vitrified blastocysts. *Hum Reprod*. 2010;25(7):1699–707.
32. Zhu J, Lin S, Li M, Chen L, Lian Y, Liu P, et al. Effect of in vitro culture period on birthweight of singleton newborns. *Hum Reprod*. 2014;29(3):448–54.
33. Mäkinen S, Söderström-Anttila V, Vainio J, Suikkari A-M, Tuuri T. Does long in vitro culture promote large for gestational age babies? *Hum Reprod*. 2013;28(3):828–34.
34. Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. *Hum Reprod Update*. 2015;21(2): 209–27.