Clinical pregnancy and live births after transfer of embryos vitrified on day 3

Nina Desai*, Faten AbdelHafez, Mohamed A Bedaiwy, Jeffrey Goldberg, Tommaso Falcone, James Goldfarb

Cleveland Clinic Fertility Center, Department of OB/GYN and Women’s Health Institute, Cleveland Clinic Foundation, 26900 Cedar Rd., Beachwood, OH 44122, USA
* Corresponding author. E-mail address: desain@ccf.org (N Desai).

Abstract Human embryo vitrification is a promising new technology but clinical outcome data is needed to gauge its effectiveness and safety. While pregnancy and live-birth data is available for blastocyst vitrification, such information is lacking for human embryo vitrification at the 6- to 8-cell stage. The current work presents clinical and obstetric outcomes from the transfer of embryos vitrified on day 3 at the cleavage stage. A total of 270 transfers were performed. The clinical pregnancy and implantation rates for patients under 38 years of age \( (n = 200) \) were 45% and 24%, respectively. Corresponding rates in patients 38–42 years old declined to 29% and 13% \( (n = 70) \). Embryonic compaction and/or blastulation by the time of transfer were excellent prognostic indicators of a successful pregnancy outcome. Of the 66 deliveries, 12 \( (18.2\%) \) were twin pregnancies and nine were preterm \( (13.6\%) \). The mean birthweight for singletons was 3281 ± 644 g, compared with 2506 ± 549 g in the twin pregnancies. A total of 78 infants have been born with no major congenital malformations. These data attest to the efficacy and safety of the vitrification technique for cryopreservation of human embryos at the 6- to 8-cell stage.

Introduction Tremendous strides have been made in IVF techniques in the past two decades. Clinical pregnancy outcomes have risen dramatically. With this increase has come a need to reduce the number of embryos transferred to avoid high-order multiple pregnancies \( \text{Fauser et al., 2005} \). There is also a
Outcomes after transfer of day-3 vitrified—warmed embryos

809

growing trend towards single embryo transfers (Gerris, 2009). Effective techniques for cryopreservation of surplus embryos at all developmental stages are therefore imperative to maximize the cumulative pregnancy rates between fresh and frozen embryos from each oocyte retrieval.

Vitrification is a promising new technology for embryo cryopreservation and is being adopted by IVF centres around the world. This ultra-rapid cooling technology transitions the embryo from 37°C to −196°C at a rate of −15,000 to −30,000°C/min (Liebermann et al., 2002). Unlike traditional slow-programmed freezing, vitrification is very quick and requires no expensive equipment.

Validation of any new technology such as vitrification clearly requires follow-up on neonatal outcomes from pregnancies established with vitrified—warmed embryos. To date, much of the accumulated information has been from blastocyst-stage vitrification (Liebemann and Tucker, 2006; Takahashi et al., 2005). Information on live-birth outcomes, much of the accumulated information has been from blastocyst-stage vitrification (Liebemann and Tucker, 2006; Takahashi et al., 2005). Information on live-birth outcomes after vitrification at the 6- to 8-cell stage is limited (Desai et al., 2007; Rama Raju et al., 2009). The current report provides much needed data on the efficacy and safety of cleavage-stage embryo vitrification.

Materials and methods

Patients

This was a retrospective analysis of prospectively collected IVF data. The study was conducted at the Cleveland Clinic Fertility Centre between January 2006 and March 2009. A total of 270 cryopreservation cycles involving embryos vitrified on day 3 at the 6- to 8-cell stage were examined. Informed consent for cryopreservation was obtained from all patients in accordance with the guidelines of the study centre’s institutional review board. All vitrification—warming cycles in the designated time interval were retrospectively analysed. Vitrification was used exclusively during the study interval so no patient selection or exclusion criteria were necessary.

Ovarian stimulation

Ovulation induction was carried out after pituitary down-regulation with leuprolide acetate (Sandoz, USA) and stimulation with recombinant FSH (Follistim; Organon, USA; or Gonad F; Serono, Switzerland) at a starting dose of 225 IU per day, unless previous stimulations indicated otherwise. The dose was subsequently individualized based on monitoring with transvaginal ultrasonography and serum oestradiol concentrations. The patients were administered 10,000 IU of human chorionic gonadotrophin (HCG; APP Pharmaceuticals LLC, Schaumburg, IL, USA) when at least two follicles reached 18 to 20 mm in mean diameter and oocyte retrieval was performed 36 h later by transvaginal ultrasound-guided needle aspiration.

Fertilization/embryo culture

The standard method of fertilization for mature oocytes was intracytoplasmic sperm injection. Immature oocytes were inseminated by conventional IVF. Zygotes were cultured individually in 20 µl media drops under an oil overlay. Human tubal fluid medium (LifeGlobal, Ontario, Canada) supplemented with 10% synthetic serum substitute (SSS; Irvine, Santa Ana, CA, USA) was used for embryo culture until day 3. All cultures were performed at 37°C with 5.5% CO₂ and air. Fresh embryo transfers were generally performed on day 3. Good-quality embryos not selected for transfer were considered for vitrification on day 3 if they were between 6 and 8 cells with less than 20% fragmentation. Day-5 transfer was offered to a select group of patients with a high number of zygotes (>12). If embryo quality was suitable, a minimum of two embryos were vitrified on day 3 and the remaining embryos were cryopreserved if they reached the blastocyst stage by day 6.

Vitrification procedure

Vitrification of embryos was carried out using a modification of the two-step protocol by Mukaida et al. (2001). Dimethylsulphoxide (DMSO) and ethylene glycol were used as cryoprotectant agents. The basal medium for vitrification solutions was global blastocyst medium (LifeGlobal) supplemented with 20% SSS. All steps were performed on a heated laminar flow hood at 37°C. Vitrification solution 1 consisted of 7.5% DMSO and 7.5% ethylene glycol. Following a 2-min incubation, the embryos were moved to vitrification solution 2 containing 15% DMSO, 15% ethylene glycol, 10 mg/ml Ficoll and 0.65 mol/l sucrose for 35 s.

Using a fine micropipette under a dissecting microscope, one or two embryos were quickly loaded on to a thin film of the same cryoprotectant solution applied to the surface of a nylon cryoloop (0.7–1.0 mm; Hampton Research, Laguna, CA, USA). The cryoloop was then immediately plunged into a vial filled with liquid nitrogen. Each vial was snapped onto a separate cane to facilitate quick transfer of the samples. All handling of the vials containing vitrified embryos was performed while keeping the samples immersed in liquid nitrogen or very briefly (less than 1 min) in the vapour phase. Canes were placed in liquid nitrogen storage tanks that were dedicated exclusively to the storage of vitrified embryos.

Warming procedure

The vitrified embryos were warmed at 37°C. The cryoloop was immersed directly in a solution containing 0.25 mol/l sucrose in basal culture medium. Embryos displaced from the cryoloop were readily visualized using the dissecting microscope. After 2 min, the recovered embryos were moved to the second solution containing 0.125 mol/l sucrose for 3 min. The final step was a 5-min incubation in basal medium before transfer to global blastocyst medium with 10% SSS for further culture until transfer. All embryos were hatched with the Octax laser (1.48 µm) during this incubation. The embryos were also photographed immediately after warming and cell damage was assessed. The study centre’s policy has been to transfer all cryopreserved embryos with any sign of vitality regardless of morphology. Only completely degenerated embryos were excluded from transfer.
Embryo replacement

Patients were prepared for cryopreserved embryo transfer using hormone replacement therapy consisting of increasing doses of estradiol 2–6 mg orally daily (Estrace; Mylan Pharmaceuticals, Morgantown, WV, USA) and progesterone, 50–100 mg i.m. (Abraix, Pharmaceutical products, Schaumburg, IL, USA) either alone or in combination with down-regulation with leuprolide acetate. Progesterone treatment was initiated on day 13 in natural override cycles and on day 15 in down-regulated cycles. A transvaginal ultrasonogram was performed prior to initiating progesterone to assure that the endometrial thickness was at least 8 mm.

Vitrified cleavage-stage embryos were warmed and cultured for 48 hours before transfer on the fifth day of progesterone. Post-warming survival and daily development were monitored. All embryos were photographed at three time points: immediately upon warming, 24 and 48 h after warming and just prior to transfer. Embryo transfer was performed under abdominal ultrasound guidance using a Wallace Sure View catheter (Irvine Scientific).

Outcome measures

The primary outcome measures were serum HCG concentrations, clinical pregnancy rate, implantation rate, gestational age at delivery, live-birth rate, birthweight, incidence of congenital malformations and miscarriage rate. Pregnancy testing was performed 15 days after the embryo transfer by measurement of serum HCG concentrations. Clinical pregnancy was confirmed by the presence of fetal cardiac activity on transvaginal ultrasonography at 6–8 weeks gestational age. The implantation rate was calculated by dividing the number of fetal hearts on ultrasonography by the number of embryos transferred.

Secondary outcome measures included the impact of age at time of embryo cryopreservation on pregnancy outcome and also the embryo stage by the time of transfer.

Results

A total of 712 vitrified embryos were warmed. The post-warming survival rate was 93.5% and 666 embryos were transferred in 270 consecutive embryo transfers. Clinical outcome data following these transfers is presented in Table 1. Outcome data was stratified according to maternal age at time of embryo transfer. The clinical pregnancy, implantation and miscarriage rates for patients under 38 years of age (n = 200) were 45.0%, 24.2% and 11.1%, respectively. The corresponding rates for women aged 38–42 years (n = 70) were 28.6%, 12.5% and 10.0%. The mean number of embryos transferred in the younger women was 2.28 ± 0.62 versus 2.5 ± 0.72 in the older age group.

Women often returned for their cryopreserved embryos several years after the initial oocyte retrieval. Figure 1 looks further at the issue of age and success rates. Transfers and outcomes are presented as a function of age at the time of initial embryo cryopreservation. Patients under the age of 32 at the time of embryo cryopreservation achieved clinical pregnancy and implantation rates of 54% and 29%, respectively. Both clinical pregnancy and implantation rates dropped sharply in patients aged 38–42 years and older, reflecting the negative impact of age on fertility.

Embryo stage by the time of transfer was also an excellent prognostic indicator of success with the transfer of vitrified–warmed embryos (Table 2). Progression to the blastocyst stage during the 48-h culture period was related to positive outcomes. In the younger age group (<38 years) and transfers where at least one vitrified–warmed embryo developed from the 6–8-cell stage to a blastocyst by the time of transfer, the pregnancy rate was 54% (76/142). The clinical pregnancy rate was further increased to 61% (45/74) if all embryos formed a blastocyst. The pregnancy rate fell to 24% (14/58) in the absence of a blastocyst. Similar results were obtained in the 38–42-year age group (Table 2). Figure 2 shows the excellent morphological characteristics of day-3 vitrified embryos on warming and at transfer.

Table 1 Clinical outcome data with vitrification of day-3 embryos stratified according to age.

<table>
<thead>
<tr>
<th>Outcome measure</th>
<th>Age &lt;38 years</th>
<th>Age 38–42 years</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age at transfer (years)</td>
<td>33 ± 2.9</td>
<td>39.4 ± 1.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Transfer cycles</td>
<td>200</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>94</td>
<td>93</td>
<td>NS</td>
</tr>
<tr>
<td>Embryos warmed</td>
<td>2.58 ± 0.91</td>
<td>2.7 ± 0.93</td>
<td>NS</td>
</tr>
<tr>
<td>Embryos transferred*</td>
<td>2.28 ± 0.62</td>
<td>2.5 ± 0.72</td>
<td>0.008</td>
</tr>
<tr>
<td>Clinical pregnancies/transfer</td>
<td>90/200 (45.0)</td>
<td>20/70 (28.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Fetal hearts/transfer</td>
<td>110/455 (24.2)</td>
<td>22/176 (12.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Deliveries/transfer</td>
<td>53/200 (26.5)</td>
<td>13/70 (18.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Infants</td>
<td>64</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Singleton pregnancies</td>
<td>42</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Twin pregnancies</td>
<td>11</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ongoing pregnancies/transfer</td>
<td>27/200 (13.5)</td>
<td>5/70 (7.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Miscarriage rate/clinical pregnancies</td>
<td>10/90 (11.1)</td>
<td>2/20 (10.0)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD, number or number/total (%), unless otherwise stated.

NS = not statistically significant.

*All warming cycles resulted in a transfer.
Figure 1 The impact of patients' age at the time of embryo vitrification on clinical outcomes.

Table 2 Clinical pregnancy rate and embryo stage according to patient age at time of transfer.

<table>
<thead>
<tr>
<th>Embryo stage</th>
<th>Clinical pregnancy rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>38 years</td>
</tr>
<tr>
<td>Morula or earlier</td>
<td>14/58 (24)</td>
</tr>
<tr>
<td>At least one</td>
<td>76/142 (54)</td>
</tr>
<tr>
<td>blastocyst</td>
<td>45/74 (61)</td>
</tr>
</tbody>
</table>

Values are number/total (%).

There have been 66 deliveries to date, 54 singleton and 12 twin pregnancies, resulting in 78 newborns (Table 3). The mean gestational age in the singleton pregnancies was 38.8 ± 1.9 weeks, compared with 35.8 ± 3.1 weeks in the twin pregnancies. All but four of the singleton pregnancies resulted in term deliveries (92.6%) while five of the 12 twin pregnancies delivered before 38 weeks. The mean birthweight for singletons was 3281 ± 644 g, compared with 2506 ± 549 g in the twin pregnancies. No major congenital malformations were observed. Thirty-two pregnancies are results of 2.8 embryos. The mean age of the patients in their study was 31.44 ± 4.75 years. Neonatal outcomes from pregnancies conceived with vitrified–warmed embryos were found to be comparable to those observed in their fresh day 3 transfers (Rama Raju et al., 2009).

Outcomes after transfer of day-3 vitrified–warmed embryos

Cryopreservation at later stages of development such as morula or blastocyst ensures that embryonic genome activation has occurred. Advanced-stage embryos also have a larger number of cells and this may enable the embryo to better withstand small degrees of cryo-injury (Veeck, 2003). However, there are data to indicate that the larger volume of blastocoelic fluid in late-stage expanding blastocysts may actually be detrimental during vitrification (Vanderzwalmen et al., 2002). Early stage human blastocysts with less blastocoelic fluid were observed to have better survival. Artificial shrinkage or collapse of expanded blastocysts by mechanical methods or laser prior to vitrification has been suggested to improve survival, reduce cell damage and increase clinical pregnancy rates (Desai et al., 2008; Hiraoka et al., 2004; Mukaida et al., 2006; Son et al., 2003).

Vitrification at the cleavage stage is attractive as it avoids the above-noted difficulties with blastocyst cryopreservation. The study centre’s approach of vitrifying on day 3, and then cultivating the warmed cleavage-stage embryos for another 48 hours to the blastocyst stage before transfer allowed us to better assess cell damage and confirm genomic activation. Embryonic compaction and/or blastulation by the time of transfer were excellent prognostic indicators of a successful pregnancy outcome. The pregnancy rate of 61% achieved if warmed embryos reached the blastocyst stage by the time of transfer was certainly comparable to that seen with blastocyst-stage vitrification (Mukaida et al., 2006).

Only one other clinic has published live-birth outcomes with day-3 embryo vitrification (Rama Raju et al., 2009). In their series of 285 transfer cycles, vitrified embryos were transferred to the patient’s uterus shortly after warming. They reported clinical pregnancy and implantation rates of 36% and 18%, respectively with the transfer of an average of 2.8 embryos. The mean age of the patients in their study was 31.44 ± 4.75 years. Neonatal outcomes from pregnancies conceived with vitrified–warmed embryos were found to be comparable to those observed in their fresh day 3 transfers (Rama Raju et al., 2009).

Exposure to high concentrations of cryoprotectants commonly used in vitrification protocols has raised the issue of safety. Continued accumulation of live-birth data from centres worldwide is necessary to validate this method of embryo preservation and to determine if vitrification poses any greater risk than traditional slow cryopreservation technologies. While neonatal outcome data from vitrification of blastocyst-stage embryos is encouraging (Liebermann and Tucker, 2006; Mukaida et al., 2003; Takahashi et al., 2005), there remains a lack of information regarding vitrification at other cell stages.

Another safety consideration for vitrification techniques is the type of carrier (Liebermann et al., 2003). Open carriers such as the cryoloop (Lane et al., 1999; Mukaida et al., 2003), cryotop (Stehlik et al., 2005) and electron microscopic grids (Son et al., 2003) have been used very successfully but raise theoretical concerns regarding the possibility of sample cross contamination since there is direct contact between the embryo and the liquid nitrogen. The miniscule volumes of fluid (less than 1 μl) associated with cryoloop vitrification certainly decrease the likelihood of cross-contamination, but ultimately the study centre hopes to adopt a

Discussion

Vitrification technology has shown great promise for cryopreservation of embryos (Vajta and Nagy, 2006) as well as oocytes (Cobo et al., 2008a). Meta-analyses comparing vitrification versus slow-programmed cooling concluded that vitrification resulted in significantly higher post-warming survival (Loutradi et al., 2008) as well as clinical pregnancy and implantation rates (AbdelHafez et al., 2010). The current findings further support the use of vitrification for human embryo cryopreservation. As far as is known, this is the first report from a US clinic on live-birth outcomes with the transfer of embryos vitrified on day 3. The optimal stage of embryo vitrification remains to be determined.
closed system. The only Food and Drug Administration-approved closed carrier system, the Cryotip (Irvine Scientific), is relatively new to the market place and has been mainly utilized for blastocyst (Kuwayama et al., 2005) and oocyte vitrification (Cobo et al., 2008b). Multicentre obstetric data are still needed to assure the safety of this device, as well as testing its effectiveness for vitrification with earlier stage embryos.

One of the limitations of this study was the lack of a matched control group with which to compare vitrification outcome data. However, it can be concluded that this technique compared very favourably with the historical data using conventional slow-freezing methodology. In the under-38 age group, conventional slow cryopreservation at the cleavage stage has yielded clinical pregnancy, implantation and miscarriage rates of 32%, 17% and 15%, respectively. The similarity in the miscarriage rates between younger and older age groups may be related to small numbers in the older age group.

In conclusion, vitrification of human embryos at the 6- to 8-cell stage is easily incorporated into the IVF regimen. Vitrification at this cell stage may be particularly helpful to new laboratories trying to establish and validate their vitrification programme. In cases with large numbers of excess embryos, cryobanking some embryos at the cleavage stage reduces the number being cultured to blastocysts, and transfer of these embryos resulted in a twin pregnancy.

**Table 3** Obstetric and neonatal outcomes following transfer of embryos vitrified on day 3 at the cleavage stage.

<table>
<thead>
<tr>
<th></th>
<th>Singleton pregnancies</th>
<th>Twin pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>38.8 ± 1.9</td>
<td>35.8 ± 3.1</td>
</tr>
<tr>
<td>Deliveries</td>
<td>54</td>
<td>12</td>
</tr>
<tr>
<td>Deliveries 34–37 weeks</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Deliveries &lt;34 weeks</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Newborn infants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>54</td>
<td>24</td>
</tr>
<tr>
<td>Females</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>3281.4 ± 644</td>
<td>2506.6 ± 549.1</td>
</tr>
<tr>
<td>Low birthweight (1500–2500 g)</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Very low birthweight (&lt;1500 g)</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Values are mean ± SD or number.
Outcomes after transfer of day-3 vitrified–warmed embryos

potentially requiring blastocoelic fluid reduction prior to vitrification. The movement towards widespread application of vitrification requires that the efficacy and neonatal outcomes with embryos at all stages of development are measured. This paper contributes reassuring preliminary data to this pool of knowledge.

References


Declaration: The authors report no financial or commercial conflicts of interest.

Received 15 September 2009; refereed 28 October 2009; accepted 18 January 2010.