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Obstetric outcomes after transfer of vitrified blastocysts

M. Wikland[1,*], T. Hardarson[1], T. Hillensjö[1], C. Westin[1], G. Westlander[1], M. Wood[1], and U.B. Wennerholm[2]

[1]Fertility Centre Scandinavia, Box 5418, SE-402 29 Göteborg, Sweden [2]Perinatal Center, Department of Obstetrics and Gynaecology, Institute for Clinical Sciences, Sahlgrenska Academy, East Hospital, Gothenburg University, SE-46 85 Göteborg, Sweden

*Correspondence address. E-mail: matts.wikland@fcivf.com

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BACKGROUND: It has been claimed that the risks to the child resulting from vitrification as compared with the slow-freezing technique, may be higher owing to the high concentrations of potentially toxic cryoprotectants. We therefore retrospectively compared the obstetric and neonatal outcomes in a cohort of children born after transfer of vitrified blastocysts, fresh blastocysts and slow-frozen early cleavage stage embryos.

METHODS: All children born after transfer of vitrified blastocysts ($n = 106$), fresh blastocysts ($n = 207$) and slow-frozen early cleavage stage embryos ($n = 206$) during the period January 2006 to May 2008 at Fertility Center Scandinavia were included. Data on obstetric and neonatal outcomes were obtained from medical records from the antenatal and delivery clinics.

RESULTS: For singletons, there were no significant differences between the groups in gestational age, mortality or birth defects. After adjustment for parity and BMI, birthweight was significantly higher in singletons born after transfer of vitrified blastocysts as compared with after transfer of fresh blastocysts (median 3560 versus 3510 g, $P = 0.0311$). More singletons born after transfer of fresh blastocysts were small for gestational age compared with singletons born after transfer of vitrified blastocysts (12.1 versus 3.0%, $P = 0.0085$). A higher rate of major post-partum haemorrhage was observed in the vitrified blastocyst group as compared with the other two groups (25.0 versus 6.0 and 7.5%).

CONCLUSIONS: No adverse neonatal outcomes were observed in children born after transfer of vitrified, as compared with fresh blastocysts or after transfer of slow-frozen early cleavage stage embryos.

Key words: vitrification / cryopreservation / blastocyst / assisted reproductive technology / obstetric outcome

Introduction

Compared with cleavage stage embryo transfer, blastocyst transfer has been shown to increase delivery rates, particular in younger women (Blake *et al*., 2007), whereas allowing for improved selection of potentially viable embryos at this late stage of development, and to reduce multiple pregnancies because fewer embryos are transferred (Henman *et al*., 2005). However, one problem with blastocyst culture has been the somewhat unpredictable results with regard to survival of embryos and the pregnancy rate after blastocysts are frozen with the conventional slow-freezing method (Liebermann and Tucker, 2006). The explanation is believed to be technical, owing to the size, multicellular structure and the presence of the blastocoele in the blastocyst (Kader *et al*., 2009). A more reliable and consistent method than conventional cryopreservation of blastocysts (slow method) seems to be vitrification (Liebermann and Tucker, 2004). Owing to the technical and clinical advantages (i.e. high pregnancy rates) associated with vitrification, many fertility clinics have included the method as routine for cryostorage of surplus blastocysts. There are, however, still concerns regarding the safety of vitrification and whether it may result in adverse effects in the offspring due to the use of high concentrations of potentially toxic cryoprotectants (Vajta and Nagy, 2006). As a result, there is no general recommendation for clinical use.

It has been estimated that nearly 4 million children have been born after assisted reproductive technology (ART), originating from gametes, early cleavage stage embryos or cryopreserved blastocysts (K.G. Nygren, ICMART 2009, personal communication). Recently, Wennerholm *et al.* (2009a, b) concluded in a review of studies dealing with follow-up of children born after transfer of cryopreserved gametes or embryos that 'infant outcome after slow-freezing of embryos was reassuring', although controlled follow-up studies with regard to vitrification of gametes and embryos were needed. Analysing the obstetric and perinatal outcomes following transfer of vitrified blastocysts is of utmost importance owing to the limited number of

reports on this subject (Takahashi et al., 2005; Liebermann, 2009; Mukaida et al., 2009).

The objective of this study was to follow-up the delivery of our first 100 children born after transfer of vitrified/warmed blastocysts and compare them with children born after transfer of fresh blastocysts and slow-frozen/thawed early cleavage stage embryos during the same time period from the same clinic.

Materials and Methods

Children born after transfer of vitrified blastocysts, fresh blastocysts and frozen thawed early cleavage stage embryos (Day 2 or 3) during the period January 2006 to May 2008 at Fertility Centre Scandinavia, Gothenburg, Sweden were followed up during pregnancy and delivery until they left the hospital.

Ovarian stimulation in fresh cycles

In the fresh cycles, ovarian stimulation was performed using recombinant FSH in combination with GnRH agonists or antagonists. Oocyte retrieval was carried out 36 h after hCG injection (Bergh et al., 1997). Standard IVF treatment was predominantly used in patients with tubal or idiopathic infertility indications, whereas ICSI was predominantly used in cases of male factor infertility.

Blastocyst culture and scoring

After oocyte retrieval, cumulus-enclosed oocytes were incubated in fertilization medium (COOK, Brisbane, Australia) prior to insemination/injection. Semen was prepared with a discontinuous gradient method (PureSperm, Nidacon AB, Gothenburg, Sweden). Fertilization was assessed 16–18 h after insemination/injection. The resulting two pronuclear embryos were cultured in cleavage medium (COOK) until Day 2 after oocyte pick-up, scored and subsequently transferred to CCM (Vitrolife AB, Gothenburg, Sweden) culture media until Day 5 or 6 in microdroplets under oil in 6% CO_2 and 5% O_2. On Day 5 the embryos were scored and those not selected for transfer or cryopreservation were moved to fresh media and cultured until Day 6.

On Day 5 or 6, each embryo which had developed to the blastocyst stage was scored using published criteria (Gardner and Schoolcraft, 1999). The blastocysts were given a numerical score (1–6) depending on their degree of development. For those blastocysts graded as 3 or higher, the inner cell mass and trophectoderm cells were graded from 0 to 2 depending on their number and cohesiveness.

Vitrification of blastocysts

The carrier chosen to contain the blastocysts was the cryoloop. The method was based on Lane et al. (1999) and adapted by Mukaida et al. (2003) adding assisted collapse by way of laser (Hardarson et al., 2007). Briefly, those blastocysts with good morphology and expansion status were vitrified. Approximately 15 min before initiating the vitrification process, the blastocysts were artificially collapsed by breaching both the zona pellucida and the trophectoderm cells using a 1.48 diode laser (Fertilase® MTG, Altdorf, Germany). Blastocysts were vitrified with two-step exposure to cryoprotectants. The solutions were incubated at 37°C for 30 min prior to the vitrification process and all work was carried out on heating stages. All solution volumes were 1 ml. Initially, blastocysts were placed in solution I, containing HEPES/Bicarbonate based base medium for 2 min and then moved to solution II containing 7.5% dimethyl sulphoxide (DMSO) and 7.5% ethylene glycol, for 2 min before being transferred to solution III, which contained 15% DMSO and 15% ethylene glycol, 12 mM ficoll and 0.58 M sucrose for 20–30 s. The blastocysts were then loaded onto the cryoloop, plunged into liquid nitrogen and stored in standard cryotanks.

Blastocyst re-warming

Blastocysts were thawed using three-step dilution with sucrose. All solutions were pre-incubated at 37°C and kept on heating stages throughout the warming procedure. The tip of the cryoloop, containing the blastocyst, was submerged into the first thaw solution containing 0.28 M sucrose. Blastocysts were moved from this solution after 2 min and transferred to base medium containing 0.17 M sucrose for an additional 3 min. Subsequently, blastocysts were washed once, incubated for 5 min in the base medium and then returned to culture.

After vitrification, the blastocysts were cultured in CCM medium for up to 4 h before being morphologically assessed based on rate of expansion, degree of cell organization and whether their blastomeres were light or dark.

Slow-freezing/thawing of early cleavage stage embryos

Embryos were frozen using the traditional protocols for cryopreservation of human embryos, with the slow-freezing technique. Briefly, embryos were exposed to a series of propandiol (PrOH) based cryoprotectants in HEPES-based medium and loaded into straws. The straws were then placed in a programmable freezing instrument (Cryologic®, Mulgrave, VIC, Australia) that insured a highly controlled slow-rate of cooling starting at room temperature and slowly (0.3°C/min) proceeding down to −6.0°C, when ice crystal formation was manually induced. After a period of 10 min the straws were further cooled (2°C/min) down to −35°C, held at that temperature for a further 10 min and thereafter allowed to fall freely down to below −100°C whereupon the straws were transferred into liquid nitrogen tanks for long-term storage.

The embryos were thawed by holding the straws in air for 40 s followed by 30 s in 30°C water and in a series of thawing solutions. The survival rate was calculated as the number of survived cells out of the total number of cells. Only embryos where 50% or more of the blastomeres had survived were transferred.

Transfer of vitrified/warmed blastocysts

Vitrified and warmed blastocysts were transferred either in hormonally supplemented or natural cycles. Supplemented cycles were used in the majority of cycles for all patients who had vitrified/warmed blastocysts transferred irrespective of whether they had regular or irregular cycles. The reason for this was that it was easier for the clinic to plan the embryo transfer.

In supplemented cycles the patients started with estradiol valerate tablets 6 mg daily (Progynon, Schering Nordiska AB, Järfälla, Sweden) from cycle Day 1 to cycle Day 12. If the endometrium was at least 8 mm as measured by vaginal ultrasound on that day, the daily estradiol dose was lowered to 4 mg. Vaginal suppositories of micronized progesterone (Progesterone vag, Apoteksbolaget AB, Umeå, Sweden) 1200 mg per day were then initiated and taken for 6 days, whereupon embryo transfer was performed. Estradiol and progesterone medication continued until the pregnancy test 2 weeks later and if positive for another 4 weeks (Simon et al., 1999).

Natural cycles were used at the end of the study period in women with regular menstrual cycles. The reason for the change from supplemented cycles to natural cycles for this group of women was that we had used the natural cycles for many years when transferring slow-frozen/thawed early cleavage stage embryos and found this more convenient to manage for the clinic and the patient.

In the natural cycle, ovarian scanning was performed on cycle Days 10–11. If there was a leading follicle of 14–15 mm, urinary luteinizing hormone (LH) sticks for identifying the LH peak were initiated. Embryo transfer was performed 6 days after a positive LH stick.

Transfer of frozen/thawed early cleavage stage embryos

In women with amenorrhoea or irregular menstrual cycles, transfer was performed in stimulated cycles according to the protocol described above for vitrified/warmed blastocysts. Transfer was performed 3 days after the initiation of progesterone.

In women with regular menstrual cycles, transfer was performed in natural cycles according to the protocol described above except for the day of transfer, which was 3 days after a positive LH stick.

Follow-up of pregnancy, delivery and perinatal period

The ethical committee at the University of Gothenburg approved the study. Details of infertility history and IVF treatment were obtained from the medical records of the fertility clinic, whereas pregnancy, obstetric and perinatal histories were obtained from the medical records of the maternity centre and the obstetrics department where the women had been followed and delivered. The majority of the patients ($n = 492$) had given written consent to retrieve their medical records. Women who did not allow us to retrieve their medical records ($n = 14$), gave us the information we needed about their maternity care and delivery themselves.

The medical records were scrutinized by two doctors (M.W. and U.B.W.). In cycles with transfer of fresh blastocysts, gestational age was determined by subtraction of the oocyte retrieval date from the date of birth and addition of 14 days. In non-stimulated cycles (in women with regular menstrual periods) with transfer of vitrified blastocysts or early cleavage stage embryos, gestational age was calculated from the date of last menstrual period to the date of birth. In stimulated (estrogen + progesterone) cycles with transfer of vitrified blastocysts or early cleavage stage embryos, gestational age was determined by subtracting the date of embryo transfer from the date of birth and addition of 14 days plus the age of the embryo in days.

During the study period, deliveries in Sweden included all live born babies and stillborns after 28 weeks of gestation. Preterm birth was defined as birth before 37 completed weeks of gestation. Low birthweight was defined as birthweight <2500 g. Small for gestational age (SGA) was defined as a birthweight $<-22\%$ of the expected mean birthweight according to gestational age in a Swedish reference population (Marsál *et al.*, 1996). Perinatal mortality included mortality in live-borns within 7 days of age and stillborns after 28 weeks of gestation. Birth defects were defined according to the Q codes in the International Classification of Diseases (ICD) 10.

Statistical methods

All statistical analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Pairwise comparisons were made between the vitrified blastocyst group and the fresh blastocyst group or the slow-frozen early cleavage stage embryo group. Fisher's exact test was used for dichotomous variables, χ^2 test was used for non-ordered categorical variables, Mantel–Haenszel χ^2 exact test for ordered categorical variables and Mann–Whitney *U*-test for continuous variables. For neonatal outcome, variables logistic regression was used to adjust for potential maternal confounding factors [parity and body mass index (BMI)]. Smoking was not included as a confounding factor owing to the small number of smokers among the women. For the specific outcome major post-partum haemorrhage, the odds ratio (OR) with 95% confidence intervals (CIs) were calculated. Adjusted ORs were calculated using multiple logistic regression analyses. Adjustment was made for parity, the weight of the baby, assisted vaginal delivery and retained placenta. Findings with a *P*-value <0.05 or a CI not including 1 was considered statistically significant.

Sample size calculation

The sample size of 300 individuals made it possible to detect a difference in birthweight of ≥ 211 g, assuming an α of 0.05 and β of 0.20 (80% power).

Results

From January 2006 to May 2008, 321 vitrified blastocyst transfers, 563 fresh blastocyst transfers and 871 transfers of slow-frozen/thawed early cleavage stage embryos were performed resulting in 172, 296 and 309 pregnancies which led to 103 (=106 children), 203 (=207 children) and 200 (=206 children) deliveries, respectively (Table I). Embryo survival rate was 81.4% in the vitrified blastocyst group and 80.8% in the slow-frozen early cleavage stage group.

The main maternal characteristics in the vitrified blastocyst, fresh blastocyst and slow-frozen early cleavage stage embryo groups resulting in deliveries are summarized in Table II. Maternal characteristics were comparable in the vitrified and fresh blastocyst groups concerning maternal age, BMI, parity, smoking habits and educational level. Women in the vitrified blastocyst group had a lower median BMI, were more often primiparous and smoked more than the slow-frozen early cleavage stage embryo group. Women in the vitrified blastocyst group had more preexisting endocrine disease (mainly thyroid

Table I Outcome after transfer of vitrified, fresh blastocysts and slow-frozen early cleavage stage embryos, January 2006 to May 2008.

	Vitrified BC	Fresh BC	SF
Embryo transfers	321	563	871
Maternal age (years)	36.1 (23.0–45.0)	36.0 (21.0–45.0)	36.4 (21.0–46.0)
SET	306 (95.3)	514 (91.4)	766 (87.9)
Pregnancies	172 (53.6)	296 (52.5)	309 (35.5)
Spontaneous miscarriages before 6 weeks	37	43	57
Spontaneous miscarriages between 6 and 12 weeks	28	42	44
Spontaneous miscarriages after 12 weeks	2	5	5
Ectopic pregnancies	1	3	2
Legal abortions	1	0	1
Deliveries	103 (32.1)	203 (36.3)	200 (23.0)
Embryo survival rate	81.4%	NA	80.8%

BC, blastocyst; SF, slow-frozen early cleavage stage embryos; SET, single embryo transfer; NA, not applicable; values are number and (%) or median and range.

Table II Maternal characteristics in pregnancies conceived after transfer of vitrified blastocysts, fresh blastocysts and slow-frozen early cleavage stage embryos.

Characteristic	Vitrified BC (n = 103)	Fresh BC (n = 203)	SF (n = 200)	Tests between groups, P-value	
				Vitrified BC versus fresh BC	Vitrified BC versus SF
Maternal age, years	35.4 (26.3–45.3)	34.7 (22.0–44.0)	35.6 (24.9–43.5)	0.2334	0.8006
BMI	22.6 (17.4–36.0)	23.2 (18.5–41.0)	24.0 (16.3–42.8)	0.1486	0.0379
Primigravida	34 (33.0)	67 (33.0)	35 (17.5)	1.000	0.0042
Primiparous	59 (57.8)	103 (50.7)	64 (32.0)	0.2930	<0.0001
Smokers in first trimester	5 (4.9)	6 (3.0)	1 (0.5)	0.5890	0.0370
Years of infertility	2.0 (0.0–20.0)	2.5 (1.0–10.0) (n = 200)	2.0 (1.0–13.0)	0.7570	0.1331
Educational level, university exam	59 (59.6)	105 (57.7)	120 (63.8)	0.8568	0.5635
Hypertension	0	0	2 (1.0)	NA	0.8698
Diabetes	2 (1.9)	0	3 (1.5)	0.2252	1.0
Endocrine disease	10 (9.7)	4 (2.0)	8 (4.0)	0.0072	0.0884
Renal disease	4 (3.9)	1 (0.5)	5 (2.5)	0.0910	0.7296
Inflammatory bowel disease	1 (1.0)	2 (1.0)	1 (0.5)	1.0	1.0
Reason for infertility					
Male factor	32 (31.1)	69 (34.0)	79 (39.5)	0.9191	0.5302
Tubal	13 (12.6)	22 (10.8)	20 (10.0)		
Unexplained	30 (29.1)	61 (30.0)	51 (25.5)		
Other	28 (27.2)	51 (25.1)	50 (25.0)		
Method					
IVF	59 (57.3)	127 (63.2)	120 (60.0)	0.3811	0.7380
ICSI	44 (42.7)	74 (36.8)	80 (40.0)		
SET	97 (94.2)	196 (96.6)	183 (91.5)	0.4908	0.5561

BC, blastocyst; SF, slow-frozen early cleavage stage embryos; BMI, body mass index; SET, single embryo transfer; NA, not applicable; values are number and (%) or median and (range).

dysfunction) than the fresh blastocyst group. In transfers that led to deliveries, single embryo transfer was performed for 94.2, 96.6 and 91.5% of the vitrified, fresh blastocyst and slow-frozen early cleavage stage embryo groups resulting in 98.1, 98 and 97% singleton deliveries, respectively (Tables II and III). One triplet delivery occurred in the vitrified blastocyst group, although the rest of the multiple births were twin births. Pregnancy complications were similar in all groups (Table III). Hypertensive disorder was the most frequent pregnancy complication and occurred in 11.8, 5.4 and 5.5% of the vitrified, fresh blastocyst and slow-frozen early cleavage stage embryo groups, respectively.

Delivery outcome in singletons

Labour was induced in 29.7, 15.6 and 15.5% of the vitrified, fresh blastocyst and slow-frozen early cleavage stage embryo groups, respectively (vitrified versus fresh blastocyst: $P = 0.0151$ and vitrified blastocyst versus slow-frozen: $P = 0.0165$ for onset of delivery; Table IV). Cesarean section was performed in 29.7% of the vitrified blastocyst group, 20.6% of the fresh blastocyst group and 20.1% of the slow-frozen early cleavage stage embryo group ($P = 0.1088$ and $P = 0.0980$ for mode of delivery).

In women with vaginal delivery, post-partum haemorrhage (>500 ml) was observed in 55.9, 30 and 28.8% ($P = 0.0005$ and $P = 0.0003$) and major post-partum haemorrhage (>1000 ml) was observed in 25, 6.0 and 7.5% ($P = 0.0003$ and $P = 0.0014$) of the vitrified, fresh blastocyst and slow-frozen early cleavage stage embryo groups, respectively. Retained placenta occurred in 12.7, 5.1 and 3.9% ($P = 0.0856$ and $P = 0.0358$), respectively. The OR for major post-partum haemorrhage in the vitrified blastocyst group as compared with the fresh blastocyst group was 5.22 (95% CI 2.19–12.5). After adjustment for potentially confounding factors (parity, the weight of the baby, assisted vaginal delivery and retained placenta) the OR was 3.09 (95% CI 1.38–6.96). The OR for major post-partum haemorrhage in the vitrified blastocyst group as compared with the early cleavage stage embryo group was 4.09 (95% CI 1.79–9.33) and after adjustment this OR was 2.16 (95% CI 0.93–5.02).

Neonatal outcome in singletons

Neonatal outcome in singletons is presented in Table V. The median gestational ages were comparable in pregnancies after transfer of vitrified blastocysts, fresh blastocysts and transfer of slow-frozen early cleavage stage embryos. Preterm birth (<37 weeks) occurred in 6.9, 7.0 and 4.6% of the vitrified, fresh blastocyst and slow-frozen early cleavage stage embryo groups, respectively ($P = 0.9356$ and $P = 0.4757$, adjusted for maternal BMI and parity). The rate of post-term birth was significantly higher in the vitrified blastocyst group

Table III Complications during pregnancy in pregnancies conceived after transfer of vitrified and thawed blastocysts, fresh blastocysts and slow-frozen early cleavage stage embryos.

	Vitrified BC (n = 103)	Fresh BC (n = 203)	SF (n = 200)	Tests between groups, P-value	
				Vitrified BC versus fresh BC	Vitrified BC versus SF
Gestational sacs >1 in first trimester	2 (1.9)	5 (2.5)	7 (3.5)	1.000	0.7164
Multiple pregnancy					
Singletons	101 (98.1)	199 (98.0)	194 (97.0)	0.7351	1.000
Twin pregnancies	1 (1.0)	4 (2.0)	6 (3.0)		
Triplet pregnancies	1 (1.0)	0	0		
Hypertensive disorder	12 (11.8)	11 (5.4)	11 (5.5)	0.0876	0.0922
Hypertension	2 (1.9)	2 (1.0)	4 (2.0)		
Mild pre-eclampsia	7 (6.8)	3 (1.5)	4 (2.0)		
Severe pre-eclampsia	3 (2.9)	6 (3.0)	3 (1.5)		
Gestational diabetes	3 (2.9)	4 (2.0)	5 (2.5)	0.8689	1.000
Placenta previa	1 (1.0)	5 (2.5)	1 (0.5)	0.6851	1.000
Abruptio placentae	2 (1.9)	3 (1.5)	1 (0.5)	1.000	0.5348
Cholestasis	0	1 (0.5)	2 (1.0)	1.000	0.8757
PPROM	0	1 (0.5)	0	1.000	NA

BC, blastocyst; SF, slow-frozen early cleavage stage embryos; PPROM, preterm prelabour rupture of the membranes; values are number and (%).

Table IV Outcome of delivery in singleton pregnancies conceived after transfer of vitrified and thawed blastocysts, fresh blastocysts and slow-freezing of early cleavage stage embryos.

	Vitrified BC (n = 101)	Fresh BC (n = 199)	SF (n = 194)	Test between groups, P-value	
				Vitrified BC versus fresh BC	Vitrified BC versus SF
Onset of delivery					
Spontaneous	63 (62.4)	147 (73.9)	146 (75.3)	0.0151	0.0165
Induction of labour	30 (29.7)	31 (15.6)	30 (15.5)		
Elective CS	8 (7.9)	21 (10.6)	18 (9.3)		
Mode of delivery					
Spontaneous vaginal delivery	61 (60.4)	144 (72.4)	141 (72.7)	0.1088	0.0980
Assisted vaginal delivery	10 (9.9)	14 (7.0)	13 (6.7)		
Cesarean section (emergency and elective)	30 (29.7)	41 (20.6)	40 (20.1)		
Post-partum haemorrhage*					
PPH > 500 ml	38 (55.9) (n = 68)	45 (30.0) (n = 150)	42 (28.8) (n = 146)	0.0005	0.0003
PPH > 1000 ml	17 (25.0) (n = 68)	9 (6.0) (n = 150)	11 (7.5) (n = 146)	0.0003	0.0014
Manual removal of the placenta**	9 (12.7) (n = 71)	8 (5.1) (n = 158)	6 (3.9) (n = 154)	0.0856	0.0358

BC, blastocyst; SF, slow-frozen early cleavage stage embryos; CS, Cesarean section; PPH, post-partum haemorrhage; *PPH in patients with vaginal deliveries (missing values for 3, 8, 8 women in the vitrified, fresh and slow-freezing group respectively); ** in patients with vaginal deliveries, values are number and (%).

as compared with the fresh blastocyst group (13.9 versus 8.0%, $P = 0.0362$).

In the crude analysis, median birthweight was similar in all three groups. The rate of low birthweight (<2500 g) was comparable in all groups (6.9, 4.5 and 3.1%, respectively, adjusted $P = 0.3728$ and 0.9559). When adjustment was made for maternal BMI and parity, singletons born after transfer of vitrified blastocysts had significantly higher median birthweights than those of the fresh blastocyst group (median birthweight 3650 versus 3510 g, adjusted P-value 0.0362). Singletons in the vitrified blastocyst group were also longer at birth and had larger head circumferences than singletons in the fresh blastocyst group. Significantly more infants in the fresh blastocyst group were SGA as compared with the vitrified blastocyst group 12.1 versus 3.0% (adjusted $P = 0.0085$). No difference in size at birth

Table V **Neonatal outcome in singleton pregnancies conceived after transfer of vitrified and thawed blastocysts, fresh blastocysts and slow-frozen early cleavage stage embryos.**

	Vitrified BC (n = 103)	Fresh BC (n = 199)	SF (n = 194)	Test between groups, P-value			
				Vitrified BC versus fresh BC		Vitrified BC versus SF	
				P-value	adj. P-value*	P-value	adj. P-value*
Gender							
Boys	48 (47.5)	102 (51.3)	107 (55.2)				
Girls	53 (52.5)	97 (48.7)	87 (44.8)	0.6252		0.2617	
Gestational age, weeks	40.3 (29.4–42.9)	40.1 (34.6–43.1)	39.9 (25.0–42.9)	0.1902	0.2050	0.0534	0.1999
GA < 37 weeks	7 (6.9)	14 (7.0)	9 (4.6)	1.000	0.9356	0.5687	0.4757
GA < 32 weeks	1 (1.0)	0	2(1.0)	0.6733	NA	1.000	0.9913
GA < 28 weeks	0	0	1 (0.5)	NA	NA	1.000	NA
GA ≥ 42 weeks	14 (13.9)	16 (8.0)	14 (7.2)	0.1699	0.0362	0.1059	0.1324
Birthweight, g	3650 (1425–5330)	3510 (1725–5130)	3615 (900–5100)	0.0985	0.0311	0.9868	0.5732
<2500 g	7 (6.9)	9 (4.5)	6 (3.1)	1.000	0.3728	0.9316	0.9559
<1500 g	1 (1.0)	0	2 (1.0)	0.6733	NA	1.000	0.9913
<1000 g	0	0	1 (0.5)	NA	NA	1.000	
SGA < −22%	3 (3.0)	24 (12.1)	15 (7.7)	0.0112	0.0085	0.1633	0.0361
LGA > 22%	7 (6.9)	14 (7.0)	23 (11.9)	1.000	0.5666	0.2582	0.7575
Length, cm	51.0 (41.0–59.0) n = 97	51.0 (42.0–57.0) n = 195	51.0 (41.0–56.0) n = 184	0.1478	0.0497	0.9832	0.5415
Head circumference, cm	35.0 (28.0–39.0) n = 98	35.0 (30.2–39.0) n = 195	35.0 (27-0-39.0) n = 185	0.0582	0.0114	0.8333	0.1782
Apgar score <7 at 5 min	5 (5.1)	2 (1.0)	2 (1.1)	0.0892	0.2097	0.1051	0.3599
Transferred to NICU	14 (13.9)	15 (7.5)	17 (8.8)	0.1269	0.4039	0.2498	0.6085
NICU > 7 days	5 (38.5) n = 14	9 (60) n = 15	3 (21.4) n = 17	0.4495	0.8093	0.5855	0.9127
Birth defects	1 (1.0)	4 (2.0)	8 (4.1)	0.9152	0-5604	0.2565	0.1569
Perinatal mortality	1 (1.0)	1 (0.5)	1 (0.5)	0.6238	0.6629	0.2948	0.9911

BC, blastocyst; SF, slow-frozen early cleavage stage embryos; GA, gestational age; NA, not applicable; SGA, small for gestational age; NICU, neonatal intensive care unit; values are number and (%) or median and (range) * adjusted for maternal BMI and parity.

was seen between singletons in the vitrified blastocyst group and singletons in the slow-frozen group.

No statistically significant difference was found for the rate of birth defects between the vitrified and fresh blastocyst groups or between the vitrified and slow-frozen groups. Birth defects were found in 1% of the children in the vitrified blastocyst group (one child with branchial cleft cyst), in 2.0% of the children in the fresh blastocyst group (three children with unilateral cryptorchidism and one child with bilateral pes equinovarus) and in 4.1% of the children in the slow-frozen early cleavage stage embryo groups (one child with bilateral syndactyly of digits 2 and 3, one child with exstrophy of the urinary bladder and anal atresia, one child with a persistent ductus arteriosus, one child with a small ventricular septum defect, one child with hydrocele testis, one child with unilateral cryptorchidism and one child with unilateral hip instability).

One intrauterine fetal death occurred in gestational week 42+ in the vitrified blastocyst group. The birthweight of the baby was 5330 g and birth length was 59 cm. The karyotype was normal. At autopsy a thrombosis was found in the umbilical cord. In the fresh blastocyst group one baby died during delivery due to intrapartum asphyxia during spontaneous labour in gestational week 42+. The birthweight was 4020 g. In the slow-frozen early cleavage stage embryo group one child was delivered by Caesarian section at 25 weeks of gestation due to severe pre-eclampsia. The child died at 7 days of age due to problems associated with immaturity. The birthweight was 900 g. The child had no birth defects.

Neonatal outcome in multiple pregnancies

In one case in the vitrified blastocyst group, the transfer of two blastocysts resulted in a dizygotic triplet pregnancy with monozygotic (MZ) twinning. In the fresh blastocyst group there were three MZ sets of twins and in the slow-frozen early cleavage stage group there was one set of MZ twins.

In the vitrified blastocyst group, the twins were delivered at 37.6 weeks of gestation and their birthweights were 2975 and 2435 g, respectively. The triplets were delivered at 33.3 weeks of gestation and their birthweights were 2215, 1829 and 2040 g. In the fresh blastocyst group, the four sets of twins were delivered at a median gestational age of 36.7 weeks (range 30.7–40.9 weeks) with a median birthweight of 2555 g (1505–3185 g). In the slow-frozen early cleavage stage group six sets of twins were delivered. Median gestational age was 35.7 weeks (34.8–38.0) and median birthweight was 2160 g (1705–3325 g). No birth defects or perinatal mortality occurred in the multiples in any of the groups.

Discussion

To the best of our knowledge our study is the first follow-up of children born after transfer of vitrified blastocysts, where the medical records from the maternity centre and the obstetrics department where the women had been followed and delivered, have been analysed.

No adverse outcome was observed in children born after transfer of vitrified blastocysts as compared with children born after transfer of fresh blastocysts or after slow-frozen of early cleavage stage embryos in terms of preterm birth, low birthweight or birth defects. Median birthweight was also comparable with data (3501 g for 2008) in the general population in Sweden (Swedish Medical Birth register, 2009). Significantly fewer newborns in the vitrified blastocyst group were SGA as compared with the fresh blastocyst group. The gestational age for the vitrified blastocyst group was comparable with that in the general population.

Blastocyst transfer has become an attractive option owing to the high implantation rate (Gardner *et al.*, 1998). In countries like Sweden where single embryo transfer has become almost mandatory for reasons of legislation this is an interesting option. However, introducing single embryo transfer of blastocysts for patients with many good quality embryos requires an effective cryopreservation method for surplus blastocysts. Vitrification has proven to be very successful with regard to survival, implantation and pregnancy rates (Mukaida *et al.*, 2003; Liebermann and Tucker, 2006). However, concerns have been raised with regard to possible toxic effects of the high concentrations of cryoprotectants (Menezo, 2004). Still, the method has been in clinical use for many years (Liebermann and Tucker, 2006). Surprisingly, there are very few studies on follow-up of children after transfer of vitrified oocytes and embryos.

Since 2006 our clinic has offered blastocyst transfer to women with more than five fertilized and cleaved embryos on culture Day 2. However, our experience with fairly poor pregnancy rates using slow-freezing of surplus blastocysts encouraged us to adopt the vitrification method which very quickly became successful in terms of pregnancy rates. When the vitrification method was introduced clinically, we also decided to perform a follow-up study on obstetric and neonatal outcome of the first 100 children and to compare them with children conceived from transfer of fresh blastocysts and children from early cleavage stage embryos frozen with the conventional slow method during the same time period. The strength of the present study is the meticulous scrutinization of the medical records and the complete follow-up of all deliveries. The limitation is the small sample size, with its inadequate power to detect differences in rare outcome measures such as birth defects.

Previous studies on follow-up of children born after vitrification of blastocysts have either been case reports or retrospective data based on questionnaires sent to parents (Choi *et al.*, 2000; Yokota *et al.*, 2001; Son *et al.*, 2003; Takahashi *et al.*, 2005; Hiraoka *et al.*, 2006, 2007; Parriego *et al.*, 2007; Mukaida *et al.*, 2009). Takahashi *et al.* performed, by means of a questionnaire to parents, a 4-year follow-up study of children born after transfer of vitrified blastocysts. They found no differences in obstetric outcomes for children born after vitrified blastocysts as compared with children born after fresh blastocysts. However, the preterm birth rate of 18.5% and low birthweight rate of 43.5% among all children in the vitrified blastocyst group were both remarkably high (Takahashi *et al.*, 2005). This was probably attributable to the multiple birth rates of 30.1%, as compared with only 2.0% for the equivalent group in our study. The Takahashi group also reported at the 25th annual meeting of the European Society of Human Reproduction and Embryology on a 9-year follow-up of children born after transfer of vitrified blastocysts. The report included 766 deliveries, 16.1% of which were multiple births. Low mean gestational age (38.1 weeks) and mean birthweight (2764 g) was again reported. Those findings differ from our study but can probably still be explained by the high multiple birth rate, due to more embryos (1.51) per transfer (Mukaida *et al.*, 2009).

Follow-up studies of children born after fresh blastocyst transfers are also relatively rare. Schwärzler *et al.* (2004) found, in 357 children born after transfer of fresh blastocysts, a high rate of preterm births and multiples (33%), probably owing to the fact that in the majority of cases they transferred more than one embryo. However, a recently published Swedish study showed a small increase in risk associated with blastocyst transfer as compared with cleavage stage transfer. In this study, 1311 children born after fresh blastocyst transfer were compared with 12 562 children born after transfer of fresh early cleavage stage embryos. After adjustment for several confounders, significantly higher risks were found for preterm births and birth defects in the blastocyst group (Källén *et al.*, 2010). Although our study only included 317 children born after transfer of blastocysts (fresh + vitrified), no such increased risks were found.

In the present study, the low rate of multiple pregnancies in all groups (2.0, 2.0 and 3.0%) is notable and is of course related to the high proportion of single embryo transfers (>90%). However, MZ twinning was more frequent, although the numbers are small, in the two blastocyst groups as compared with the slow-frozen early cleavage stage group. This is in line with other recent studies (Chang *et al.*, 2009; Vitthala *et al.*, 2009). In the meta-analysis by Chang *et al.* (2009) of nine studies, they found a 3-fold increased risk of MZ twinning after blastocyst transfer as compared with early cleavage stage embryo transfer. Another meta-analysis included 27 studies and found blastocyst transfer to be associated with a 4-fold higher risk of MZ twinning as compared with spontaneous conception (1.7 versus 0.4%; Vitthala *et al.*, 2009).

Concerns have been raised about gender selection in blastocyst transfer, i.e. that more boys are born. Combined data of four studies including 2587 infants presented in a meta-analysis showed that blastocyst transfer appeared to be associated with a sex ratio skewed in favour of males (OR 1.29 95% CI 1.10–1.51; Chang *et al.*, 2009). In our study the numbers of females and males were identical when the vitrified and fresh blastocyst groups were combined. In the vitrified blastocyst group there were more females than males, although the situation was the reverse for the fresh blastocyst group, although the differences were not statistically significant, possibly due to the small sample size. Correspondingly, Lin *et al.* found, in children born after transfer of vitrified blastocysts, using exactly the same method as in our study, a significant difference in sex ratio between children born after fresh and vitrification-thawed blastocyst transfer, with more females in the vitrified blastocyst group. They postulated that it is neither the blastocyst culture itself nor the vitrification process that alters the sex ratio but the selection criteria for embryos (Lin *et al.*, 2009).

The rate of post-term pregnancies was highest in the vitrified blastocyst group, where it was about twice the rate in the general population. In Sweden, the optimal management of post-term pregnancies is a subject of debate (Wennerholm *et al.*, 2009a, b). Some clinics recommend induction of labour between 41 and 42 weeks of gestation while others, recommend expectant management with close surveillance up to 43 weeks of gestation. However, it is noteworthy that in this study two perinatal deaths occurred in post-term pregnancies. More post-term pregnancies and a higher rate of hypertensive disorders in the vitrified blastocyst group contributed to more induced labours in this group as compared with the fresh blastocyst and early cleavage stage groups.

We observed a higher rate of post-partum haemorrhage in the vitrified blastocyst group as compared with the other two groups. The underlying mechanism for this effect is not clear.

Several studies have reported higher frequencies of placenta previa in pregnancies after ART as compared with the general population (Jackson *et al.*, 2004, Shevell *et al.*, 2005; Källen *et al.*, 2005; Romundstad *et al.*, 2006; Schieve *et al.*, 2007; Healy *et al.*, 2010), with ORs varying between 2.3 and 6.0. Undiagnosed low-lying but not total placenta previa may result in increased risk of post-partum haemorrhage in vaginal deliveries. In a recent large Australian study comprising 6730 singleton births, an increased rate of primary post-partum haemorrhage was observed in the ART group as compared with the general population. Endometriosis and ovulation disorders were risk factors for post-partum haemorrhage in the ART population, and there was a trend towards more post-partum haemorrhage after cryopreservation as compared with fresh cycles (Healy *et al.*, 2010). Further studies are needed to verify or refute the findings of increased risk of post-partum haemorrhage after transfer of vitrified blastocysts. It could be speculated that the vitrification process itself somehow affects the trophectoderm cells in a way that may negatively influence placentation.

In conclusion, transfer of vitrified and warmed blastocysts does not seem to have any adverse effect on neonatal outcome. Continuous follow-up of obstetric and neonatal outcomes as well as long-term follow-up children is recommended until larger series have been evaluated.

Authors' roles

All authors: conception and design. M.W. and U.B.W.: collection and assembly of data, data analysis, interpretation and drafting the manuscript. All authors critically reviewed and approved the final version of the manuscript.

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