Human Reproduction, Vol.28, No.11 pp. 2950–2957, 2013
Advanced Access publication on September 12, 2013 doi:10.1093/humrep/det361

human reproduction

ORIGINAL ARTICLE *Embryology*

The time aspect in storing vitrified blastocysts: its impact on survival rate, implantation potential and babies born

B. Wirleitner[1,*], P. Vanderzwalmen[1,2], M. Bach[1], B. Baramsai[1], A. Neyer[1], D. Schwerda[1], M. Schuff[1], D. Spitzer[3], A. Stecher[1], M. Zintz[1], and N.H. Zech[1]

[1]IVF Centers Prof. Zech – Bregenz, Römerstrasse 2, 6900 Bregenz, Austria [2]Centre Hospitalier Inter Régional Cavell (CHIREC), Braine-l'Alleud, Bruxelles, Belgium [3]IVF Centers Prof. Zech – Salzburg, Innsbrucker Bundesstrasse 35, 5020 Salzburg, Austria

*Correspondence address. Tel: +43-(0)5574/44-836; Fax: +43-(0)5574/44-836-9; E-mail: b.wirleitner@ivf.at

Submitted on April 8, 2013; resubmitted on July 31, 2013; accepted on August 9, 2013

STUDY QUESTION: Does the storage time of vitrified human blastocysts negatively impact their survival, the implantation potential of embryos or the malformation rate of babies born?

SUMMARY ANSWER: There was no evidence that storage times of up to 6 years after vitrification (VIT) had a negative impact on blastocyst survival, the implantation potential of embryos or the malformation rate of babies born.

WHAT IS KNOWN ALREADY: Although several thousand children have been born after blastocyst VIT, many aspects of this technique remain to be elucidated. New applications, such as fertility preservation, lead to long storage times of vitrified gametes or embryos but it remains to be determined if these vitrified embryos are stable over time.

STUDY DESIGN, SIZE, DURATION: A retrospective study including 603 transfers was conducted between January 2009 and April 2012. Blastocysts were vitrified using a closed system.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All patients underwent the transfer of aseptically vitrified/warmed blastocysts in a cryo-cycle. A total of 1077 blastocysts were transferred. Survival rates (SRs), implantation potential, birth rates and characteristics of the children born were evaluated.

MAIN RESULTS AND THE ROLE OF CHANCE: We found that the storage of vitrified blastocysts in aseptic conditions neither impaired blastocyst viability (SR after warming during the first year of storage was 83.0% compared with 83.1% after 5–6 years of storage: NS) nor decreased pregnancy rates (clinical pregnancy rate after 1 year of storage was 40.0 versus 38.5% after 6 years: NS). In addition, no increase in the malformation rate over time was observed.

LIMITATIONS, REASONS FOR CAUTION: Our study only included the transfer of blastocysts which had been vitrified aseptically (i.e. using a closed system). Therefore, our results might not be applicable to 'open' VIT systems. The long-term follow-up of children born will be necessary to confirm our findings.

WIDER IMPLICATIONS OF THE FINDINGS: The results suggest that vitrified human blastocysts can be stored for long periods of time without significant negative consequences for the offspring. Therefore, the method should be of benefit to those patients who need to consider taking measures for fertility preservation.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was sought for this study and the authors have no conflict of interest to declare.

Key words: aseptic vitrification / period of storage / blastocyst / live birth rate / malformation rate

Introduction

The cryopreservation of gametes and embryos has become an essential method in assisted reproduction technology (ART). This technique is not only applied for surplus embryos after fresh embryo transfer (ET) but also is used in patients with inappropriate build-up of the endometrial lining, for women with signs of severe ovarian hyperstimulation syndrome or for fertility preservation in cancer patients. Additionally, the number of frozen embryos is increasing due to a change in the transfer policy towards single ET (SET). Further, according to the legislation in place, the storage of cryopreserved embryos is now possible for several years or even decades.

Owing to its widespread application, different cryopreservation techniques have been established and are still constantly improving. In particular, over the last 5 years, there has been a dramatic shift from slow freezing (SF) to vitrification (VIT), due to its reliability in blastocyst and oocyte cryopreservation. Although there are a wide range of articles studying the influence of SF on embryo viability and the health of babies born, little is known about VIT despite the fundamental differences between the two methodologies.

In SF, which has been applied since the 1980's, embryos are exposed to lower concentrations of cryoprotectants (CP) compared with VIT. During SF, the temperature is gradually lowered until extracellular crystal formation is artificially induced at the seeding temperature, which is just below the freezing point (mostly -6 to $-7^{\circ}C$), with the aim of dehydrating the cells and concentrating the intracellular CP. In contrast to SF, VIT works (theoretically) by complete avoidance of intra- or extracellular ice formation. Therefore, the viscosity is increased and higher concentrations of CP in the incubation solutions are inevitable. The frequently used CP dimethylsulfoxide (DMSO) was reported to change the epigenetic profile, whereas 1,2-propandiol (PrOH) has known cytotoxic effects, induces DNA damage and leads to chromosomal mutations (Iwatani *et al.*, 2006; Aye *et al.*, 2010; Berthelot-Ricou *et al.*, 2011; Lawson *et al.*, 2011). In VIT very fast cooling and warming rates are required to avoid ice crystal formation and allow conversion of a supercooled liquid into a glassy state where molecules are solidified in a disorganized unstable state, whereby no energy is released and entropy does not decrease. Therefore, VIT probes might be more vulnerable to temperature variations and the length of time in the liquid nitrogen (LN_2) tank might have a greater impact on them.

As a technologically young innovation in VIT, closed carrier devices were implemented to circumvent the direct contact of embryos with LN_2, with the goal of preventing infection with pathogens during VIT, as well as cross contamination with reactive chemical compounds during storage. Damage of probes in the tank by chemical reactions is avoided. This aspect of aseptic storage is of particular interest for long-term storage. Nevertheless, in contrast to the SF method, VIT raises concern over the possible toxic effects of CP related to exposure to higher concentrations of CP in the incubation solutions. Furthermore, there is increased susceptibility to molecular changes in the vitrified cell structures due to the glassy state, and thereby a higher sensitivity to deviations in storage temperature is present.

The effect of cryopreservation, and especially VIT, on the human embryo and subsequent offspring is still a matter of debate. It is still not completely clear whether cryopreservation itself is linked with increased malformation rates (Wada *et al.*, 1994; Belva *et al.*, 2008; Wennerholm *et al.*, 2009; Li *et al.*, 2010). Although there are several case reports describing successful ET and subsequent deliveries of healthy babies after a prolonged period of cryostorage, it is still unknown how long an embryo can remain frozen and result in a successful transfer and live birth (Revel *et al.*, 2004). There are few studies in the literature about the viability of embryos after long-term cryopreservation and, to the best of our knowledge, there have been no reports about the cryostorage period of vitrified human blastocysts, the embryo quality and competence after warming, and the health of babies born.

The objective of this study was to retrospectively analyze the effect of storage time on aseptically vitrified human blastocysts over a time period of 6 years in 603 cryocycles. We analyzed the survival rate (SR) of embryos after warming, as well as the implantation rate (IR), pregnancy and clinical pregnancy rates (PR and cPR), and the weight and malformation rate in children born in relation to the storage time in LN_2 tanks.

Materials and Methods

Patients and study design

Our study included data from 603 autologous cryo-cycles with aseptically vitrified blastocysts between January 2009 and April 2012. Informed consent to use the outcome data was signed by all patients. We retrospectively analyzed the SR of warmed blastocysts 3 h post-warming, as well as the implantation potential, and the birth rate (BR). Either 1 (SET) or 2 blastocysts (double ET: DET) were transferred and the mean number of transferred embryos is given in Table II. Birthweight and any malformations reported with respect to the length of time of blastocyst storage in LN_2 tanks were collected. Seven 'storage-groups' were defined: Group I storage was 0–3 months, Group II was 3–6 months, Group III was 6–12 months, Group IV was 12–24 months, Group V was 24–36 months, Group VI was 36–48 months and Group VII was 48–72 months.

Ovarian stimulation

For ovarian stimulation, the long protocol was applied (Zech *et al.*, 2007). Thirty-six hours after hCG administration the oocytes were retrieved and kept in human tubal fluid media (LifeGlobal, Ontario, Canada) for 2–4 h before denudation.

Embryo culture

Oocytes were fertilized using standard insemination (IVF, ICSI or intracytoplasmic morphologically selected injection) (Vanderzwalmen *et al.*, 2008). Embryo culture was performed in Global embryo culture medium (LifeGlobal, Ontario, Canada) supplemented with human serum albumin (HSA) (LifeGlobal, ON, Canada) in four-well dishes (Nunc A/S, Roskilde, Denmark). On Day 5, embryo quality was evaluated, and the best one or two blastocyst(s) were selected for ET. Blastocyst quality was assessed according to the degree of blastocoele expansion, and the quality of both the inner cell mass and trophectoderm, which were classified according to the Gardner blastocyst grading scale (Gardner *et al.*, 2000). Blastocysts with a degree of expansion of Grades 2–6, and with Grade A for the inner cell mass and trophectoderm, or a combination of Grades A and B, were classified as top blastocysts. Surplus blastocysts were aseptically vitrified in the afternoon of Day 5. Criteria for VIT were good quality early blastocysts, or blastocyst with the degree of expansion of 2–6 with trophectoderm or/and inner cell mass quality of at least BC or CB grading. Exclusion criteria were not reaching the blastocyst stage, only CC grading for inner cell mass and trophectoderm, or early blastocysts c–d.

Aseptic VIT and storage

Aseptic VIT using Vitrisafe devices (VitriMed, Austria) was performed as previously described (Vanderzwalmen et al., 2009). All initial steps were performed at room temperature (22–25°C). Specifically, blastocysts were exposed to 5% ethylene glycol (EG) (v/v) with 5% DMSO (v/v) for 5–7 min and to 10% EG (v/v) with 10% DMSO (v/v) for 4 min. Hence, blastocysts were transferred to a solution containing 20% EG (v/v) with 20% DMSO (v/v), 25 μmol/l (10 mg/ml) Ficoll (70 000 MW) and 0.75 mol/l sucrose, and placed on the Vitrisafe gutter. The exposure time in the last solution before plunging into LN_2 did not exceed 1 min. All VIT solutions were prepared in HEPES-buffered Global medium containing 20% HSA. After placing the embryos on the gutter of the Vitrisafe, it was inserted into a 0.3 ml CBS straw (Cryo Bio System, France) that was immediately sealed and plunged into the LN_2. In this manner, the blastocysts were aseptically vitrified, which prevented direct contact with LN_2. Soon after, the straws were transferred to conventional LN_2 storage tanks and kept for up to 6 years until ET.

Warming of vitrified blastocysts

The Vitrisafe straws were transferred from the storage tank to small cans containing LN_2. Keeping the part of the device containing the embryos below the LN_2 surface, the upper end of the straw was cut open and the Vitrisafe gutter was retrieved with a small extractor straw. The gutter with the blastocysts was immediately plunged into 1 mol/l sucrose solution. After 1 min, the cells were transferred to 0.75, 0.5, 0.25 and 0.125 mol/l sucrose solution for 1, 2, 2 and 2 min, respectively. Warming took place at room temperature. Subsequently, blastocysts were placed in 500 μl Global medium supplemented with 7.5% HSA for 15 min, before being transferred to new medium. The ET was performed 3–6 h post-warming. The estimate of survival of vitrified/warmed blastocysts was based on the re-expansion and further development of blastocysts 3 h after warming. Blastocysts with the best morphological appearance were chosen for transfer, while surplus re-expanded embryos were re-vitrified. All vitrified blastocysts in this study derived from the first VIT cycle: no re-vitrified embryos were included.

Preparation of the endometrium in the cryo-cycle

After confirmed steroid down-regulation for 2–3 weeks, estrogen therapy with increasing doses of estradiol valerate (Progynova, Schering) starting with 4 mg per day. After 10 days the dosage was increased to 6 mg and the first ultrasound was performed. When the endometrium was <6 mm thick the next ultrasound was scheduled 1 week later and stimulation prolonged to achieve an appropriate endometrium thickness (>8 mm) for ET. From 5 days before ET and onwards until Week 7 when a pregnancy was achieved, i.m. progesterone (Prontogest, IBSA) was administered, followed by intravaginal progesterone until Week 16.

Clinical outcome

PR was determined by testing urinary β-hCG 14 days after ET. The cPR was defined as observation of the fetal heartbeat by ultrasound 8–12 weeks after ET. For IR, we calculated the number of fetal heartbeats divided by the number of embryos transferred. BR was calculated as live birth/ET.

Statistical analysis

Differences in IR between groups were evaluated using Pearson's χ^2-test. Differences in PR, cPR and BR were analyzed using the Wishart distribution. A one-way analysis of variance test was applied to test for significant differences in the age of patients at the time of VIT or ET between the groups. A two-tailed *t*-test was used to test for differences in SR and top-blastocyst rate. Differences between the groups were considered statistically significant when the *P*-value was <0.05. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 17.0 software for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Patient's demographics

A total of 603 ETs after blastocyst VIT were included in this study and grouped according to storage time. Number of cycles per group, storage time, mean age of women at the time of VIT and at time point of cryo-ET are listed in Table I. The qualities of blastocysts after VIT was comparable among groups: the mean rate of ETs with top blastocysts was (69.5%).Whereas there was no significant difference in patient age between groups at the time when VIT was performed or in the percentage of ETs with at least one top blastocyst, patient age at the time of ET increased with longer storage time ($P < 0.05$; Table I).

Blastocyst survival

No differences between the numbers of VIT blastocysts warmed per ET (mean 3.2–3.6 embryos) were observed between groups (Table I). Three hours post-warming, the SR was evaluated and found to range between 81.8 and 89.9% with no significant differences between groups and no decrease over time (Table II).

Table I Patient characteristics.

	Storage groups							*P*-value
	I	II	III	IV	V	VI	VII	
Storage time (months)	0–3	3–6	6–12	12–24	24–36	36–48	48–72	
Storage time (days) mean ± SD	74 ± 8	135 ± 25	254 ± 53	558 ± 118	903 ± 97	1281 ± 121	1728 ± 196	
No. of cycles	100	169	99	92	90	27	26	
Age of patients at VIT, mean ± SD	34.5 ± 4.4	35.0 ± 4.7	35.2 ± 4.3	34.4 ± 4.7	34.4 ± 3.4	33.6 ± 5.2	32.4 ± 4.2	n.s.
Age of patients at ET, mean ± SD	34.7 ± 4.4	35.4 ± 4.7	35.9 ± 4.3	35.8 ± 4.7	36.7 ± 3.4	36.9 ± 5.2	36.9 ± 4.2	<0.05
No. of embryos warmed, mean/cycle	341 (3.4)	543 (3.2)	325 (3.3)	305 (3.3)	297 (3.3)	98 (3.6)	83 (3.2)	n.s.
ET with top-blastocysts (%)	69 (69.0%)	120 (71.0%)	67 (67.7%)	56 (60.9%)	64 (71.1%)	23 (85.2%)	16 (61.5%)	n.s.

Cryo-cycles were grouped (I–VII) according to the storage time of blastocysts after VIT.

Table II SRs of blastocysts and clinical outcome following transfer of vitrified blastocysts after different storage times.

	Storage groups							*P*-value
	I	II	III	IV	V	VI	VII	
SR	283/341 (83.0%)	488/543 (89.9%)	274/325 (84.3%)	250/305 (82.0%)	243/297 (81.8%)	86/98 (87.8%)	69/83 (83.1%)	n.s.
embryos transferred (*n*)	186 (1.9/ET)	316 (1.9/ET)	186 (1.9/ET)	153 (1.7/ET)	149 (1.7/ET)	47 (1.7/ET)	40 (1.5/ET)	$P < 0.001$
IR (*n*; %)	46 (24.7)	65 (20.6)	36 (19.4)	39 (25.5)	47 (31.5)	15 (31.9)	12 (30.0)	n.s.
PR (*n*; %)	48 (48.0)	65 (38.5)	39 (39.4)	46 (50.0)	48 (53.3)	13 (48.2)	12 (46.2)	n.s.
cPR (*n*; %)	40 (40.0)	51 (30.2)	33 (33.3)	31 (33.7)	43 (47.8)	11 (40.7)	10 (38.5)	n.s.
BR (*n*; %)	33 (33.0)	47 (27.8)	28 (28.3)	30 (32.6)	35 (38.9)	11 (40.7)	7 (26.9)	n.s.

SR, survival rate; IR, implantation rate; PR, pregnancy rate; cPR, clinical PR; BR, birth rate. PR, cPR and BR are calculated 'per cycle'.

Implantation rates

The number of blastocysts transferred per ET decreased with storage time; starting with a mean of 1.9 blastocysts per ET in Groups I–III, the highest percentage of SETs was observed in Group VII with a mean of 1.5 blastocysts per ET ($P < 0.001$; Table II). This observation can be explained by the fact that there was an increased percentage of patients who had already given birth in the groups with longer storage time and who accepted an SET (see green bars in Fig. 1). This transfer strategy led to an increased IR per blastocyst transferred in groups V–VII, although the differences were not statistically significant. Furthermore, the IR might be elevated additionally because there were more patients with a good prognosis.

Pregnancy and birth rates

Regarding the clinical outcome, no difference in the PR between the groups was observed, and no decline from Group I (48.0%) to Group VII (46.2%) was detected (Table II). A decline in PR in Groups II and III was observed (38.5 and 39.4%, respectively) but this was not significant. The same deviations were observed in the cPR. Whereas Groups I, VI and VII showed a very similar cPR of 40.0, 40.7 and 38.5%, respectively, Groups II, III and IV reached only 30.2, 33.3 and 33.7%, respectively. To find an explanation for this observation, we analyzed patient history and found that the decline in cPR in Groups II, III and IV could be due to the existence of a higher proportion of patients with lower implantation potential in those groups (Fig. 1). In these groups a high percentage of patients with implantation failure (blue bars), or either biochemical pregnancy (bPR) or miscarriage (yellow bars) were experienced in the fresh cycle. However, with respect to BR, there was no significant difference between the groups with prolonged storage time, although a small decline in Groups II, II and VII was found. Similarly, no significant differences in miscarriage rates were observed between groups.

Weight, gestational age and malformation rate of children born

In Table III, the number of babies born, multiple BRs and conspicuous medical notes are listed. Two severe cases of multiple malformations were reported. In Group IV, one girl was born with major malformations, including stenosis, polysyndactyly with six toes, a palatine cleft and ventricular septum defects. A deletion on chromosome 7 was diagnosed.

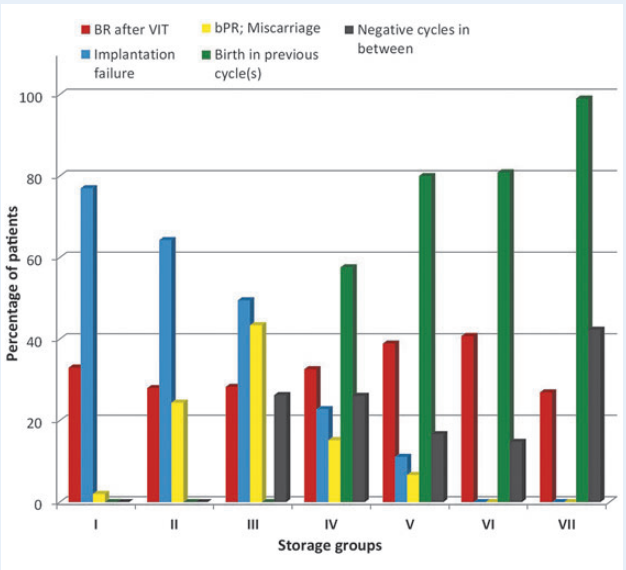

Figure 1 BR after human blastocyst VIT and history of previous cycle(s). Storage groups I–VII represent increasing periods of time for blastocyst storage in LN_2 (see Table I for storage times). Implantation failure, biochemical prengnacy rate (bPR) and miscarriage refer to the fresh cycle. Birth in previous cycle(s) refers to the fresh cycle, while 'negative cycles in between' refers to patients with at least one failed cycle in between fresh and cryo-embryo transfer.

The child died at 4 weeks of age. The second case was reported in Group VII; namely, a case of induced abortion after diagnosis of multiple malformations, including hydronephrosis, club feet and a single umbilical artery. In this case, no genetic background for the malformations was found. One single major malformation was reported, a girl born with a cleft palate after twin pregnancy in Group VI. Two induced terminations of pregnancies due to trisomy 21 were reported, both in Group II. In addition, two stillbirths of singletons were reported, both in Group I. One occurred in gestation Week 34 in a 37-year-old patient with a history of preterm delivery of a healthy child in gestation week 33 in a previous cycle. In a subsequent cycle, the same patient delivered a healthy child in

Table III Characteristics of the children born.

	Group						
	I	II	III	IV	V	VI	VII
Children born	46	55	30	35	37	12	9
Singletons	20	41	26	26	33	10	5
Multiples	26	14	4	9	4	2	4
Medically induced abortions	–	2[a]	–	–	–	–	1[b]
Stillbirths	2	–	–	–	–	–	–
Single malformation	–	–	–	–	–	1[c]	–
Multiple malformations	–	–	–	1[d]	–	–	–

Singletons and multiples born after blastocyst VIT.
[a]Both due to trisomy 21.
[b]After diagnosis of multiple malformations
[c]One twin child born with cleft palatine.
[d]Child born with multiple malformations, died at the age of 4 weeks.

Table IV Obstetric outcome after VIT of blastocysts.

Parameter	Singleton gestation	Multiple gestation
Live birth	147/147 babies	38/79 babies
Male (*n*)	67 (45.6%)	41 (51.9%)
Female (*n*)	80 (54.4%)	38 (48.1%)
Mean birthweight (g) ± SD	3400.01 ± 562.48	2508.13 ± 406.45
Mean gestation age ± SD	39.07 ± 1.89	35.97 ± 2.05
Very low birthweight (<1500 g)	2 (1.4%)	1 (1.3%)
Low birthweight (<2500 g)	2 (1.4%)	38 (48.1%)
Birthweight (2500–4000 g)	124 (84.3%)	40 (50.6%)
Birthweight (>4000–4499 g)	16 (10.9%)	–
Birthweight (>4500 g)	3 (2.0%)	–
Mean gestation age ± SD	39.07 ± 1.89	35.97 ± 2.05
Extreme preterm birth (<28 weeks)	1 (0.7%)	–
Very preterm birth (<32 weeks)	1 (0.7%)	1 (2.6%)
Preterm birth (< 37 weeks)	13 (8.8%)	19 (50.0%)
Term birth	132 (89.8%)	18 (47.4%)

All children born after VIT were analyzed according to birthweight and gestational age.

the 38th week of gestation. No medical reason for the stillbirth was found. In the second case, the stillbirth took place in the 24th week of gestation in a 30-year-old woman, and here also, no medical indication for the event in either mother or child was found. These were the only cases of stillbirths reported in our study.

For all babies born, without reference to storage time, the birthweight and gestational age were analyzed (Table IV). Among all singleton babies born after VIT, four were born with very low or low birthweight. On the other hand 16 singletons weighed >4000 g, and 3 were born with macrosomia (>4500 g). One singleton was born extremely preterm (before gestational Week 28) and one very preterm, before Week 32 after pre-eclampsia but both are developing very well. With regard to multiple births, only one child was born with a very low birthweight and one very preterm delivery was reported. Hence, weight and gestational age were directly compared in siblings born after fresh ET, and then after the subsequent ET of VIT blastocysts. Forty-two patients were identified with this pattern (Table V). The rate of males born from fresh ET tended to be higher than those born from VIT blastocysts (57.1 versus 47.2%, NS). With a similar gestational age, the mean birthweight was slightly lower after fresh ET (3198 versus 3406 g, respectivley) but this was also not statistically significant.

Discussion

In this study we found that there were no significant differences in PR or BR after transfer from vitrified blastocysts in relation to storage time. A period of up to 6 years in the LN_2 tank was evaluated. This result suggests that VIT is a safe method for the cryopreservation of human embryos for a longer time period, which is of particular interest, for example in the preservation of fertility in specific groups of patients.

The aim of cryopreservation in ART is to preserve gametes or embryos for long periods, making their stability over time an important issue. Even though VIT is widely applied today, to the best of our knowledge, this is the first wider study to analyze whether the prolonged storage time of VIT blastocysts in LN_2 impairs SR, PR and BR, or influences malformation rate and weight of children born. The question of the stability over time of an object which has undergone VIT, even at very low temperatures, is reasonable as the VIT state is the solidification of a fluid without formation of crystalline structures in a disorganized unstable state. In this state, structural changes are more likely to occur, increasing the risk for cryopreserved gametes or embryos.

The single serious risk in SF of embryos is the accumulation of radiation over time; however, radiation doses equivalent to 2000 years normal background radiation levels did not impair mouse embryo survival or increase the incidence of mutations in offspring (Whittingham, 1977; Glenister *et al.*, 1984, 1990). Reports on the effect of storage time are still controversial: whereas one study found no reduction in PR over time, others published a tendency towards lower PR with prolonged cryopreservation (Schalkoff *et al.*, 1993; Machtinger *et al.*, 2002). A decline in SR after 6–15 months of storage was observed (Testart *et al.*, 1987).

For SF embryos it was shown that the risk of birth defects is comparable with fresh ET (Wada *et al.*, 1994; Wennerholm *et al.*, 2009; Li *et al.*, 2010). However, other reports have shown a significantly higher rate of malformation after frozen ET (Belva *et al.*, 2008). Regarding VIT, data are still limited. In summary eight publications on VIT blastocysts, amongst them four case reports, showed no statistically significant differences in mean gestational age, birthweight, preterm birth or congenital birth defects compared with fresh ET (summarized in Wennerholm *et al.*, 2009).

In VIT, additional risks due to use of higher dosages of CP are to be considered, although EG often replacing PrOH seems to have a lower genotoxicity (Aye *et al.*, 2010; Berthelot-Ricou *et al.*, 2011; Lawson *et al.*, 2011). In a recent study testing the intracellular CP concentrations it was shown that only a small quantity of CP crosses the cell membrane (Vanderzwalmen *et al.*, 2013). A second potential risk in VIT when using

Table V Comparison of siblings born after fresh ET and VIT.

Parameter	Singleton gestation after fresh IVF cycle	Singleton gestation after VIT	*P*-value[a]
Cycles	42		
Male (*n*)	24 (57.1%)	20 (47.6%)	
Female (*n*)	18 (42.9%)	22 (52.4%)	
Mean birthweight (g) ± SD	3198.1 ± 436.2	3405.5 ± 548.6	n.s.
Low birthweight (<2500 g)	4 (9.5%)	2 (4.8%)	
Birthweight (2500–4000 g)	37 (88.1%)	34 (80.9%)	
Birthweight (>4000–4499 g)	1 (2.4%)	4 (9.5%)	
Birthweight (>4500 g)	–	2 (4.8%)	
Mean gestation age ± SD	38.6 ± 1.5	38.8 ± 1.8	n.s.
Very preterm birth (<32 weeks)	–	1 (2.4%)	
Preterm birth (<37 weeks)	4 (9.5%)	2 (4.8%)	
Term birth	38 (90.5%)	39 (92.8%)	

Forty-two patients in all groups (I–VII) first gave birth to a singleton in the fresh cycle and then to a sibling singleton in the following cryo-cycle, after transfer of VIT blastocysts.
[a]Birthweight and gestational age among the siblings were compared.

open systems are physico-chemical reactions with, for example, reactive oxygen and nitrogen compounds present in the LN_2 and the risk of cross contamination. This can be avoided by using closed devices (Vanderzwalmen *et al.*, 2009, 2012). Finally, concerns arise about the stability of the glassy state [especially near the glass transition temperature (Tg)], which is particularly advantageous for reducing thermo-mechanical stresses that can cause fracturing. Furthermore, considerable molecular mobility persists near and under Tg. Residual molecular mobility below the Tg allows glass to very slowly contract, release heat and decrease entropy during relaxation toward equilibrium. Although diffusion is practically non-existent below Tg, small local movements of molecules may have consequences for cryobiology (Wowk, 2010). Recent studies found time-dependent deterioration in seeds, possibly owing to a degradation mechanism driven purely by vibrations of adjacent molecules (Walters *et al.*, 2004; Ballesteros and Walters 2007).

In our analysis, we found no negative effects of prolonged storage for up to 6 years on SR. The viability of blastocysts was analyzed 3 h post-warming and ranged between 82.0 and 89.9%. This observation is in accordance with a study on VIT porcine embryos that reported no impairment of embryo viability after 3 years of storage in LN_2 (Sánchez-Osorio *et al.*, 2010). Similarly, Eum *et al.* (2009 reported no differences between VIT or SF mouse embryos after storage in LN_2 vapour tanks; however, this group analyzed storage only up to 6 months using open VIT devices (Eum *et al.*, 2009).

In addition, we evaluated the clinical outcome after ET of blastocysts following VIT in relation to storage time. No significant differences over time in cPR, PR, BR or MR were observed, although cPR, PR and BR were reduced in storage Groups II, III and IV (6 months, 1 year and 2 years, respectively) compared with very short and prolonged storage times. This effect was not related to time but rather was due to the characteristics of patients in the different groups. Women with a successful pregnancy in the fresh cycle showed up later for cryo-ET compared with women who experienced no implantation or had an early abortion after fresh ET.

The finding that the implantation potential of VIT blastocysts was not reduced with prolonged storage time is underlined by the fact that there was an increase in IR with longer storage in LN_2. This observation can be explained by the increase in women with 'good implantation prognosis' (Fig. 1) in the groups with prolonged storage time, and by a reduction in the number of blastocysts transferred per ET. Larger-scale studies on the effect of storage after VIT on blastocysts in IVF are still missing. However, our findings are in line with a recent study on rabbit embryos, which showed that embryos cryopreserved using VIT can be stored in LN_2 for at least 15 years, without impairing PR, fertility or survival at birth (Lavara *et al.*, 2011).

Although thousands of healthy babies have been born after SF or VIT, there are still concerns about the safety of these IVF procedures in terms of the health of the children born (Mukaida *et al.*, 2003; Noyes *et al.*, 2009). In many studies, the risk of birth defects associated with the transfer of SF embryos was found to be comparable with fresh ET.

Similarly, reports on VIT of cleavage-stage embryos are promising. In a recent study, the neonatal outcome of 196 ETs after VIT of cleavage-stage embryos was compared with 500 fresh ETs (Aflatoonian *et al.*, 2010). Although the authors found a statistically significant higher abortion rate after VIT (14.5 versus 9%), the neonatal outcome was comparable for both groups in terms of prematurity, birthweight, stillbirth, neonatal death and major malformations.

In our study we did not find statistically significant differences in BR or abortion rate after VIT of blastocysts during the analyzed time period of 6 years. The overall abortion rate was 4.3% (after detection of a positive heartbeat). The incidence of stillbirth was 2 in 191 births. Severe malformations were reported in three terminated pregnancies and multiple malformations in one live birth and a single malformation in 1 child out of 224 children. Taking together the stillbirths, medically induced abortion and all malformations in children born, a total malformation rate of 3.1% was observed. In comparison, in fresh cycles after ICSI a malformation rate of 4.2% was reported and after transfer of slow frozen embryos a rate of 8.4% was observed (Bonduelle *et al.*, 2002; Belva *et al.*, 2008). Our findings correspond well with an earlier report, which showed that VIT of blastocysts had no adverse effect on neonatal outcome compared with fresh blastocyst ET or SF cleavage-stage embryos (Wikland *et al.*, 2010).

In the literature, there is an accumulation of evidence showing that the rate of children born large for gestational age or even with macrosomia increases after SF, most likely due to epigenetic changes associated with the freeze/thaw procedure (Sazonova *et al.*, 2013). In our study, three singletons were born with a birthweight >4500 g, giving a rate of 2% for singletons and thereby much lower than the reported 4.5% for SET after SF or 4.3% after ET of two SF embryos as reported by Sazonova *et al.* (2013). Even for fresh ET, Sazonova *et al.* (2013) reported a higher percentage of macrosomia children with 2.9% after SET and 3.3% after DET.

Even though VIT is widely applied today, to the best of our knowledge, this is the first broader study to evaluate whether blastocyst VIT followed by a prolonged storage time in LN_2 impairs SR, PR, BR or the birthweight and malformation rate in children born. Our results are reassuring in that storage time did not have a negative impact on these outcomes. However, it has to be kept in mind that our data only included blastocysts stored in closed devices that avoided any direct contact with LN_2 during the actual VIT procedure and the storage period. This hermetical protection is also achieved in SF. The reassuring results from our analysis, showing no negative impact of storage for up to 6 years, might not apply directly to VIT in open straws where this hermetic protection of the embryos is not given. In open straws, the constant contact with LN_2 and reactive chemical compounds leads to an increased risk of damage.

In our study, neither SR nor the developmental potential of blastocysts transferred after VIT was impaired by storage time for up to 6 years. Additionally, no indication of a potential risk to BR, birthweight or malformation rate in children born after blastocyst VIT was observed. These results indicate that VIT in closed carriers is a safe technique with regard to storage time. Further analyses are necessary to evaluate the long-term health of infants born following blastocyst VIT plus long-term storage.

Authors' roles

B.W., M.B., B.B., A.N., D.S., A.S. and M.Z. collected the data. B.W. and P.V. designed the study, interpreted the data and wrote the manuscript. B.W. performed the statistical analysis of the data. M.S., N.H.Z. and D.Sp. reviewed the manuscript.

Funding

No external funding was sought for this study.

Conflict of interest

There are no conflicts of interest to declare.

References

Aflatoonian A, Moghaddam Mansoori F, Mashaekhy M, Mohamadian F. Comparison of early pregnancy and neonatal outcomes after frozen and fresh embryo transfer. *J Assist Reprod Genet* 2010;**27**:695–700.

Aye M, Di Giorio C, De Mo M, Botta A, Perrin J, Courbiere B. Assessment of the genotoxicity of three cryoprotectants used for human ooocyte vitrification: dimethylsulfoxide, ethylene glycol and propylene glycol. *Food Chem Toxicol* 2010;**48**:1905–1912.

Ballesteros D, Walters C. Calorimetric properties of water and triacylglycerols in fern spores relating to storage at cryogenic temperatures. *Cryobiology* 2007;**55**:1–9.

Belva F, Henriet S, Van den Abbeel E, Camus M, Devroey P, Van der Elst J, Liebaers I, Haentjens P, Bonduelle M. Neonatal outcome of 937 children born after transfer of cryopreserved embryos obtained by ICSI and IVF and comparison with outcome data of fresh ICSI and IVF cycles. *Hum Reprod* 2008;**23**:2227–2238.

Berthelot-Ricou A, Perrin J, Di Giorgio C, De Meo M, Botta A, Courbiere B. Comet assay on mouse oocytes: an improved technique to evaluate genotoxic risk on female germ cells. *Fertil Steril* 2011;**95**:1452–1457.

Bonduelle M, Liebaers I, Deketelaere V, Derde MP, Camus M, Devroey P, Van Streiteghem A. Neonatal data on a cohort of 2889 infants born after ICSI (1991–1999) and of 2995 infants born after IVF (1983–1999). *Hum Reprod* 2002;**17**:671–694.

Eum JH, Park JK, Lee WS, Cha KR, Yoon TK, Lee DR. Long-term liquid nitrogen vapor storage of mouse embryos cryopreserved using vitrification or slow cooling. *Fertil Steril* 2009;**91**:1928–1932.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;**73**:1155–1158.

Glenister PH, Whittingham DG, Lyon MF. Further studies on the effect of radiation during the storage of frozen 8-cell mouse embryos at −196°C. *J Reprod Fertil* 1984;**70**:229–234.

Glenister PH, Whittingham DG, Wood MJ. Genome cryopreservation: a valuable contribution to mammalian genetic research. *Genet Res* 1990; **56**:253–258.

Iwatani M, Ikegami K, Kremenska Y, Hattori N, Tanaka S, Yagi S, Shiota K. Dimethyl sulfoxide has an impact on epigenetic profile in mouse embryoid body. *Stem Cells* 2006;**24**:2549–2556.

Lavara R, Baselga M, Vincente JS. Does storage time in LN2 influence survival and pregnancy outcome of vitrified rabbit embryos? *Theriogenology* 2011; **76**:652–657.

Lawson A, Ahmad H, Sambanis A. Cytotoxicity effects of cryoprotectants as single-component and cocktail vitrification solutions. *Cryobiology* 2011; **62**:115–122.

Li H, Qiao J, Chi H, Chen X, Liu P, Ma C. Comparison of the major malformation rate of children conceived from cryopreserved embryos and fresh embryos. *Chinese Med J* 2010;**123**:1893–1897.

Machtinger R, Dor J, Levron J, Mashiach S, Levran D, Seidman DS. The effect of prolonged cryopreservation on embryo survival. *Gynecol Endocrinol* 2002;**16**:293–298.

Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, Takahashi K. Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. *Hum Reprod* 2003;**18**:384–391.

Noyes N, Porcu E, Borini A. Over 900 oocyte cryopreservation babies born with no apparent increase in congenital anomalies. *Reprod Biomed Online* 2009;**18**:769–776.

Revel A, Safran A, Laufer N, Lewin A, Reubinov BE, Simon A. Twin delivery following 12 years of human embryo cryopreservation: case report. *Hum Reprod* 2004;**19**:328–329.

Sánchez-Osorio J, Cuello C, Gil MA, Parrilla I, Alminana C, Caballero I, Roca J, Vázquez JM, Rodríguez-Martínez H, Martínez EA. *In vitro* postwarming viability of vitrified porcine embryos: Effect of cryostorage length. *Theriogenology* 2010;**74**:486–490.

Sazonova A, Källen K, Thurin-Kjellberg A, Wennerholm UB, Bergh C. Neonatal and maternal outcomes comparing women undergoing two *in vitro* fertilization (IVF) singleton pregnancies and women undergoing one IVF twin pregnancy. *Fertil Steril* 2013;**99**:731–737.

Schalkoff ME, Oskowitz SP, Powers RD. A multifactorial analysis of the pregnancy outcome in a successful embryo cryopreservation program. *Fertil Steril* 1993;**59**:1070–1074.

Testart J, Lassalle B, Forman R, Gazengel A, Belaisch-Allart J, Hazout A, Rainhorn JD, Frydman R. Factors influencing the success rate of human embryo freezing in an *in vitro* fertilization and embryo transfer program. *Fertil Steril* 1987;**48**:107–112.

Vanderzwalmen P, Hiemer A, Rubner P, Bach M, Neyer A, Stecher A, Uher P, Zintz M, Lejeune B, Vanderzwalmen S *et al.* Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. *Reprod Biomed Online* 2008;**17**:617–627.

Vanderzwalmen P, Ectors F, Grobet L, Prapas Y, Panagiotidis Y, Vanderzwalmen S, Stecher A, Frias P, Liebermann J, Zech N. Aseptic vitrification of blastocysts from infertile patients, egg donors and after IVM. *Reprod Biomed Online* 2009;**19**:700–707.

Vanderzwalmen P, Zech NH, Ectors F, Stecher A, Lejeune B, Vanderzwalmen S, Wirleitner B. Blastocyst transfer after aseptic vitrification of zygotes an approach to overcome an impaired uterine environment. *Reprod Biomed Online* 2012;**6**:591–599.

Vanderzwalmen P, Connan D, Grobet L, Wirleitner B, Remy B, Vanderzwalmen S, Zech N, Ectors F. (2013) Lower intracellular concentration of cryoprotectants after vitrification than after slow freezing despite exposure to higher concentration of cryoprotectant solutions. *Hum Reprod* 2013;**28**:2101–2110.

Wada I, Macnamee MC, Wick K, Bradfield JM, Brinsden PR. Birth characteristics and perinatal outcome of babies conceived from cryopreserved embryos. *Hum Reprod* 1994;**9**:543–546.

Walters C, Wheeler L, Stanwood PC. Longevity of cryogenically stored seeds. *Cryobiology* 2004;**48**:229–244.

Wennerholm UB, Söderström-Anttila V, Bergh C, Aittomäki K, Hazekamp J, Nygren KG, Selbing A, Loft A. Children born after cryopreservation of embryos or oocytes: a systematic review of outcome data. *Hum Reprod* 2009;**24**:2158–2172.

Whittingham DG. Fertilization *in vitro* and development to term of unfertilized mouse oocytes previously stored at −196°C. *J Reprod Fertil* 1977;**49**:89–94.

Wikland M, Hardarson T, Hillensjo T, Westin C, Westlander G, Wood M, Wennerholm UB. Obstetric outcomes after transfer of vitrified blastocysts. *Hum Reprod* 2010;**25**:1699–1707.

Wowk B. Thermodynamic aspects of vitrification. *Cryobiology* 2010;**60**:11–22.

Zech NH, Lejeune B, Puissant F, Vanderzwalmen S, Zech H, Vanderzwalmen P. Prospective evaluation of the optimal time for selecting a single embryo for transfer: day 3 versus day 5. *Fertil Steril* 2007;**88**:244–246.