Outcomes of closed blastocyst vitrification in relation to blastocyst quality: evaluation of 759 warming cycles in a single-embryo transfer policy


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BACKGROUND: In order to optimize blastocyst cryopreservation, vitrification was introduced as the routine procedure instead of slow freezing. The outcome of a closed blastocyst vitrification system was evaluated in relation to the blastocyst score before cryopreservation in single embryo transfers (SETs).

METHODS: Supernumerary blastocysts of IVF/ICSI patients with a fresh Day 5 transfer were vitrified using CBS-VIT High Security (HS) straws. In 759 warming cycles, morphological survival and transfer rates were assessed in relation to the blastocyst score and the day of vitrification. Pregnancy rates were assessed in 530 SET and 156 double embryo transfer (DET) cycles. Implantation rates per embryo transferred in SET cycles were analysed according to blastocyst quality and day of cryopreservation.

RESULTS: Immediate morphological survival was 77.8% (921/1185) and the transfer rate per warmed blastocyst was 70.7% (838/1185). Survival rates were higher for Day 5 early blastocysts (86.7%) compared with full (78.7%) or expanded blastocysts (72.7%). A reduced survival rate of 70.1% was found for Day 6 blastocysts compared with Day 5 blastocysts (80.6%, P < 0.001). The overall clinical/ongoing pregnancy rate after SET was 16.4/14.2% and 24.4/20.5% after DET with an ongoing multiple pregnancy rate of 21.8% (7/32) after DET. Significantly lower implantation rates were found for Day 5 early blastocysts (10.6%) compared with advanced blastocysts (17.5%, P < 0.05). Similar implantation potentials for Day 5 and 6 blastocysts (14.3 versus 13.7%) were found.

CONCLUSIONS: Successful cryopreservation of blastocysts from the early cavitating up to expanded blastocyst stages is possible using a closed HS device. The choice between single or double frozen blastocyst transfer should depend on blastocyst expansion after vitrification.

Key words: IVF / vitrification / blastocyst / cryopreservation / SET

Introduction

A better implantation potential of fresh blastocysts compared with cleavage-stage embryos has been reported in several studies (Gardner et al., 1998; Marek et al., 1999; Milki et al., 2000; Langley et al., 2001; Papanikolaou et al., 2005). Especially in younger patients, single Day 5 blastocyst transfer has been recommended (Papanikolaou et al., 2006). The contribution of cryopreservation in terms of cumulative reproductive outcome is considered even more important in single-embryo transfer (SET) practice. The proportion of SETs has increased substantially at our centre (Van Landuyt et al., 2006) in view of a government-controlled programme in order to reduce multiple pregnancies. Consequently, the impact of the cryopreservation programme on the IVF/ICSI success rate has gained importance since more embryos become available for cryopreservation. Furthermore, the application of SET is now becoming common practice in transfer of frozen—thawed embryos as well. Successful slow freezing and thawing of blastocysts with high survival rates (from 76 up to 94%), originating from both Day 5 and Day 6 good-quality blastocysts, has been reported in several studies (Langley et al., 2001; Behr et al., 2002; Anderson et al., 2003; Veeck et al., 2004; Desai and Goldfarb, 2005; Liebermann and Tucker, 2006).
Slow freezing was shown to be successful also when unselected blastocysts at all developmental stages, i.e. compacted, expanded and hatching blastocysts, were cryopreserved (Virant-Klun et al., 2003). Results of our slow-freezing programme for blastocyst cryopreservation have been reported by Van den Abbeel et al. (2005). In this study, two blastocyst slow-freezing strategies were evaluated in a Day 2/3 fresh embryo transfer programme. It was found that early blastocysts showed a better morphological survival than advanced or hatching blastocysts, but this benefit was lost by a lower in vitro developmental capacity. An overall morphological survival rate of 71.9% was obtained. In the case of blastocyst cryopreservation, one is confronted with a cohort of blastocysts at different developmental stages to be frozen, ranging from early cavitating to expanded and hatching blastocysts. Because of the complex structure of blastocysts containing inner cell mass (ICM) cells and a fluid-filled cavity that is surrounded by a barrier of trophectoderm cells, the cryopreservation of expanded blastocysts remains a challenge. Therefore, in order to optimize blastocyst cryopreservation outcome at our centre, slow freezing was replaced by vitrification. Since the pioneering reports on the vitrification of mouse embryos (Rall and Fahy, 1985), this method has been modified towards using minimal volume methods (Vajta et al., 1997; Lane et al., 1999; Kuwayama et al., 2005a,b) and has as such become an interesting alternative to slow freezing for cryopreservation of mammalian embryos, as vitrification has many advantages: it does not require ice-seeding, slow cooling rates or a programmable freezer, and thus cooling is instant. Vitrification is expected to achieve a high rate of survival because of the absence of ice. This is crucial for the cryopreservation of full and expanded blastocysts which are less permeable to water and have a fluid-filled cavity very sensitive to lethal ice crystal formation (Tucker and Liebermann, 2007). Therefore, in the literature, vitrification has become the method of choice to cryopreserve blastocysts (Cho et al., 2002; Son et al., 2003; Mukaida et al., 2003, 2006; Vanderzwalmen et al., 2003; Kuwayama et al., 2005a,b; Liebermann and Tucker, 2006; Stachecki et al., 2008).

Although so far there are no proven viral transmissions between embryos or to the patient at embryo transfer, the use of an open system for vitrification potentially introduces a risk of cross-contamination during liquid nitrogen storage (Bielanski and Vajta, 2009). Therefore, a feasible closed method vitrification system was preferentially introduced in our unit. It was reported in the review by Bielanski and Vajta (2009) that regarding plastic straws, it appears that the CBS High Security Straws (CryoBioSystem, Paris, France) which are made from ionomeric resin are the most suitable storage containers for cryopreservation and storage of germplasm. These straws are heat sealed on both ends and are impermeable to pathogens. Their safety for HIV-1 contamination under cryopreservation conditions has been examined by Letur-Könirsch et al. (2003). Ionomeric resin straws appeared to be safe in contrast with polyvinylchloride and polyethylene terephthalate glycol straws which could probably be attributed to splashing during ultrasonic sealing. Therefore and because of its validation in a preclinical study at our centre (Guns et al., 2008, abstract nr O-134 ESHRE), the closed vitrification system using CBS straws, with dimethylsulphoxide (DMSO) and ethylene glycol as the cryoprotectants, was chosen as the vitrification system for routine clinical use.

The aim of the present study was to assess the efficiency of closed blastocyst vitrification in relation to embryo development at the time of cryopreservation and clinical transfer practice in a large series of consecutive vitrification cycles. The survival after warming and the transfer rates of Day 5 and 6 vitrified blastocysts of different morphological quality and at different developmental stages were analysed. Clinical and ongoing pregnancy rates were assessed in both SET and DET cycles. In SET, the implantation rate was assessed in relation to the day of vitrification and the blastocyst quality on Day 5.

### Materials and Methods

#### Patients

Blastocyst vitrification was implemented at our centre in March 2008 for patients with a fresh Day 5 blastocyst transfer. All blastocyst warming cycles (n = 759) of IVF/ICSI patients performed between April 2008 and February 2010 were analysed retrospectively. The mean female age was 31.5 years (range 22–42 years).

#### Ovarian stimulation and oocyte retrieval

In the fresh cycle, female patients underwent ovarian stimulation using uninary (Menopur, Ferring Pharmaceuticals A/S, Copenhagen, Denmark) or recombinant FSH (Puregon, NV Organon, Oss, The Netherlands; Gonal F, Merck-Serono, Geneva, Switzerland) in combination with GnRH antagonist (Orgalutran, NV Organon) or agonist (Suprefact, Aventis Pharma, Frankfurt, Germany). Final oocyte maturation was induced by injection of 10 000 IU hCG (Pregnyl; Schering-Plough, Oss, The Netherlands, or Profasi, Merck-Serono), as soon as three follicles of 17 mm were seen on ultrasound. Oocyte retrieval was carried out using vaginal ultrasound-guided puncture of ovarian follicles 36 h after hCG administration.

#### IVF/ICSI treatment, embryo culture and embryo selection

IVF and ICSI procedures were carried out as described by Van Landuyt et al. (2005). For *in vitro* culture of oocytes and embryos, sequential media formulations were used and embryos were cultured at 37°C in an atmosphere of 6% CO₂, 5% O₂ and 89% N₂. Oocytes and embryos were cultured individually in 25 μl medium droplets covered with mineral oil, from oocyte retrieval (Day 0) until transfer or cryopreservation (Day 5/6). All embryos were evaluated daily.

Embryo selection for transfer or cryopreservation was done in the morning of Day 5. Blastocysts were scored according to the grading system of Gardner and Schoolcraft (1999). Embryos were considered for fresh transfer on Day 5 if they reached the stage of full compaction, early blastocyst (Bl1 or Bl2), full (Bl3), expanded (Bl4) or hatching (Bl5–6) blastocyst. Cavitated blastocysts fulfilled the criteria for transfer if they had at least an ICM type C and trophectoderm quality type B. On Day 5, early or full, expanded and hatching blastocysts with ICM and trophectoderm type A or B (Gardner’s grading system) were considered eligible for cryopreservation. On Day 6, only full, expanded or hatching blastocysts with ICM type A and trophectoderm quality A or B were cryopreserved.

Embryo transfer was performed using a soft catheter (K-Soft 5100, Cook, Brisbane, Australia).

#### Vitrification and warming protocols

Vitrification was performed using closed CBS-VIT High Security (HS) straws (CryoBioSystem) in combination with DMSO-EG-S as the cryoprotectants (Irvine Scientific® Freeze Kit). Blastocysts were vitrified one by one. The vitrification procedure was carried out at room temperature (between 22 and 27°C). The blastocyst was first incubated for 2 min in a 50 μl droplet of HEPES-buffered culture medium. Then, the blastocyst was...
brought in a 50 μl droplet of equilibration solution containing 7.5% (v/v) DMSO and 7.5% (v/v) ethylene glycol and incubated for 10 min. The blastocyst was then transferred consecutively into four 25-μl droplets with vitrification solution containing 15% (v/v) DMSO and 15% (v/v) ethylene glycol. The blastocyst was incubated for 5 s in droplets 1 and 2 and for 10 s in the third droplet. The blastocyst was then transferred to the fourth droplet and immediately loaded onto the CBS-HS straw. The straw was heat sealed and plunged into liquid nitrogen. The total time needed to vitrify the blastocyst starting from the first vitrification droplet to the loading of the straw and plunging into liquid nitrogen did not exceed 90 s.

On the day of transfer, blastocysts were warmed one by one until one or two blastocysts were suitable for transfer. The choice to transfer one or two embryos in the frozen cycle was decided by the clinician at consultation mainly depending on the patient’s age and the number of embryos replaced in the previous treatment cycles. SET was planned in patients younger than 36 years in their first warming cycle after the first fresh treatment cycle. Blastocysts were warmed randomly, independently of the blastocyst stage or quality prior to vitrification. For warming, the Irvine Scientific® Thaw Kit was used. A Petri dish containing two 25 μl droplets with thawing solution [TS; 1 M sucrose in HEPES-buffered human tubal fluid (HTF) medium supplemented with 20% DSS] was kept at 37°C. For warming the straw, the straw was transferred from the LN2 storage container to a transport dewar filled with liquid nitrogen. After cutting the straw and pulling the capillary from the straw, the gutter was placed in the first droplet with TS and the blastocyst was released from the gutter. The blastocyst was incubated for two times 1 min at room temperature in the two TS droplets. The blastocyst was then transferred to the first of two dilution solution (DS) droplets of 25 μl (0.5 M sucrose in HEPES-buffer HTF medium supplemented with 20% DSS) and after that incubated for 2 min in a second DS droplet. Finally, the blastocyst was washed in three droplets (25 μl) of washing solution (HEPES-buffered HTF medium supplemented with 20% DSS), each for 3 min. After warming, the blastocyst was transferred to a culture dish with blastocyst medium to assess its morphological survival. If the blastocyst was severely or completely damaged, a new one was warmed immediately. If the blastocyst was fully intact or showed moderate damage, expansion and re-expansion was assessed 1–2 h later. If the morphological quality of the blastocyst was regressing or no signs of re-expansion were present, an additional blastocyst was warmed until one blastocyst was suitable for transfer, i.e. with good survival (less than half of the blastocyst being intact cells were transferred (633/662, 95.6%) compared with the moderately damaged ones (202/259, 78.0%) with \( P < 0.0001 \). In Table I, the survival and transfer rates are presented according to the day of vitrification and to blastocyst scoring. Survival rates and transfer rates were significantly higher for blastocysts cryopreserved on Day 5 (80.6 and 74.0%) than on Day 6 of embryo culture (70.1 and 62.0%). Early Day 5 blastocysts showed a significantly higher survival rate (86.7%) and transfer rate (81.8%) than blastocysts types 3 (78.7 and 70.6%) and 4 (72.7 and 64.9%). Top-quality full and expanded blastocysts with ICM type A that were cryopreserved on Day 5 had a similar survival and transfer rate compared with Day 5 blastocysts with ICM type B.

**Clinical outcome of 759 warming cycles**

In total, 759 warming cycles were analysed in which 1185 blastocysts were warmed, i.e. a mean number of 1.6 per warming cycle. In Fig. 1, a flow diagram presents the clinical outcome of 759 warming cycles. In 686 cycles, the patient received a transfer. The transfer rate per warming cycle was 90.4% (686/759). SET was performed in 530 warming cycles and DET in 156 warming cycles. The clinical pregnancy rate after vitrified/warmed SET was 16.4% (87/530) and significantly lower than after vitrified/warmed DET 24.4% (38/156, \( P < 0.05 \)). The ongoing pregnancy rates were not significantly different between the two groups (14.2%, 75/530 and 20.5%, 32/156). One monozygotic twin was obtained after SET (1/75, 1.3%). The multiple pregnancy rate after transfer of two vitrified—warmed blastocysts was 21.8% (7/32) per ongoing pregnancy and higher than after SET (\( P < 0.01 \)). The implantation rate (with FHB) per embryo transferred was 14.3% (76/530) after SET and similar to the 12.8% implantation rate after DET (40/312).

The clinical pregnancy rate and implantation rate with FHB according to the day of cryopreservation and to the type of blastocyst cryopreserved in the SET group and are presented in Table II. The implantation rate for Day 5-vitrified blastocysts was 14.3% per transfer and this was not different from the implantation rate of Day 6-vitrified blastocysts (13.7%). Of the blastocysts vitrified on Day 5, early blastocysts resulted in a significantly lower implantation rate of 10.6% than full and expanded blastocysts (17.5%). Day 5 full and expanded blastocysts with ICM type A quality showed an implantation rate of 19.0% per transfer, which was similar to the implantation rate of blastocysts with ICM type B (15.6%). The best results were observed after transfer of Day 5-vitrified advanced blastocysts with ICM/TE type AA with an implantation rate per transferred embryo of 23.9% (16/67).

Furthermore, fully intact blastocysts showed an implantation rate of 16.2% (68/421) per transferred embryo compared with 6.4% (7/109) for moderately damaged blastocysts (\( P < 0.05 \)).

**Results**

**Immediate morphological survival and embryo transfer rates**

In all 759 warming cycles, the overall immediate morphological survival rate (=survival rate) and transfer rate per warmed embryo was 77.8% (921/1185) and 70.7% (838/1185). Out of the 1185 warmed embryos, 662 (55.9%) were fully intact and 259 (21.9%) blastocysts were moderately damaged. Significantly more blastocysts with 100% intact cells were transferred (633/662, 95.6%) compared with the moderately damaged ones (202/259, 78.0%) with \( P < 0.0001 \).

**Outcome parameters**

The embryological outcome measures were the immediate morphological survival after warming (percentage of fully intact and moderately damaged blastocysts/number of warmed blastocysts) and the transfer rate (percentage of blastocysts transferred/number of warmed blastocysts). Immediate morphological survival was evaluated according to the day of vitrification, according to the blastocyst stage prior to vitrification [early (Bl1–2) or advanced blastocysts (Bl3–4)] and according to the blastocyst quality on Day 5 (ICM type A or B).

The following clinical outcome measures were analysed: clinical pregnancy rate per transfer (i.e. a pregnancy with an intratubal gestational sac seen at transvaginal ultrasound scan at least 5 weeks after embryo transfer (Zegers-Hochschild et al., 2009), ongoing pregnancy rate per transfer (i.e. a clinical pregnancy with a fetal heartbeat (FHB) at \( \geq 12 \) weeks (Bonduelle et al., 2002) and implantation rate (with FHB) per transferred embryo.

**Statistics**

Outcome parameters were compared using the \( \chi^2 \) test with the significance level set at \( P < 0.05 \).
**Table I** Morphological survival after warming in 759 warming cycles according to the day of vitrification and according to the blastocyst quality on Day 5.

<table>
<thead>
<tr>
<th>Day of cryopreservation</th>
<th>No. of warmed</th>
<th>No. of survived (%)</th>
<th>No. of transferred (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 5</td>
<td>864</td>
<td>696 (80.6)</td>
<td>639 (74.0)</td>
</tr>
<tr>
<td>Day 6</td>
<td>321</td>
<td>225 (70.1)</td>
<td>199 (62.0)</td>
</tr>
<tr>
<td>Blastocyst stage on Day 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bl1/Bl2</td>
<td>384</td>
<td>333 (86.7)</td>
<td>314 (81.8)</td>
</tr>
<tr>
<td>Bl3</td>
<td>235</td>
<td>185 (78.7)</td>
<td>166 (70.6)</td>
</tr>
<tr>
<td>Bl4</td>
<td>245</td>
<td>178 (72.7)</td>
<td>159 (64.9)</td>
</tr>
<tr>
<td>ICM score on Day 5 (Bl3 and Bl4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICM A</td>
<td>267</td>
<td>204 (76.4)</td>
<td>184 (68.9)</td>
</tr>
<tr>
<td>ICM B</td>
<td>213</td>
<td>159 (74.6)</td>
<td>139 (65.3)</td>
</tr>
</tbody>
</table>

*P < 0.001.

*P < 0.05.

*P < 0.001.

*P < 0.001.

*P < 0.01.

*P < 0.001.

**Figure I** Flow diagram of 759 blastocyst warming cycles in regular IVF/ICSI patients.
Discussion

The aim of the present study was to assess the implementation of a closed vitrification and storage system as the cryopreservation method for Day 5–6 embryos. The choice for closed vitrification was mandatory in order to comply with the European Union directives on tissues and cells storage (European Union, 2006), requiring safe storage of gametes and embryos.

Principle variables of a vitrification procedure consist of cooling and warming rates and cryoprotectants, the probability of vitrification being determined as a correct interplay between cooling and warming rates and the concentration of cryoprotectants. Many of the latest methods used for vitrification revolve around a technique that utilizes minimal volumes of cryoprotectants in order to maximize heat transfer and thus create a very rapid cooling/warming environment. Among these are Cryotop and Cryoloop methods. These methods although very efficient have been brought into question by the possibility of cross-contamination from direct contact with liquid nitrogen. In an experiment to test liquid nitrogen as a vector for contamination, a contaminated tank was used to hold samples in both closed and open systems, showing that the open system tested positive. In an experiment to test liquid nitrogen as a vector for contamination, a contaminated tank was used to hold samples in both closed and open systems, showing that the open system tested positive. Among these are Cryotop and Cryoloop methods. These methods although very efficient have been brought into question by the possibility of cross-contamination from direct contact with liquid nitrogen. In an experiment to test liquid nitrogen as a vector for contamination, a contaminated tank was used to hold samples in both closed and open systems, showing that the open system tested positive.

<table>
<thead>
<tr>
<th>Blastocyst stage on Day 5</th>
<th>% Clin P per ET</th>
<th>% FHB per ET</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1/B2</td>
<td>12.2 (23/189)</td>
<td>10.6 (20/189)*</td>
</tr>
<tr>
<td>B3/B4</td>
<td>20.3 (44/217)</td>
<td>17.5 (38/217)*</td>
</tr>
<tr>
<td>ICM A</td>
<td>20.7 (25/121)</td>
<td>19.0 (23/121)</td>
</tr>
<tr>
<td>ICM B</td>
<td>20.8 (20/96)</td>
<td>15.6 (15/96)</td>
</tr>
</tbody>
</table>

*M p < 0.05

Table II Clinical outcome after SET (n = 530) according to the day of vitrification and blastocyst quality on Day 5.

Morphological survival after warming

The overall morphological survival of the 1185 warmed blastocysts was 77.8%, regardless of their quality or Day of cryopreservation. In our vitrification policy, advanced blastocysts with ICM A and B as well as good-quality early blastocysts on Day 5 of in vitro culture were vitrified. On Day 6, only blastocysts with ICM type A were selected for vitrification. However, the Day 6 vitrified blastocysts always originated from early blastocysts that were not selected for vitrification on Day 5 because of their doubtful quality. Before warming, there was no selection concerning the blastocyst quality, i.e. always the first blastocyst that was vitrified was chosen for warming. Hence, different types of blastocyst qualities could be included in this observational analysis. Early blastocysts survived better than advanced Day 5 blastocysts as was demonstrated in other studies on blastocyst vitrification (Cho et al., 2002; Vanderzwalmen et al., 2002; Mukaida et al., 2003, 2006; Zech et al., 2005; Ebner et al., 2009). The risk of detrimental ice crystal formation is higher in expanded blastocysts compared with early blastocysts due to the large fluid-filled cavity. Furthermore, it has been observed that later blastocyst stages are less permeable to the cryoprotectant (Cho et al., 2002; Vanderzwalmen et al., 2002), with a higher risk of intracellular ice formation. Several groups perform artificial shrinkage to collapse the fluid-filled blastocoelic cavity before vitrification in order to obtain higher survival rates in expanded blastocysts (Vanderzwalmen et al., 2002, 2003; Son et al., 2003; Hiraoka et al., 2004; Mukaida et al., 2006). A survival rate of 78.7 and 72.7% in the present study for Day 5 full and expanded blastocysts in a closed vitrification system without artificial shrinkage is acceptable. However, we recognize that improvements should be made to make the procedure more robust, e.g. increasing the volume of the droplets with warming solution may optimize the warming rate.

A lower survival was observed for Day 6 versus Day 5 blastocysts. However, only full and expanded blastocysts were chosen for vitrification on Day 6 and thus when we compared their survival to Day 5 advanced blastocysts only, the survival rates were similar. These results are in line with the findings of Hiraoka et al. (2004), Stehlik et al. (2005), Liebermann and Tucker (2006) and Liebermann (2009). Compared with the results obtained after slow cooling of blastocysts at our centre reported by Van den Abbeel et al. (2005), we obtained similar survival for Day 5 advanced blastocysts but a significant improvement in survival for Day 6 blastocysts (70.1 versus 55.8%) by performing vitrification. Furthermore, transfer rates per...
warmed embryo were improved both for Day 5 and Day 6 blastocysts when compared with our conventional slow-freezing strategy.

In the literature, other groups have recently moved forward to closed vitrification in order to avoid danger of contamination. In the update on human blastocyst vitrification reported by Liebermann (2009), closed vitrification using CBS HS-VIT straws was used, yielding an excellent survival of 96.8% which was similar to the survival rate obtained with the open CryoTop system (96.3%). Vanderzwalmen et al. (2009) recently developed a closed vitrification technique (Vitri-Safe) to replace their ultrarapid open vitrification procedure (Vanderzwalmen et al., 2002, 2003). It consists of an open hemi-straw that is inserted into an HS 0.3 ml straw (CryoBioSystem) before plunging it into liquid nitrogen. To compensate for the decrease in cooling and warming speed, blastocysts were gradually exposed to increasing concentrations of cryoprotectants. Survival rates of 82% were obtained for couples with male and/or female factor infertility.

Clinical outcome after vitrified blastocyst transfer

The overall ongoing pregnancy rate per transfer was 14.3% after SET and 20.5% after DET and was not significantly different. Note that this is the result of the specific vitrification and warming strategy used for the current observational study in which random selection of embryos for warming was used. Warming the best-quality blastocyst first would probably further increase the immediate additional value of vitrified transfer cycles. These results were obtained in an unselected group of 759 consecutive warming cycles with a large age distribution (up to 42 years) of the patients included. Several other reports document implantation rates after vitrified and warmed blastocyst transfer ranging from 29.0 to 44.6% (Takahashi et al., 2005; 34.7%, Mukaida and Takahashi, 2007; Ebner et al., 2009; Liebermann, 2009) and ongoing implantation rates ranging from 20.7 to 46.2% (Choi et al., 2000; Yokota et al., 2001; Cho et al., 2002; Hiraoka et al., 2004, 2009; Stachecki et al., 2008; Vanderzwalmen et al., 2009). The meta-analysis of Sunkara et al. (2010) suggested that the discrepancy in literature reports can be explained by the clinical heterogeneity among published studies. Liebermann (2009) indicated that clinical success with cryopreservation seems to be highly variable from laboratory to laboratory and may depend on many factors, including patient characteristics, quality of embryos selected for cryopreservation or developmental stage at cryopreservation.

In the present study, the multiple pregnancy rate after transfer of two blastocysts was 21.9% per ongoing pregnancy with random choice of the blastocyst quality being warmed. Patient age was similar in SET and DET cycles but DET was planned according to the patient history (number of previous failed fresh and frozen cycles). The multiple pregnancies occurred in cycles where excellent-quality full or expanded blastocysts that were fully intact after warming were transferred. In order to decrease this relatively high twinning/triplet rate, it should be considered to perform SET or DET according to the quality of the blastocyst at the time of cryopreservation and/or according to its post-warming survival.

Implantation potential of different blastocyst qualities in frozen SETs

Ebner et al. (2009) found that partial damage of the blastocysts (in either ICM or TE) after vitrification did not reduce the implantation potential. However, we found that for blastocysts with moderate damage, the chance of being transferred was significantly reduced because of the lack of re-expansion. Furthermore, the implantation potential was lower compared with that of fully intact blastocysts. This observation would justify transferring two blastocysts when no intact blastocyst is obtained after warming.

Despite the better immediate morphological survival of early blastocysts in the present study, the implantation rate per transferred embryo was lower than for advanced blastocysts. These findings support the warming of two blastocysts instead of one if only early blastocysts were vitrified for the patient and to select at the time of transfer the blastocyst that has most progressed during post-thaw culture.

In the present study, an implantation rate of 13.7% was obtained for Day 6 blastocysts and this was not different from the overall implantation rate of Day 5 blastocysts, which was also observed by other groups (Mukaida et al., 2003; Hiraoka et al., 2004, 2009; Liebermann and Tucker, 2006). The meta-analysis of Sunkara et al. (2010) showed no significant difference in pregnancy rates in studies where blastocysts were frozen at the same developmental stage on Days 5 and 6. In this particular cryopreservation strategy, the additional vitrification of advanced Day 6 blastocysts that derived from early blastocysts with doubtful quality on Day 5 seemed a good approach to maximize the additive value of the supernumerary frozen embryos.

Conclusion

This evaluation of a closed blastocyst vitrification system without artificial shrinkage shows reassuring results for IVF/ICSI patients receiving fresh transfer on Day 5. Successful cryopreservation of early cavitating up to expanded blastocyst stages is possible using a closed HS device. The choice between single or double frozen blastocyst transfer should depend on the degree of survival immediately after warming and on the blastocyst stage at the time of cryopreservation since early cavitating blastocysts showed lower implantation capacity than full and expanded blastocysts. Future strategies to optimize cryosurvival and implantation rates could include the use of increasing concentrations of cryoprotectants or using other aseptic vitrification systems. For instance, the use of a vitrification system in which the device that holds the embryo is wrapped by a pre-cooled straw and where the sealing is performed after vitrification or on the other hand open system vitrification with sterile nitrogen followed by closed storage. In general, to achieve a more robust and efficient vitrification method for embryos or blastocysts, one should carefully investigate the interactions between the cryoprotectant concentration, cooling and warming rates. All experimental protocols should be validated in a preclinical evaluation or randomized controlled comparison before introducing them as the standard vitrification protocol.

Authors’ roles

L.L.: conception and design, acquisition of data, analysis and interpretation of data, writing of the article, critical review of the article and final approval of the version to be published. D.S.: analysis and interpretation of data, critical review of the article and final approval of the version to be published. G.V.: critical review of the article and final approval of the version to be published. W.V.: critical
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