Article

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

Dr Nina Desai received her doctorate in microbiology/cell biology from the University of Medicine and Dentistry of New Jersey, USA and then completed a post-doctoral fellowship in infertility at The Ohio State University, Columbus. She joined the faculty of Case Western Reserve University, Cleveland, Ohio in 1993 and was subsequently appointed Director of the IVF/Andrology Laboratories at University MacDonald Women's Hospital. In 2000, she became Director of IVF and Clinical Research at the Cleveland Clinic Foundation and designed a new state-of-the-art IVF laboratory there. Her research has focused on growth factors, extracellular matrices, co-culture systems and the study of embryonic growth and apoptosis, and oocyte and embryo vitrification.

Dr Nina Desai

Nina Desai[1,3], Heather Blackmon[2], Julia Szeptycki[1], James Goldfarb[1]
[1]Cleveland Clinic Fertility Centre/Cleveland Clinic Foundation, Beachwood, OH; [2]Carolina Conceptions, Raleigh, NC, USA
[3]Correspondence: Cleveland Clinic Fertility Centre, 26900 Cedar Road, Beachwood, OH 44122, USA. Tel: +1 216 8392907; e-mail: desain@ccf.org

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 cryoloops 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 (Liebermann *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; Liebermann 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% CO_2 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% CO_2. 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

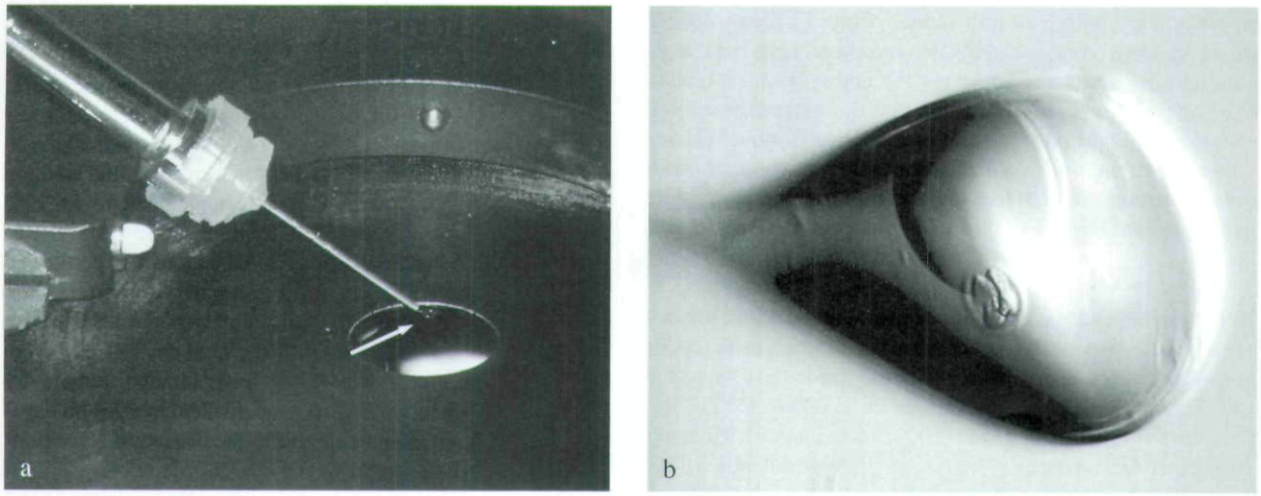

Figure 1. (**a**) Loading of an embryo onto a cryoloop during the vitrification procedure. (**b**) Embryo on a cryoloop viewed under an inverted microscope. Original magnification ×100.

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. Clinical pregnancy 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/compacting 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.

Parameter	*Value*
No. of warming cycles	77
No. of transfers	77
Patient age (years)[a]	34.1 ± 4.5
Post-warming survival rate (%)	201/236 (85)
No. of embryos warmed per cycle[a]	3.1 ± 1.0
No. of embryos transferred per patient[a]	2.7 ± 0.9
Clinical pregnancy rate (%)	34/77 (44)
Implantation rate (%)	40/201 (20)

[a]Mean ± SD.

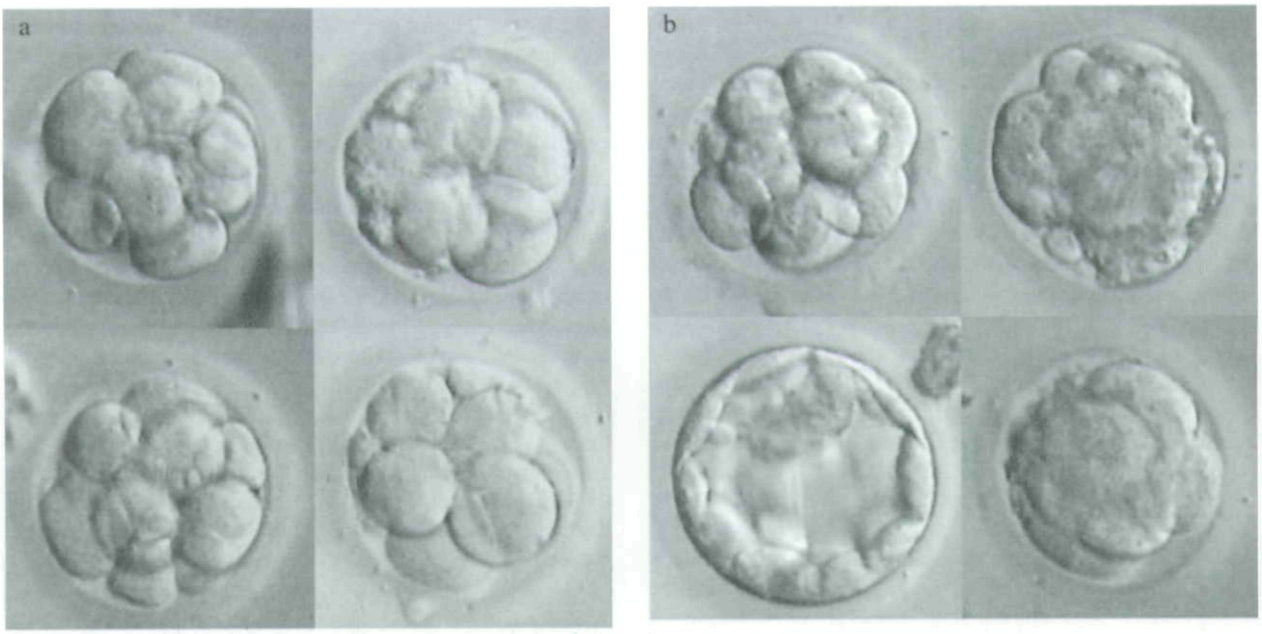

Figure 2. (**a**) Vitrified human embryos immediately after warming. (**b**) Warmed embryos after 24 h in culture. In **a** and b, original magnification ×300.

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

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.*, 2003).

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 cane). 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.

Acknowledgements

The authors thank the Cleveland Clinic Fertility Centre team of doctors, nurses and embryologists. We are also grateful to Dr Faten Abdel-Hafez for her assistance in manuscript preparation.

References

Archer J, Gook DA, Edgar DH 2003 Blastocyst formation and cell numbers in human frozen–thawed embryos following extended culture. *Human Reproduction* **18**, 1669–1673.

Bielanski A, Bergeron H, Lau PC, Devenish J 2003 Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology* **46**, 146–152.

Bielanski A, Nadin-Davis S, Sapp T, Lutze-Wallace C 2000 Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology* **40**, 110–116.

Chian RC, Kuwayama M, Tan L *et al.* 2004 High survival rate of bovine oocytes matured in vitro following vitrification. *Journal of Reproductive Development* **50**, 685–696.

Chung HM, Hong SW, Lim JM *et al.* 2000 In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. *Fertility and Sterility* **73**, 545–551.

Cohen J, Simons RF, Edwards RG *et al.* 1985 Pregnancies following the frozen storage of expanding human blastocysts. *Journal of In Vitro Fertilization and Embryo Transfer* **2**, 59–64.

Desai N, Blackmon H, Goldfarb J 2003 Can vitrification on cryoloops be used effectively for cryopreservation of dDay 3 cleavage stage embryos? *Fertility and Sterility* **80**, 142.

El-Danasouri I, Selman H 2001 Successful pregnancies and deliveries after a simple vitrification protocol for day 3 human embryos. *Fertility and Sterility* **76**, 400–402.

Fugger EF, Bustillo M, Katz LP *et al.* 1988 Embryonic development and pregnancy from fresh and cryopreserved sibling pronucleate human zygotes. *Fertility and Sterility* **50**, 273–278.

Hiraoka K, Kinutani M, Kinutani K 2004 Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Human Reproduction* **19**, 2884–2888.

Hong SW, Chung HM, Lim JM *et al.* 1999 Improved human oocyte development after vitrification: a comparison of thawing methods. *Fertility and Sterility* **72**, 142–146.

Huang CC, Lee TH, Chen SU *et al.* 2005 Successful pregnancy following blastocyst cryopreservation using super-cooling ultra-rapid vitrification. *Human Reproduction* **20**, 122–128.

Kasai M, Nishimori M, Zhu SE *et al.* 1992 Survival of mouse morulae vitrified in an ethylene glycol-based solution after exposure to the solution at various temperatures. *Biology of Reproduction* **47**, 1134–1139.

Kuleshova LL, Lopata A 2002 Vitrification can be more favorable than slow cooling. *Fertility and Sterility* **78**, 449–454.

Kuwayama M 2006 Highly efficient vitrification for cryopreservation of human oocytes and embryos: *Theriogenology*, 2006 Oct 19; [Epub ahead of print].

Kuwayama M, Vajta G, Ieda S, Kato O 2005a Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reproductive BioMedicine Online* **11**, 608–614.

Kuwayama M, Vajta G, Kato O, Leibo SP 2005b Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive BioMedicine Online* **11**, 300–308

Lane M, Schoolcraft WB, Gardner DK 1999 Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertility and Sterility* **72**, 1073–1078.

Lassalle B, Testart J, Renard JP 1985 Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. *Fertility and Sterility* **44**, 645–651.

Liebermann J, Tucker MJ 2006 Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertility and Sterility* **86**, 20–26.

Liebermann J, Tucker MJ 2004 Vitrifying and warming of human oocytes, embryos, and blastocysts: vitrification procedures as an alternative to conventional cryopreservation. *Methods of Mollecular Biology* **254**, 345–364.

Liebermann J, Tucker MJ 2002 Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. *Reproduction* **124**, 483–489.

Liebermann J, Dietl J, Vanderzwalmen P, Tucker MJ 2003 Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now? *Reproductive BioMedicine Online* **7**, 623–633.

Liebermann J, Tucker MJ, Graham JR *et al.* 2002 Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette. *Reproductive BioMedicine Online* **4**, 146–150.

Mukaida T, Nakamura S, Tomiyama T *et al.* 2003a Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. *Human Reproduction* **18**, 384–391.

Mukaida T, Takahashi K, Kasai M 2003b Blastocyst cryopreservation: ultrarapid vitrification using cryoloop technique. *Reproductive BioMedicine Online* **6**, 221–225.

Mukaida T, Nakamura S, Tomiyama T *et al.* 2001 Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertility and Sterility* **76**, 618–620.

Mukaida T, Wada S, Takahashi K *et al.* 1998 Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Human Reproduction* **13**, 2874–2879.

Park SP, Kim EY, Oh JH *et al.* 2000 Ultra-rapid freezing of human multipronuclear zygotes using electron microscope grids. *Human Reproduction* **15**, 1787–1790.

Rama Raju GA, Haranath GB, Krishna KM *et al.* 2005 Vitrification of human 8-cell embryos, a modified protocol for better pregnancy rates. *Reproductive BioMedicine Online* **11**, 434–437.

Reed ML, Lane M, Gardner DK *et al.* 2002 Vitrification of human blastocysts using the cryoloop method: successful clinical application and birth of offspring. *Journal of Assisted Reproduction and Genetics* **19**, 304–306.

Saito H, Ishida GM, Kaneko T *et al.* 2000 Application of vitrification to human embryo freezing. *Gynecology and Obstetrics Investigation* **49**, 145–149.

Son WY, Yoon SH, Park SJ *et al.* 2002 Ongoing twin pregnancy after vitrification of blastocysts produced by in-vitro matured oocytes retrieved from a woman with polycystic ovary syndrome: case report. *Human Reproduction* **17**, 2963–2966.

Stehlik E, Stehlik J, Katayama KP *et al.* 2005 Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reproductive BioMedicine Online* **11**, 53–57.

Takahashi K, Mukaida T, Goto T, Oka C 2005 Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. *Fertility and Sterility* **84**, 88–92.

Testart J, Lassalle B, Belaisch-Allart J *et al.* 1986 High pregnancy rate after early human embryo freezing. *Fertility and Sterility* **46**, 268–272.

US Department of Health and Human Services and the Center for Disease Control and Prevention 2006 *Assisted Reproductive Technology Success Rates 2004*. Published December 2006. CD-Rom.

Vajta G, Kuwayama M 2006 Improving cryopreservation systems. *Theriogenology* **65**, 236–244.

Vanderzwalmen P, Bertin G, Debauche C *et al.* 2003 Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. *Human Reproduction* **18**, 1504–1511.

Vanderzwalmen P, Bertin G, Debauche C *et al.* 2002 Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Human Reproduction* **17**, 744–751.

Walker DL, Tummon IS, Hammitt DG *et al.* 2004 Vitrification versus programmable rate freezing of late stage murine embryos: a randomized comparison prior to application in clinical IVF. *Reproductive BioMedicine Online* **8**, 558–568.

Yoon TK, Kim TJ, Park SE *et al.* 2003 Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertility and Sterility* **79**, 1323–1326.

Zech NH, Lejeune B, Zech H, Vanderzwalmen P 2005 Vitrification of hatching and hatched human blastocysts: effect of an opening in the zona pellucida before vitrification. *Reproductive BioMedicine Online* **11**, 355–361.

Zhu GJ, Jin L, Zhang HW *et al.* 2005 [Vitrification of human cleaved embryos in vitro fertilization-embryo transfer]. *Zhonghua Fu Chan Ke Za Zhi* **40**, 682–684.

Zhu SE, Kasai M, Otoge H *et al.* 1993 Cryopreservation of expanded mouse blastocysts by vitrification in ethylene glycol-based solutions. *Journal of Reproductive Fertility* **98**, 139–145.

Presented at the Conjoint Annual Meeting of the American Society of Reproductive Medicine and the Canadian Fertility Association, Montreal, Canada October 15–19, 2005.

Received 11 October 2006; refereed 6 November 2006; accepted 27 November 2006.