Background: The aim of this study was to analyse the outcome of closed blastocyst vitrification of embryos biopsied at the cleavage stage.

Methods: Vitrification of supernumerary blastocysts was performed using the closed CBS-VIT High Security straws. Warming cycles (n = 100) for patients with preimplantation genetic diagnosis (PGD) and/or aneuploidy screening in the fresh cycle were analysed. The outcome parameters were morphological survival and transfer rates after warming, clinical pregnancy rate and implantation rate (with fetal heart beat). Clinical outcome was compared with two control groups of (i) vitrified/warming transfer cycles without embryo biopsy and (ii) fresh Day 5 transfer of biopsied embryos.

Results: In total, 131 blastocysts were warmed with a morphological survival of 83.2% (109/131) and a transfer rate of 79.4% (104/131). Day 5 blastocysts survived significantly better (90.4%) than Day 6 blastocysts (70.8%, P < 0.01). No difference in survival rate was observed between early cavitating (89.2%) and full/expanded blastocysts (93.3%). In nine cycles, no blastocyst was available for transfer. The clinical pregnancy rate was 19.2% (15/78) after single-embryo transfer (SET) and 38.5% (5/13) after double-embryo transfer (DET). In SET, the implantation rate for blastocysts frozen on Day 5 was 13.7% (7/51), which was not different from the implantation rate of Day 6 blastocysts (18.5%, 5/27). The overall implantation rate of vitrified PGD biopsied blastocysts (14.4%) was comparable with that of vitrified blastocysts without biopsy (20.4%), but lower than the implantation rate obtained in the fresh PGD cycles (24.4%).

Conclusion: Blastocysts on Day 5 and Day 6 of development derived from biopsied embryos can be successfully vitrified using a closed system.

Key words: preimplantation genetic diagnosis / vitrification / blastocyst / cryopreservation / single embryo transfer

Introduction

Preimplantation genetic diagnosis (PGD) allows the genetic diagnosis of embryos before transfer and freezing for couples who are at risk of transmitting a serious genetic disorder to their children (Handyside et al., 1990; Verlinsky et al., 1990). Since the start of the PGD programme in Brussels in 1992, the number of indications for PGD and the number of patients treated have increased substantially, and cryopreservation of supernumerary biopsied embryos is an essential part of the programme. Since only genetically normal embryos can be considered for transfer and freezing, the number and the choice of embryos available for transfer and freezing are limited when compared with regular IVF/ICSI cycles. Embryos with an opened zona pellucida and with one or two blastomeres removed may be more susceptible to damage during freezing and thawing. The need for an optimized cryopreservation programme for patients undergoing PGD is therefore of crucial importance.

After standard slow freezing of mouse embryos, lower survival rates have been reported for cleavage-stage biopsied embryos compared with non-biopsied embryos (Krzyminska and O’Neill, 1991). This lower survival rate however could not be confirmed in the study of Liu et al. (1993) as the survival of biopsied mouse embryos was not different from non-biopsied embryos and independent of the number of blastomeres biopsied. In human embryos, the studies of Joris et al. (1999) and Magli et al. (1999) showed lower success rates after cryopreservation of biopsied versus non-biopsied embryos. In our PGD and aneuploidy screening programme, only five children were born so far after slow freezing of biopsied embryos at the blastocyst stage (personal communication). The first birth after human blastocysts vitrification of biopsied embryos for
Materials and Methods

Patient population and oocyte collection cycle characteristics

Blastocyst vitrification was performed in the Brussels Centre for Reproductive Medicine in March 2008 for patients with fresh Day 5 blastocyst transfer, including patients having embryos biopsied on Day 3. The oocyte collection cycle characteristics were assessed for all PGD cycles (n = 941) performed between March 2008 and January 2010. The mean age of the patients was 32.9 ± 4.7 years. The number of cumulus-oocyte-complexes retrieved was 12.8 ± 7.1, the mean number of embryos biopsied was 5.3 ± 3.6. The mean embryo cryopreservation rate per fertilized oocyte in all 941 fresh cycles with embryo biopsy was 3.5% ± 8.8. In 18.0% (169/941) of fresh cycles, at least one blastocyst could be cryopreserved. Between April 2008 and February 2010, 100 consecutive blastocyst warming cycles that originated from 72 fresh treatment cycles with embryo biopsy were performed. The mean age of the patients was 30.5 ± 3.4 years at fresh treatment cycle and 30.7 ± 3.4 years at frozen embryo transfer. In the 72 fresh cycles, the mean numbers of cumulus-oocyte-complexes, oocytes fertilized, embryos biopsied and blastocysts vitrified were 19.4 ± 10.8, 12.3 ± 6.2, 9.5 ± 4.6 and 2.3 ± 1.6, respectively. In total, 164 blastocysts were vitrified of which 131 were warmed in 100 warming cycles.

The indications for PGD were as follows: 47 cycles with an autosomal dominant disorder, 16 cycles with an autosomal recessive disorder, 7 and 17 cycles with an X-linked dominant and X-linked recessive disease and 2 cycles with translocations. Furthermore, 11 cycles with aneuploidy screening were included.

Control groups

In order to compare the clinical outcome obtained after vitrified blastocyst transfer of biopsied embryos (=study group), two control groups were retrospectively analysed: (i) vitrified /warmed blastocyst transfer without embryo biopsy and (ii) fresh blastocyst transfer of biopsied embryos. These patient groups were control-matched for age and number of biopsied embryos transferred and were treated during the same study period.

Ovarian stimulation and oocyte retrieval

Female patients underwent ovarian stimulation using urinary (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 agonist (Orgalutran, NV Organon, Oss, The Netherlands) 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, Geneva, Switzerland), when at least 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.

ICSI, embryo culture and embryo biopsy

The ICSI procedure was carried out as described previously by Joris et al. (1998). 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 the day of oocyte retrieval (Day 0) until the day of cryopreservation (Day 5/6). The embryos were evaluated daily.

Embryos were selected for biopsy in the morning of Day 3. Embryos from the 5-cell stage onwards for FISH analysis and embryos from the six-cell stage onwards for PCR analysis with <50% fragmentation were biopsied using a 1.48-μm diode laser as previously described by De Vos et al. (2009). Embryos were biopsied in G-PGD decompensation medium (Vitrolife, Kungsbacka, Sweden).

Embryo selection for transfer and freezing

Embryo selection for transfer or cryopreservation was done in the morning of Day 5. Blastocysts were graded according to the scoring system of Gardner and Schoolcraft (1999). Blastocysts that were artificially hatching through the biopsy hole were scored as BL7. Embryos were suitable for fresh transfer on Day 5 if they were compacted embryos, early blastocysts (BL1 or BL2), full (BL3), expanded (BL4) or hatching (BL7) blastocysts. Advanced and hatching blastocysts were transferred if they had at least an inner cell mass (ICM) type C and trophoderm quality type B. Embryo transfer was performed using a soft catheter (K-Soft S100, Cook, Brinsbane, Australia).

Early blastocysts were cryopreserved on Day 5. Early blastocysts of doubtful quality (e.g. when presenting small cell numbers, fragments or cells not participating in the blastocyst formation) were not frozen on Day 5 but left in culture for re-evaluation on Day 6. Full, expanded and artificially hatching blastocysts with ICM and trophoderm type A or B
Results

Immediate morphological survival and embryo transfer rates

In 100 warming cycles of biopsied embryos, 131 blastocysts were warmed, i.e. 1.3 per cycle (Fig. 1). The mean number of blastocysts warmed was 1.2 in SET cycles and 2.3 in DET cycles. The overall immediate morphological survival after warming was 83.2% (109/131). In Table I, the survival and transfer rates are presented according to the blastocyst quality. Significantly more blastocysts survived and were transferred if cryopreserved on Day 5 (survival, 90.4%) compared with blastocysts cryopreserved on Day 6 (survival, 70.8%, P < 0.01) (Table I). For blastocysts frozen on Day 5, early cavitating blastocysts (Bl1 and Bl2) showed a similar survival and transfer rate as full or expanded blastocysts (89.2% and 89.2 versus 93.3% and 86.7%). Of the 45 full or expanded blastocysts, 39 were artificially hatching (BL7) at the time of cryopreservation on Day 5. The survival and transfer rates of blastocysts with ICM type A (90.0 and 85.0%) were not different from the blastocysts with ICM type B (96 and 84.9%).

Clinical outcome of vitrified—warming cycles of biopsied embryos

In Fig. 1 a flow diagram is presented of the 100 warming cycles of patients who underwent PGD or aneuploidy screening in the fresh cycle. In nine patients, no blastocyst was available for transfer after warming. These patients had only one blastocyst vitrified. The transfer rate per warming cycle was 91% with 78 SET and 13 DET cycles. In 71 out of 78 SET cycles, it was planned to perform a single-embryo transfer. In the other seven cases, a DET cycle was intended but only one blastocyst was transferable after warming. The clinical and ongoing pregnancy rate was 19.2% (15/78) and 15.4% (12/78) after SET and 38.5% (5/13) and 23.1% (3/13) after DET. No multiple pregnancies were observed.

The overall ongoing implantation rate for the 100 warming cycles (SET and DET cycles) was 14.4% (15/104) per embryo transferred and 11.5% (15/131) per embryo warmed. The implantation rates were further calculated in single-embryo transfers (n = 78) in order to assess the impact of the day of vitrification, blastocyst expansion and ICM score on the implantation potential. The implantation rate per embryo transferred was not different between blastocysts vitrified on Day 5 (13.7%, 7/51) and Day 6 of development (18.5%, 5/27). For the blastocysts that were vitrified on Day 5 (n = 83), we did not find a different implantation rate between early (19.0%, 4/21) and advanced blastocysts (10.0%, 3/30). For the advanced blastocysts vitrified on Day 5 (n = 30), no difference in implantation rate was found between blastocysts with ICM type A (91.1%, 11/11) or B (105.0%, 2/19). Clinical results obtained after vitrified/warmed blastocyst transfer of biopsied (study group) and non-biopsied embryos (control group I) and the results after fresh blastocyst transfer of biopsied embryos (control group II) are shown in Table II. No difference in clinical pregnancy rate per transfer was found between the study group (22.2%) and control group I (26.9%) and II (28.6%). A higher implantation rate (with fetal heart beat) per embryo transferred was found for fresh blastocyst transfer of biopsied embryos (24.4%) compared with the vitrified/warmed study group (14.4%, P < 0.05).
Discussion

In this study, the clinical efficiency of closed blastocyst vitrification of embryos biopsied on Day 3 of development was assessed by analysing the first 100 warming cycles of patients with PGD or aneuploidy screening in their fresh cycle since April 2008. Morphological survival and transfer rates were assessed for 131 vitrified and warmed blastocysts. Overall morphological survival was 83.2% regardless of the blastocyst quality or day of cryopreservation. This survival rate was more optimal than the survival rate obtained in the report of Escriba et al. (2008), which was 48.8% using the 0.25 ml French straws as a closed device for blastocyst vitrification after PGD. Despite the limited survival, the authors did not change the vitrification protocol, considering the straws as a safety device option for vitrification. It has been shown that high survival rates can be obtained for blastocysts that were biopsied at the cleavage stage using open vitrification. Using the cryoloop system, Keskinitepe et al. (2009) reported a higher survival rate (95%) after vitrification than after slow cooling (71%). However, especially for PGD embryos, containing an opening in the zona pellucida, it can be motivated to prevent any risk of contamination using aseptic vitrification. In an experimental setting using a contaminated tank, the open system was found positive for contamination in contrast to the samples using a closed system (Bielanski et al., 2000).

Figure 1 Flow diagram of 100 blastocyst warming cycles in patients with embryo biopsy (study group). P, pregnancy; ET, embryo transfer; SET, single ET; DET, double ET.

Table I Morphological survival after warming in 100 PGD warming cycles according to the day of vitrification and according to the blastocyst quality on Day 5.

<table>
<thead>
<tr>
<th></th>
<th>n, warmed</th>
<th>n, survived (%)</th>
<th>n, transferred (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day of cryopreservation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>83</td>
<td>75 (90.4)*</td>
<td>72 (86.7)**</td>
</tr>
<tr>
<td>Day 6</td>
<td>48</td>
<td>34 (70.8)*</td>
<td>32 (66.7)**</td>
</tr>
<tr>
<td>Blastocyst stage on Day 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bl1/Bl2</td>
<td>38</td>
<td>33 (89.2)</td>
<td>33 (89.2)</td>
</tr>
<tr>
<td>Bl3/Bl4/Bl7</td>
<td>45</td>
<td>42 (93.3)</td>
<td>39 (86.7)</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>20</td>
<td>18 (90.0)</td>
<td>17 (85.0)</td>
</tr>
<tr>
<td>ICM B</td>
<td>25</td>
<td>24 (96.0)</td>
<td>22 (84.9)</td>
</tr>
</tbody>
</table>

*Bl1/Bl2, early blastocysts.
*B3/Bl4/Bl7, full, expanded and artificially hatching blastocysts.
*P < 0.05.
**P < 0.01.
In the present study, early and advanced blastocysts obtained from biopsied embryos showed a similar survival rate. No artificial shrinkage of the blastocoelic cavity was done. Several reports on vitrification of non-biopsied embryos found a better survival of earlier stage blastocysts with a more expanded cavity (Cho et al., 2002; Vanderzwalmen et al., 2002; Mukaida et al., 2003, 2006; Zech et al., 2005; Ebner et al., 2009). Because of the higher risk of ice crystal formation in blastocysts with a large fluid-filled cavity, some groups perform artificial shrinkage to collapse the blastocyst before vitrification (Vanderzwalmen et al., 2002, 2003; Son et al., 2003; Hiraoka et al., 2004; Mukaida et al., 2006). In the present study, most of the advanced blastocysts were artificially hatching through the biopsy hole and this phenomenon probably resulted in a beneficial effect in terms of survival. It was observed by Zech et al. (2005) that hatching blastocysts and intact blastocysts responded differently to the cryoprotectant solution and they postulated that this was due to the faster expulsion of water out of the blastocoelic cavity and a more uniform decrease in the volume of the blastocoel. They found an increase in the survival rate of almost 26% for hatching or completely hatched blastocysts. In the present study, vitrification of blastocysts derived from biopsied embryos has certainly resulted in a better outcome compared with our standard slow freezing protocol. Out of 119 thawing cycles performed after slow cooling of PGD blastocysts, only 62 (52.1%) resulted in a frozen embryo transfer (unpublished observations). The benefit of vitrification when compared with slow cooling for artificially or naturally hatching blastocysts specifically lies probably in the absence of intracellular and extracellular ice crystal formation. The slow cooling process for example allows a controlled growth of ice crystals in the extracellular cryopreservation solution to prevent dehydrating the embryo. To ensure that ice is formed outside the embryo before it is formed inside the cells, extracellular ice formation is induced by seeding between −6 and −9°C. In case of hatching blastocysts, the risk exists that these ice crystals will damage the protruding trophoblast cells and severely damage the embryo. It has also been observed that in cleavage stage embryos, the blastomeres nearest the biopsy hole are more prone to undergo post-thaw lysis following slow freezing (Zheng et al., 2005).

In the present study group of vitrified and warmed biopsied blastocysts, a clinical pregnancy rate per transfer of 22.2% was obtained after transfer of 1.1 blastocysts on average. This was comparable with the clinical pregnancy rates from our control groups of non-biopsied (26.9%) and non-vitrified blastocysts (28.6%). The overall implantation rate (ongoing with FHB) was 14.4% per blastocyst transferred and was not found to be different from the implantation potential of vitrified and warmed blastocysts in our regular IVF/ICSI patients without embryo biopsy (20.4%). However, vitrified biopsied blastocysts seemed to have a lower implantation capacity compared with the fresh ones. Whether the viability of the blastocyst is affected by the vitrification and warming procedure itself or (also) by the fact that in most cycles only second best embryos are cryopreserved is not known. Especially in PGD cycles with autosomal inheritance of the disease, theoretically only half of the embryos can be selected for transfer and even in the fresh transfer, not always the best embryo can be replaced. Furthermore, the present cohort of warming cycles only included patients who did not become pregnant in the fresh cycle and did not include patients who came back for a second child.

In a small cohort of PGD vitrification warming cycles using a closed system, Escriba et al. (2008) reported a high clinical pregnancy rate per transfer of 44.0% (11/25) with a mean number of 1.5 embryos transferred. However, reduced survival after warming and overnight culture resulted in an increased transfer cancellation rate (37.5%) per started warming cycle and an implantation rate (gestational sacs) of 19.5% (16/82) when calculated per warmed blastocyst, which is not significantly different from the implantation rate (gestational sacs) per blastocyst warmed in the present study (14.5%, 19/131). The lower transfer rate reported by Escriba et al. (2008) can be attributed to the extra selection after overnight culture and evaluation of the expansion/re-expansion of the blastocoelic cavity resulting in fewer blastocysts available for transfer. This selective vitrification and warming policy probably provides a stronger selection of the blastocyst with the highest implantation potential and may explain the high implantation rate of 40.0% per blastocyst transferred.

Using the open cryoloop system for vitrification of biopsied embryos at the blastocyst stage, Keskinetepe et al. (2009) obtained a higher ongoing pregnancy rate (37%) per transfer when compared with the slow cooled biopsied blastocysts (23%).

After standard slow freezing, optimal survival (87%), transfer (74%) and implantation rates (35%) of cryo-thawed PGD blastocysts were reported in a recent study by El-Toukhy et al. (2009). In that study, 32 PGD warming cycles were compared with 191 IVF/ICSI blastocyst thawing cycles and a similar survival and implantation potential was observed for both groups. Also the study of Magli et al. (2006) reported a similar cumulative outcome for both PGD blastocysts and blastocysts obtained in regular IVF/ICSI cycles, but the results were less optimal especially in terms of survival compared with the results obtained by El-Toukhy et al. (2009). These better results could, according to the authors, be explained by the fact that no

### Table II: Clinical outcome of vitrified warmed blastocyst transfer of biopsied and non-biopsied embryos and of biopsied embryos transferred in the fresh cycle.

<table>
<thead>
<tr>
<th>Study group vitrified/warmed transfers with biopsy (n = 91)</th>
<th>Control group I vitrified/warmed transfers without biopsy (n = 182)</th>
<th>Control group II fresh transfers with biopsy (n = 182)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at stimulation (years)</td>
<td>Mean no of blastocysts transferred (n)</td>
<td>Clinical pregnancy rate (n)</td>
</tr>
<tr>
<td>30.5 ± 3.4</td>
<td>1.1 ± 0.5 (104)</td>
<td>22.2 (20)</td>
</tr>
<tr>
<td>30.6 ± 2.9</td>
<td>1.1 ± 0.5 (201)</td>
<td>26.9 (49)</td>
</tr>
<tr>
<td>30.5 ± 2.9</td>
<td>1.2 ± 0.4 (225)</td>
<td>28.6 (52)</td>
</tr>
<tr>
<td>Implantation rate (FHB) per transferred embryo (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.4 (15)*</td>
<td>20.4 (41)</td>
<td>24.4 (55)*</td>
</tr>
</tbody>
</table>

*P < 0.05.
patients were included with aneuploidy screening and by differences in selection of fresh embryos for biopsy and cryopreservation.

In the present study, a significantly lower survival rate was found for Day 6 blastocysts compared with Day 5 blastocysts. However, when being transferred a similar implantation potential was found. The present data did not demonstrate a difference in implantation potential between early and advanced blastocysts or between top-quality (ICM A) and good quality (ICM B) blastocysts. Therefore, our freezing strategy to cryopreserve good quality early and advanced blastocysts on Day 5 and good quality advanced blastocysts on Day 6 (that were derived from early blastocysts with doubtfull quality on Day 5) will be continued.

**Conclusion**

The present evaluation has shown that the closed system vitrification is a feasible method for cryopreserving Day 5 and Day 6 blastocysts that were biopsied at the cleavage stage.

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