Article

Cryoloop vitrification of human day 3 cleavage-stage embryos: post-vitrification development, pregnancy outcomes and live births

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Abstract

Vitrification technology has shown great promise for cryopreservation of human embryos. The majority of this work has been with blastocyst-stage embryos. This report describes initial clinical results following vitrification of human embryos on day 3 of culture at the 6- to 8-cell stage. A total of 236 embryos were cryopreserved on cryokits using a vitrification protocol. Warmed embryos were cultured until day 5 before transfer to the patient. The post-warming survival rate was 85%. The clinical pregnancy rate was 44% (34/77), and the implantation rate was 20% (40/201). In transfers where at least one warmed embryo reached the blastocyst stage by the day of transfer, the clinical pregnancy rate was 58% (28/48). The cryoloop was an excellent vessel for ultra-rapid cryopreservation of embryos. This study supports the effectiveness of a dimethylsulphoxide/ethylene glycol cryoprotectant combination for vitrification of human embryos at the 6- to 8-cell stage.

Keywords: cryoloop, cryopreservation, IVF, post-vitrification survival, pregnancy outcome, vitrification

Introduction

Clinical pregnancy rates associated with IVF have increased tremendously since the birth of the first IVF baby in 1978. With this increase has come a movement to reduce the number of embryos being transferred to avoid high order multiple pregnancies. Effective techniques for cryopreservation of supernumerary embryos for a future cycle are vital to avoid embryo wastage and to augment the pregnancy rate from a single oocyte retrieval.

Cryopreservation results have been quite variable amongst clinics depending on embryo stage and cryopreservation protocol used. Based on the 2004 national registry data for IVF clinics (see US Department of Health and Human Services and the Center for Disease Control and Prevention, 2006), cryopreservation cycles resulted in a live birth rate of 28–30% for patients under 38 years of age. Slow freeze protocols using controlled rate freezers that slowly lower the temperature to below −30°C have traditionally been used for embryo freezing in the clinical laboratory. Different cryoprotectant solutions have been utilized for embryo dehydration depending on cell stage. Propanediol/sucrose-based protocols have been the most commonly used methodology for slow freezing of pronuclear and early cleavage (2- to 8-cell) stage embryos (Lassalle et al. 1985; Testart et al. 1986; Fugger et al. 1988). Glycerol-based cryoprotectant solutions have been favoured for blastocyst cryopreservation (Cohen et al., 1985).

Application of vitrification technology to human embryo cryopreservation has received much attention in recent years. Vitrification is an ultra-rapid method of cryopreservation...
whereby the embryo is transitioned from 37°C to -196°C in < 1 s. High concentrations of cryoprotectants and high cooling rates are necessary to get the embryos into a 'glass-like state' (Vajta and Kuwayama, 2006). Direct exposure of embryos to liquid nitrogen and introduction of novel carrier systems that minimize vitrification solution volumes have been instrumental in achieving the rapid temperature shift necessary for this technique to be successful (Park et al., 2000).

Human embryo vitrification has been attempted with a variety of vessels such as electron microscope grids (Park et al., 2000; Son et al., 2002), open pulled and hemi-straws (El-Danasouri and Selman, 2001; Vanderzwalmen et al., 2002, 2003), the flexipipet (Lieberman et al., 2002), the cryotop (Kuyama, 2006) and the cryoloop (Lane et al., 1999; Mukaida et al., 2001, 2003a,b; Reed et al., 2002; Rama Raju et al., 2005).

To date, clinical reports using vitrification have been limited and mainly involve oocyte (Yoon et al., 2003; Kuwayama et al., 2005b; Kuwayama, 2006) or blastocyst-stage cryopreservation (Hiraoka et al., 2004; Huang et al., 2005; Kuwayama et al., 2005a; Stehlik et al., 2005; Takahashi et al., 2005; Zech et al., 2005; Kuwayama, 2006; Lieberman and Tucker, 2006). Cryopreservation of human embryos at the 6- to 8-cell stage using vitrification methodology has not been widely reported (Mukaida et al., 1998; Saito et al., 2000; El-Danasouri and Selman, 2001; Rama Raju et al., 2005; Zhu et al., 2005).

This report presents initial clinical results following vitrification of human embryos on day 3 of culture at the 6- to 8-cell stage. Post-warming embryo morphology and its relationship to pregnancy outcome were assessed. The paper also describes experience with the cryoloop as a carrier for the vitrification procedure.

Materials and methods

Patients

Vitrification was introduced into the authors' clinical practice in November 2002 for cryopreservation of cleavage-stage embryos at the 6- to 8-cell stage, immediately following fresh transfer. Seventy-seven couples undergoing initial IVF treatment between November 2002 and April 2006, returned for a subsequent warming-cycle. All vitrification—warming cycles were retrospectively analysed. No patient selection or exclusion criteria were used.

Ovarian stimulation

Ovulation induction was carried out after down-regulation with leuprolide acetate (Lupron; TAP Pharmaceuticals, Lake Forest, IL, USA). FSH (Follistim; Organon, USA or Gonal F; Serono, Italy) was initiated at a dose of 225 IU per day, unless previous stimulations indicated otherwise. When at least two follicles were of 18 mm mean diameter, 10,000 IU of human chorionic gonadotrophin (HCG) was administered 36 h before scheduled oocyte retrieval. Oocytes were recovered by transvaginal aspiration of follicles under ultrasound guidance.

Embryo culture

Patients' oocytes were fertilized by intracytoplasmic sperm injection 3–4 h after retrieval. Fertilization check was performed the following morning. Zygotes were cultured individually in 20 μl media drops under an oil overlay. Human tubal fluid medium (HTF; Life Global, Ontario, Canada) supplemented with 10% synthetic serum substitute (SSS; Irvine Scientific, USA) was used for embryo culture until day 3. All culture was performed at 37°C with 5.5% CO₂ and air. Fresh embryo transfers were 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 <20% fragmentation. Laboratory practice has been to vitrify 2–6 good quality embryos on day 3 and culture all remaining embryos to the blastocyst stage prior to cryopreservation.

Vitrification procedure

Vitrification of embryos was carried out by the two-step protocol of Mukaida et al. (2001). This technique uses dimethylsulphoxide (DMSO), ethylene glycol and sucrose as the cryoprotectant agents. The basal medium was Global Blastocyst Medium (Life Global) 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 and the aid of a dissecting microscope, the embryos were quickly loaded onto a cryoloop (Hampton Research, Laguna, CA, USA) covered with a thin film of the same cryoprotectant (Figure 1a,b). The cryoloop with embryos was immediately plunged into a vial filled with liquid nitrogen. The tiny amount of fluid used for cryoloop vitrification (<1 μl) necessitated careful handling of the vitrified sample to prevent inadvertent warming during transfer to and from the large nitrogen storage tank. Each vial was snapped onto a separate cane to facilitate quick transfer of samples. All handling of vials containing vitrified embryos was done while keeping the samples immersed in liquid nitrogen or very briefly (<1 min) in the vapour phase. Canes were placed in a liquid nitrogen storage tank that was dedicated exclusively to vitrified embryos.

Warming procedure

Warming of vitrified embryos was also performed at 37°C. The cryoloop was directly immersed in a warming solution containing 0.25 mol/l sucrose. The embryos that fell off the cryoloop were visualized using the dissecting microscope. Following a 2-min rinse, the recovered embryos were moved to the second warming solution, which contained 0.125 mol/l sucrose. The embryos were incubated for 3 min. The final rinse was in culture media for 5 min before placement in an incubator at 37°C with 5.5% CO₂. The warmed embryos were cultured in Global Blastocyst Medium with 10% SSS until transfer. Cell damage was assessed immediately upon warming. Embryos were considered to be damaged if fewer than half of their blastomeres were intact upon warming. The authors' policy has been to transfer all
Figure 1. (a) Loading of an embryo onto a cryoloop during the vitrification procedure. (b) Embryo on a cryoloop viewed under an inverted microscope. Original magnification ×100.

warmed embryos with any sign of vitality regardless of morphology. Only completely degenerated embryos were excluded from transfer.

Embryo replacement

Patients were prepared for vitrified-warmed embryo transfer using hormone replacement therapy consisting of increasing doses of estrace (2–6 mg p.o. daily) and progesterone (50–100 mg i.m.) starting on day 13. The only monitoring in the replacement cycle was the evaluation of endometrium prior to progesterone administration. Warming cycles were cancelled if the endometrial thickness was <7 mm. Vitrified cleavage-stage embryos were warmed and cultured for 48 h before transfer on day 5 of progesterone administration. Post-warming survival and daily development were monitored. All embryos were photographed at three time points: at vitrification, immediately upon warming and just prior to transfer. Embryo transfer was performed under ultrasound guidance using a Wallace Sure View catheter (Irvine Scientific). Pregnancy testing was performed 15 days after the embryo transfer. Morphology was confirmed by the presence of a fetal heart beat on ultrasound examination at 6–8 weeks. The implantation rate was derived from the number of fetuses with a heart beat divided by the total number of embryos transferred.

Results

Table 1 presents results for human embryos vitrified at the 6- to 8-cell stage. The data set represents consecutive vitrification-warming cycles performed over a 2.5-year interval. A total of 236 embryos were warmed. The average number of embryos transferred per patient was 2.66 ± 0.86. All warming cycles resulted in a transfer.

The clinical pregnancy rate was 44% (34/77) and the implantation rate was 20% (40/201). The embryos were evaluated for blastomere number and cytoplasmic integrity. The post-warming survival rate was 85% (201/236). Only 4.5% of surviving embryos had outward signs of injury and considerable blastomere cell loss on thawing. Figure 2 shows the excellent morphology typically seen following warming of vitrified human embryos.

It was found that 78% (184/236) of warmed embryos showed signs of embryonic compaction and/or blastulation by the time of transfer. The blastulation rate from warmed cleavage-stage embryos was 44% after 48 h in culture. Predominantly early stage blastocysts were noted during the morning observation but by late afternoon expansion was clearly evident in high quality embryos. The timely post-warming development of vitrified embryos appeared to be an excellent indicator of subsequent developmental potential. Morphology on the day of vitrified-warmed embryo transfer was associated with pregnancy outcome. In transfers where at least one warmed embryo reached the blastocyst stage by the day of transfer, the pregnancy rate was 58% (28/48). In contrast, when warmed embryos developed only to the morula/compact stage by the transfer day, the pregnancy rate was notably lower, at 26% (6/23). No pregnancies occurred when the embryos failed to show signs of compaction by the day of transfer (0/6).

Thirty singleton pregnancies and two sets of twins and triplets were conceived. To date, there have been 11 deliveries of healthy infants and two miscarriages. No congenital anomalies have been reported.
Table 1. Results of vitrification–warming cycles of 236 human embryos vitrified at the 6–8-cell stage.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of warming cycles</td>
<td>11</td>
</tr>
<tr>
<td>No. of transfers</td>
<td>11</td>
</tr>
<tr>
<td>Patient age (years)</td>
<td>34.1 ± 4.5</td>
</tr>
<tr>
<td>Post-warming survival rate (%)</td>
<td>201/236 (85)</td>
</tr>
<tr>
<td>No. of embryos warmed per cycle</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>No. of embryos transferred per patient</td>
<td>2.7 ± 0.9</td>
</tr>
<tr>
<td>Clinical pregnancy rate (%)</td>
<td>34/77 (44)</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>40/201 (20)</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Discussion

The present series, achieved exclusively with cryoloop vitrification, represents the largest clinical series to date showing successful vitrification of day 3 cleavage-stage embryos.

Vitrification technology has been shown to be a very promising alternative to slow-freezing methodology for cryopreservation of chill-sensitive cells such as oocytes (Hong et al., 1999; Chung et al., 2000; Kuleshova and Lopata, 2002; Liebermann et al., 2003; Yoon et al., 2003; Chian et al., 2004; Liebermann and Tucker, 2004; Kuwayama et al., 2005b; Kuwayama, 2006) and even for blastocyst-stage embryos with large fluid volumes (Lane et al., 1999; Reed et al., 2002; Mukaida et al., 2003b; Vanderzwalmen et al., 2003; Liebermann and Tucker, 2004, 2006; Walker et al., 2004; Kuwayama et al., 2005a, 2006; Stehlik et al., 2005). Numerous publications describe blastocyst-stage vitrification and clinical outcomes (Mukaida et al., 2001, 2003a; Reed et al., 2002; Vanderzwalmen et al., 2002, 2003; Hiraoka et al., 2004; Huang et al., 2005; Kuwayama et al., 2005a; Stehlik et al., 2005; Takahashi et al., 2005; Liebermann and Tucker, 2006) using a variety of different techniques. Pregnancy outcomes in these early trials with vitrified human blastocysts have ranged from 23 to 53%.

Clinical application of vitrification technology to human cleavage embryos at the 6- to 8-cell stage has not been widely explored. Mukaida and colleagues (Mukaida et al., 1998) reported high

Figure 2. (a) Vitrified human embryos immediately after warming. (b) Warmed embryos after 24 h in culture. In a and b, original magnification ×300.
survival of 8-cell embryos vitrified with 40% ethylene glycol combined with Ficoll and sucrose, but pregnancy rates were only 5.5%. Saito et al. (2000) obtained three clinical pregnancies and two deliveries in 31 transfers with vitrified cleavage-stage embryos. Two subsequent studies (El-Danasouri and Selman, 2001; Rama Raju et al., 2005) used a similar two-step protocol with 10% ethylene glycol, followed by a 40% ethylene glycol–sucrose step for vitrification, but omitted Ficoll from their vitrification solution. Pregnancy rates were 31 (11/36) and 35% (14/40) using the open straw method (El-Danasouri and Selman, 2001) and a nylon loop (Rama Raju et al., 2005) respectively. The implantation rate per warmed embryo transferred was 10 and 15% respectively.

The present clinical trial, in contrast to the aforementioned studies, used a combination of DMSO and ethylene glycol. It was thus possible to use lower concentrations of individual cryoprotectants, thereby possibly reducing any cytotoxic effects (Kasai et al., 1992; Zhu et al., 1993; Archer et al., 2003). The current two-step protocol adapted from Mukaida et al. (2001, 2002) involved exposing embryos to 7.5% DMSO/ethylene glycol for 2 min followed by 35 s in the final vitrification solution containing 15% DMSO/ethylene glycol, sucrose, and Ficoll. This formulation was originally developed for blastocyst-stage cryopreservation, and to date there have been no clinical reports on day 3 cleavage-stage embryos using this protocol.

One positive attribute following vitrification–warming with this protocol was the remarkably low percentage of embryos with considerable cellular damage (4.5%). Historically, with the conventional slow freezing protocol using cryovials, almost a quarter of human embryos surviving the freeze–thaw process had greater than 50% damage, resulting in blastomere loss and degeneration (Desai et al., 2003). Higher damage rates with conventional slow freezing compared with vitrification have been observed by others (Walker et al., 2004). Cell loss on thawing has also been related to reduced pregnancy rates (Archer et al., 2005).

Another advantage of the vitrification procedure was that it was extremely efficient, taking <5 min as compared with the more lengthy programmed slow freezing protocols, which required a minimum of 2 h per run and several freezer units to accommodate cryopreservation for all patients. Introduction of cryoloop vitrification to the clinical laboratory did, however, require extensive technician training with mouse embryos. The embryologist’s speed and skill in performing the vitrification–warming procedure were paramount to success.

This series was also unique in that warmed cleavage-stage embryos were cultured until the morula/early blastocyst stage prior to transfer. Culture for this additional 48-h interval allowed better assessment of post-warming damage and embryonic potential after vitrification. This work demonstrates a clear relationship between embryonic compaction after warming and subsequent developmental potential. Lieberman and Tucker (2002) using embryos derived from abnormally fertilized zygotes cultured to day 3, found that only 39% of vitrified–warmed embryos were capable of compaction. The high compaction rate (78%) observed in the present study was reflective of overall embryo quality on warming. It also attests to the suitability of this vitrification procedure for human embryo cryopreservation. Vitrification at the cleavage stage allowed more embryos to be vitrified per patient and alleviated the need for extended culture of large numbers of spare embryos. The patient also benefited by having embryos frozen at both early and late stages.

The pregnancy and implantation rates achieved in this series are indicative of the tremendous potential of vitrification in the clinical setting. The clinical pregnancy rate of 44% and the embryo implantation rate of 20% were higher than historical results with slow programmed freezing (Desai et al., 2003). Introduction of this technology into the laboratory allowed for more efficient cryopreservation of patient embryos with good success rates.

One issue with vitrification on cryoloops is that it involves direct exposure to liquid nitrogen. The risk of cross-contamination in liquid nitrogen storage containers even at −196°C has been widely debated (Bielanski et al., 2000, 2003). This concern was addressed in part by keeping all vitrified embryos in a separate, newly purchased tank. With cryoloops, the volume of fluid in each vial was less than 1 μl and at its maximum capacity the liquid nitrogen storage Dewar could handle 320 vials (one vial per case). With less than 320 μl of fluid in the entire 79 l tank, it was felt that the risk of possible cross-contamination was minimal. Other ‘closed’ systems for vitrification are being considered, but most involve heat-sealing of the carrier vessel. If heat is transferred to a sample, it could have a negative impact, especially with vitrification, since fluid volumes are so small.

In conclusion, initial experiences with vitrification of human embryos were positive. The vitrification procedure has been adapted successfully to the authors' IVF laboratory after a period of employee training, and the clinical pregnancy and implantation rates were promising. Moreover, it was found that the cryoloop was an excellent carrier for ultra-rapid cryopreservation of embryos. This study supports the effectiveness of a DMSO/ethylene glycol cryoprotectant combination for vitrification of human embryos at the 6- to 8-cell stage. Moreover, ethylene glycol could be used at far lower concentrations than has previously been reported for cleavage-stage vitrification. It remains to be determined if this same protocol can be applied to other cell stages with equal success.

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