# Cryotop Vitrification as Compared to Conventional Slow Freezing for Human Embryos at the Cleavage Stage: Survival and Outcomes

Tseng-Kai Lin[1,2]*, Jin-Tsung Su[1], Fa-Kung Lee[1,2], Yu-Ru Lin[1], Hsiao-Ching Lo[1]

*[1]Department of Obstetrics and Gynecology, Hsinchu Cathy General Hospital, and [2]Department of Medical Laboratory Science and Biotechnology, Yuanpei University, Hsinchu, Taiwan.*

**SUMMARY**

**Objective:** This study was conducted to compare the efficacy of cryotop vitrification of human cleavage-stage embryos to that of conventional slow freezing of these embryos with respect to survival. A second objective was to compare the two cryopreservation techniques with respect to outcomes for a cohort of women.

**Materials and Methods:** Cleavage-stage embryos from 102 patients were cryopreserved either by vitrification (57 patients) or by traditional slow freezing (45 patients). After thawing, rates of embryo survival, implantation, and clinical pregnancy were determined.

**Results:** Survival of embryos was significantly higher with the vitrification procedure as compared to traditional slow freezing [287/298 (96.3%) *vs.* 294/446 (65.9%); $p < 0.05$). Rates of implantation and clinical pregnancy were also significantly higher using vitrification procedure as compared to the slow freezing procedure (24.3% *vs*. 7.1% and 35.6% *vs*. 15.6% respectively, $p < 0.05$).

**Conclusion:** As compared to conventional slow freezing, cryopreservation of human cleavage-stage embryo using vitrification results in higher rates of embryo survival, implantation, and clinical pregnancy. Vitrification therefore represents the superior cryopreservation technique for cleavage-stage embryos. [*Taiwan J Obstet Gynecol* 2010;49(3):272–278]

**Key Words:** cryopreservation, human cleavage-stage embryo, slow freezing, vitrification

## Introduction

Since the first report of a pregnancy following replacement of frozen-thawed human embryos [1], embryo cryopreservation has become a routine procedure. Embryonic cryopreservation has been found to increase cumulative pregnancy rates and to prevent the risk of multiple pregnancies. However, cryopreservation of human embryos significantly reduces their capacity for implantation [2].

Successful cryopreservation depends, at least in part, on the modality of the freeze-thaw procedure. Over the last decade, methods involving slow [1] and rapid [3] rates of cooling have been used successfully to cryopreserve embryos from a wide range of species. Slow-rate freezing protocols employing controlled rate freezers for gradual reduction of temperatures below –30°C have traditionally been used to freeze embryos in the clinical laboratory. Various cryoprotectant solutions have been utilized for dehydration; however, the efficacies of these solutions depend on the stage of the embryo. In 1985, Lassalle et al [4] described successful cryopreservation with a solution containing 1,2-propanediol (PROH) and cooling to –30°C. Since then,

*Correspondence to: Dr Tseng-Kai Lin, Department of Obstetrics and Gynecology, Hsinchu Cathay General Hospital, 678 Section 2, Chung-Hua Road, Hsin-Chu 300, Taiwan.
E-mail: ce140@cgh.org.tw
Accepted: March 4, 2010

slow cooling has become the most commonly used approach for cryopreservation of pronuclear and early cleavage (2- to 8-cell) stage embryos [4–6]. Glycerol-based cryoprotectant solutions have been found to be helpful for blastocyst cryopreservation [7]. However, slow-rate freezing procedures require expensive equipment and are considerably time-consuming. Moreover, in clinically-assisted reproductive technology programs, only a small number of embryos can be cryopreserved for an individual patient. For that reason, a faster and simpler method for cryopreservation of human embryos is desirable.

Recent attention has been focused on the application of vitrification technology to human embryo cryopreservation. With this technology, embryos suspended in a highly concentrated solution are preserved in liquid nitrogen without the formation of ice crystals [8]. Vitrification requires the addition of cryoprotectants at high concentrations and the use of high cooling rates such that the embryo is transformed into a "glasslike state" [9]. 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 shifts necessary for the success of this technique [10]. Carrier systems that have been developed for the vitrification procedure include the Open Pulled Straw [11], electron microscope grid [10,12], Flexipet [13], Cryoloop [14,15] and Cryotop [16].

The advantages of vitrification have been reviewed [9,17–19]. The elimination of ice crystal formation, made possible through the increased speed of temperature conduction such that chill-associated injuries are reduced, represents the principal benefit. A practical advantage is that the speed of the process minimizes the time during which the embryo is outside the incubator. The vitrification procedure involves minimal set-up time, can be performed as needed, and does not require expensive programmable freezing equipment.

To date, clinical reports of findings with the vitrification procedure are limited and have mainly described oocyte- [20,21] or blastocyst-stage [22–29] cryopreservation. By contrast, cryopreservation of human embryos at the cleavage stage using vitrification methodology has not been widely studied [15,30–33]. The present study was therefore undertaken to evaluate the efficacy of vitrification of human embryos at the cleavage stage in terms of embryo survival rate and to determine the outcomes of this procedure with respect to rates of implantation and clinical pregnancy. For comparison purposes, the efficacy and outcomes of cryopreservation of human cleavage-stage embryos by conventional slow freezing were also evaluated.

## Material and Methods

### *Patients*

A total of 102 subjects were enrolled in this retrospective study, which was conducted between January 2003 and August 2009. Prior to January 2007, a slow-freezing protocol was routinely employed for cryopreservation of human cleavage-stage embryos. The practice of vitrification of day 2–3 surplus embryos was initiated in the clinic in April 2007. The policy of the clinic was to perform day 2–3 embryo transfers to patients who have undergone *in vitro* fertilization/intracytoplasmic sperm injection treatments with cryopreservation of the surplus good quality embryos performed on the day of transfer. Good quality embryos not selected for transfer were considered for cryopreservation on day 2–3 if they were 2–4 blastomeres with less than 20% fragmentation for day 2 and 6–8 blastomeres with less than 20% fragmentation for day 3.

### *Ovarian stimulation procedure*

Subcutaneous injection of leuprolide acetate (Lupron; Abbott Laboratories, Chicago, IL, USA) for pituitary desensitization was initiated on the 20$^{th}$ day of the menstrual cycle with daily injections continued until human chorionic gonadotrophin (hCG; Pregnyl; Organon, Oss, the Netherlands) was administered. Gonadotrophins, a combination of menotrophin (Menopur; Ferring, GmbH, Germany) and recombinant follicle stimulation hormone (r-FSH; Gonal-F; Serono, Aubonne, Switzerland), were administered intramuscularly from the third day of the menstrual cycle (once daily). The r-FSH and menotrophin dosages were adjusted for each subject according to the ovarian response to stimulation. Beginning with the seventh day of the menstrual cycle, transvaginal ultrasonography was performed once daily or every other day to monitor follicular growth. When two or three dominant follicles reached a mean diameter of 19 mm, ovulation was induced with 10,000 IU of hCG. Transvaginal ovum pick-up was then performed under ultrasonographic guidance 34–36 hours later.

### *Slow-freezing and thawing procedures*

Embryos were first placed in equilibration solution comprising 1.5 M PROH in phosphate-buffered saline, supplemented with 20% (v/v) maternal serum at room temperature for 5 minutes and then transferred to a freezing solution (1.5 M PROH and 0.2 M sucrose) in phosphate-buffered saline supplemented with 20% maternal serum for an additional 5 minutes. Following exposure to the final freezing solution, embryos were transferred to 0.25 mL plastic straws and placed in a programmable freezer (Kryo 10 series; Planar Products,

Sunbury Thames, UK) for cooling. Embryos were cooled at rate of −2°C/min to −7°C. Ice crystal formation was then induced by placing the straw in contact with a metal object that had been cooled in liquid nitrogen for seeding. Cooling was continued at a rate of −0.3°C/min to −30°C, and the straw was then plunged into liquid nitrogen for storage. For the thawing process, the straw was removed from storage, held at ambient temperature for 6 seconds, and immersed in a water bath at 37°C for 30–40 seconds. For the dilution procedure, embryos were transferred to the following solutions in series and held in each for 5 minutes at room temperature: 1.0 M PROH containing 0.2 M sucrose, 0.5 M PROH containing 0.2 M sucrose, and 0.2 M sucrose. Embryos were then transferred to sucrose-free medium and held at room temperature for 5 minutes.

### *Vitrification and thawing procedure*

Beginning in March 2007, vitrification was employed as the routine strategy for cryopreservation of human cleavage-stage embryos. Vitrification of surplus embryos was performed according to the two-step protocol of Mukaida et al [30]. This technique involves the use of dimethylsulphoxide (DMSO), ethylene glycol, and sucrose as cryoprotective agents. All steps were performed in a laminar flow hood at room temperature. Embryos were incubated in Vitrification Solution #1, which consisted of 7.5% (v/v) DMSO and 7.5% (v/v) ethylene glycol, for 5–6 minutes during which the appropriate morphological changes were observed. Embryos were then moved to Vitrification Solution #2, which was comprised of 15% (v/v) DMSO, 15% (v/v) ethylene glycol, and 0.65 M sucrose and were held for 30 seconds. With the aid of a fine micropipette and a dissecting microscope, the embryos were quickly placed onto a Cryotop (Kitazato Supply Co., Fujinomiya, Japan), followed by aspiration of excess medium with a fine pipette. The Cryotop with embryos was then quickly immersed in liquid nitrogen and the plastic cap was pulled over the film portion of the Cryotop, followed by continued storage in liquid nitrogen. For warming, the protective cover was removed from the Cryotop while it was submerged in liquid nitrogen, and the polypropylene strip of the Cryotop was immersed directly into 3 mL of 1.0 M sucrose at 37°C for 1 minute. Embryos were retrieved and held for 3 minutes in 1 mL of a dilution solution consisting of 0.5 M sucrose in TCM199 medium containing 20% serum substitute supplement, followed by two washes with TCM199 medium supplemented with 20% serum substitute supplement for 5 minutes each at room temperature. Embryos were then subjected to culture as detailed below.

### *Assessment of embryo survival*

After thawing, embryos were placed in culture medium (Cleavage medium; SAGE, BioPharma, USA) supplemented with 10% serum protein substitute under oil at 37°C in an atmosphere containing 5% $O_2$, 5% $CO_2$, and 90% $N_2$ and immediately evaluated for integrity and number of surviving blastomeres. Embryos were considered to have survived if at least half of their blastomeres were intact after thawing. Surviving embryos were incubated at 37°C for an additional 4–6 hours during which time subjects were prepared for transfer.

Patients were prepared for frozen-warmed embryo transfer using hormone replacement therapy consisting of increasing doses of estromon (2.5–5 mg p.o. daily) and progesterone (Utrogestan, 200 mg, 3 times daily) for luteal supplementation. Pregnancy was confirmed by measuring serum β-hCG levels 12–13 days after embryo transfer. An intrauterine gestational sac with active fetal heart beat as visualized on ultrasound examination 6 weeks after embryo transfer was considered to be a clinical pregnancy. The implantation rate was derived from the number of gestational sacs divided by the total number of embryos transferred. Multiple pregnancies were defined as a gestation with more than one gestational sac. Ongoing pregnancy was defined as pregnancy proceeding beyond the 12$^{th}$ gestational week. Abortion was defined as fetal loss at less than 12 weeks of gestation.

### *Statistical analysis*

The vitrification and slow freezing protocols were compared with respect to rates of post-thaw embryo survival, implantation, and pregnancy using $\chi^2$ analysis. Patient age and number of embryos transferred per cycle were compared using the Student's *t* test. A *p* value of $< 0.05$ was considered statistically significant.

## Results

Table 1 presents the characteristics of patients in their last fresh assisted reproductive technology (ART) cycle for the vitrification and slow-freezing groups. The percentages of *in vitro* fertilization and intracytoplasmic sperm injection cycles were found to be similar between the two groups. Moreover, no significant differences in the percentages of patients with each infertility indication or in the number of previous ART cycles were observed between the two groups. However, the mean age of patients was significantly higher in the vitrification group ($33.1 \pm 4.0$ years *vs.* $31.1 \pm 3.9$ years; $p = 0.010$). The clinical outcomes of the frozen-thawed cleavage-stage embryos for the vitrification and slow-freezing groups are

**Table 1.** Characteristics of patients in the last fresh assisted reproductive technology cycle*

| Variable | Vitrification ($n=57$) | Slow-freezing ($n=45$) | $p$ |
|---|---|---|---|
| Age (yr) | 33.1±4.0 | 31.1±3.9 | 0.010 |
| IVF cycles | 23 (40.4) | 13 (28.9) | 0.320 |
| ICSI cycles | 34 (59.6) | 32 (71.1) | 0.320 |
| Infertility factor | | | |
| Male | 5 (8.8) | 8 (17.8) | 0.165 |
| Female | 14 (24.6) | 18 (40.0) | 0.199 |
| Combined | 38 (66.7) | 19 (42.2) | 0.019 |
| Previous ART cycles | 0.4±0.6 | 0.3±0.6 | 0.858 |
| Supernumerary embryos | 6.0±3.1 | 10.3±4.8 | <0.001 |

**Data are presented as n (%) or mean±standard deviation, analyzed by $\chi^2$ test and student's t test, respectively. ART=assisted reproductive technology; IVF=in vitro fertilization; ICSI=intracytoplasmic sperm injection.*

**Table 2.** Clinical outcomes of human cleavage-stage embryo transfer after cryopreservation with the vitrification and slow-freezing methods*

| Outcome | Vitrification | Slow-freezing | $p$ |
|---|---|---|---|
| Cycles | 59 | 45 | |
| Embryos warmed | 298 (5.05±1.86) | 446 (9.91±3.83) | <0.001 |
| Cryosurvival | 287/298 (96.4) | 294/446 (65.9) | <0.001 |
| Embryos transferred | 169 (2.86±1.11) | 155 (3.44±0.89) | <0.005 |
| Implantation rate | 41/169 (24.3) | 11/155 (7.1) | <0.001 |
| Clinical pregnancy rate | 21/59 (35.6) | 7/45 (15.6) | 0.040 |
| Multiple pregnancy rate | 13/21 (61.9) | 3/7 (42.9) | 0.659 |
| Ongoing pregnancies | 20/59 (33.9) | 6/45 (13.3) | 0.030 |
| Abortions | 1/21 (4.8) | 1/7 (14.3) | 0.397 |
| Deliveries | 8 (3 twins, 5 singletons) | 5 (5 singletons) | |

**Data are presented as n (mean±standard deviation) or n (%), analyzed by student's t test and $\chi^2$ test, respectively.*

summarized in Table 2. The mean number of thawed embryos was significantly lower in the vitrification as compared to the slow-freezing group (5.05±1.86 *vs.* 9.91±3.83; $p<0.001$) whereas the survival rate per thawed embryo was significantly higher in the vitrification as compared to the slow-freezing group (96.4% *vs.* 65.9 %; $p<0.001$). Despite the lower mean number of embryos selected for transfer in the vitrification group (2.86±1.11 *vs.* 3.44±0.89; $p=0.005$), the clinical pregnancy and implantation rates were significantly higher as compared with those for the slow-freezing group (35.6% *vs.* 15.6%, $p=0.040$; and 24.3% *vs.* 7.1%, $p<0.001$). To date, 16 healthy infants have been born and two miscarriages have occurred in each group, and no congenital anomalies have been observed.

## Discussion

Relatively few studies evaluating the clinical outcomes for human cleavage-stage embryos subjected to vitrification as compared to slow freezing have been performed [15,33,34]. Zheng et al [34] reported higher survival of biopsied 8-cell stage embryos following vitrification as compared with slow freezing (94% *vs.* 15%). However, no statistical difference was observed between the groups with respect to development to the blastocyst stage. In a larger clinical study, Kuwayama et al [25] observed significant improvement of 4-cell stage embryo survival following cryotop vitrification as compared with slow freezing (98% *vs.* 91%). However, these investigators reported similar pregnancy rates per transfer for vitrification and slow freezing (27% and 32%, respectively). Rama Raju et al [15] reported higher survival of human 8-cell embryos following cryoloop vitrification as compared to slow freezing (95% *vs.* 60%) and observed significantly better implantation and pregnancy rates for the vitrification as compared with the slow freezing group. In the present study, an overall embryo survival rate of 96.4%, a clinical pregnancy rate of 35.6%, and an implantation rate of 24.3% was observed using the cryotop vitrification procedure. Moreover, warming was required for fewer embryos in the vitrification was compared to the slow freezing group;

patients in the vitrification group had an increased opportunity to receive all post-thawed embryos because of the higher survival rate per total warmed embryo (96.4% for the vitrification as compared to 65.9% for the slow freezing group). To compensate for the poor survival rate of the slow frozen-thawed embryos and to increase the chances of successful pregnancy with these embryos, it was necessary to increase the number of embryos for the transfer. Regardless, the clinical outcomes of pregnancy and implantation rates were found to increase significantly in the vitrification group (35.6% and 25.3%, respectively) as compared to the slow freezing group (15.6% and 7.1%, respectively).

Successful vitrification mandates that ice crystal formation be avoided. Factors necessary for successful vitrification have been previously determined to include a faster cooling rate, a high viscosity cryoprotectant solution, and a small vitrification solution volume [8,35]. Theoretically, minimizing the volume of the vitrification solution containing the embryos not only permits increases in both cooling and warming rates but also decreases the chance of ice crystal formation [8]. To decrease the volume of the vitrification solution, Arav et al [36] developed a vitrification method for immature bovine oocytes that involved a "minimum drop size" of 0.1–0.5 μL droplets which were loaded onto glass coverslip strips and immersed in liquid nitrogen or nitrogen slush. Cryotops can also be adapted for reduction in vitrification solution volume [16]. The solution volume within the thin film strip in a Cryotop is less than 0.1 μL. In the present study, the Cryotop was modified for cleavage-stage embryo vitrification and the cryopreservation solution volume used during the vitrification process was made sufficiently small to reduce the potential damage to the embryo during the freezing process [16], thereby increasing the chances for a successful outcome.

A notable advantage of vitrification as compared to the conventional slow-freezing methods is the very short time period (seconds) required for embryos to cool; with the vitrification procedure embryos freeze almost immediately, well before extracellular crystal formation can occur. Osmotic effects and chilling injuries are therefore virtually eliminated [11]. In contrast, programmed slow-freezing procedures require a minimum of 2 hours per run and several freezer units to accommodate cryopreservation for a large number of patients.

A major finding of the present study was the remarkably low percentage (3.6%) of embryos with significant cellular damage after application of the vitrification technique. By contrast, with the conventional slow-freezing protocol using cryostraws, almost one-third of the surviving embryos exhibited damage in excess of 50%. This degree of damage was associated with blastomere loss and degeneration. In this regard, it should be noted that blastomere loss in frozen-thawed early cleavage-stage embryos is strongly associated with a reduction in implantation potential [37]. Frozen embryo transfer cycles in which all transferred embryos remain fully intact at thawing achieve a better outcome than those in which at least one partially damaged embryo is transferred [38,39]. Moreover, reduced rates of pregnancy [40] and implantation [41] have been linked to transfer of embryos that are partially damaged after thawing.

Despite the consistently good findings with the vitrification technique, certain concerns remain. For example, contamination of embryos by viruses present in liquid nitrogen storage vessels is possible [42–45]. However, the findings of Kyuwa et al [43] revealed that such cross-contamination is unlikely. These investigators observed no viral contamination of mouse 2-cell embryos stored for one year in cryotubes in a liquid nitrogen vessel in which cryotubes containing mouse hepatitis virus or *Pasteurella pneumotropica* were also stored. Although the potential for microbial contamination of embryos stored in liquid nitrogen vessels also exists, current findings are inconsistent with the likelihood of such contamination [43,45].

Because vitrification is a relatively new cryopreservation method, prenatal outcome is a major concern. At the time of this writing, eight of the 21 pregnancies resulting from embryo transfers performed in this study have resulted in the births of 11 healthy babies. In the single largest study addressing this issue [46], the neonatal outcome of 817 nylon loop vitrified-warmed day 3 embryo transfers was compared with that of 1,576 fresh day 3 embryo transfers. No statistical differences were observed with respect to mean gestational age, mean birth weight, congenital birth defect rate, and karyotypic abnormalities. Although these findings are encouraging, follow-up studies are necessary to ensure that the vitrification approach for cryopreservation is truly safe.

In conclusion, the high rates of embryo survival, implantation, and clinical pregnancy that follow cryopreservation of human cleavage-stage embryos by vitrification render this approach superior to cryopreservation of these embryos by conventional slow-freezing.

## Acknowledgments

This work was supported by a research grant CMRI-09801 from the Cathay General Hospital, Taipei, Taiwan.

## References


1. Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* 1983;305:707–9.
2. Edgar DH, Bourne H, Speirs AL, McBain JC. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum Reprod* 2000;15:175–9.
3. Gordts S, Roziers P, Campo R, Noto V. Survival and pregnancy outcome after ultrarapid freezing of human embryos. *Fertil Steril* 1990;53:469–72.
4. Lassalle B, Testart J, Renard JP. Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. *Fertil Steril* 1985;44:645–51.
5. Testart J, Lassalle B, Belaischallart J, et al. High pregnancy rate after early human embryo freezing. *Fertil Steril* 1986; 46:268–72.
6. Fugger EF, Bustillo M, Katz LP, et al. Embryonic development and pregnancy from fresh and cryopreserved sibling pronucleate human zygotes. *Fertil Steril* 1988;50:273–8.
7. Cohen J, Simons RF, Edwards RG, Fehilly CB, Fishel SB. Pregnancies following the frozen storage of expanding human blastocysts. *J In Vitro Fert Embryo Transf* 1985;2:59–64.
8. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at –196 degrees C by vitrification. *Nature* 1985; 313:573–5.
9. Vajta G, Kuwayama M. Improving cryopreservation systems. *Theriogenology* 2006;65:236–44.
10. Park SP, Kim EY, Oh JH, et al. Ultra-rapid freezing of human multipronuclear zygotes using electron microscope grids. *Hum Reprod* 2000;15:1787–90.
11. Vajta G, Holm P, Kuwayama M, et al. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev* 1998;51:53–8.
12. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol Reprod* 1996;54:1059–69.
13. Liebermann J, Tucker MJ, Graham JR, et al. Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette. *Reprod Biomed Online* 2002; 4:146–50.
14. Lane M, Schoolcraft WB, Gardner DK. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril* 1999;72:1073–8.
15. Rama Raju GA, Haranath GB, Krishna KM, et al. Vitrification of human 8-cell embryos, a modified protocol for better pregnancy rates. *Reprod Biomed Online* 2005;11:434–7.
16. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology* 2007;67:73–80.
17. Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. *Fertil Steril* 2002;78:449–54.
18. Liebermann J, Tucker MJ. Vitrifying and warming of human oocytes, embryos, and blastocysts: vitrification procedures as an alternative to conventional cryopreservation. *Methods Mol Biol* 2004;254:345–64.
19. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online* 2006;12:779–96.
20. Yoon TK, Kim TJ, Park SE, et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertil Steril* 2003;79:1323–6.
21. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online* 2005;11:300–8.
22. Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology* 2007;67:73–80.
23. Hiraoka K, Hiraoka K, Kinutani M, Kinutani K. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Hum Reprod* 2004;19:2884–8.
24. Huang CC, Lee TH, Chen SU, et al. Successful pregnancy following blastocyst cryopreservation using super-cooling ultra-rapid vitrification. *Hum Reprod* 2005;20:122–8.
25. Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reprod Biomed Online* 2005;11:608–14.
26. Stehlik E, Stehlik J, Katayama KP, et al. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reprod Biomed Online* 2005;11:53–7.
27. Takahashi K, Mukaida T, Goto T, Oka C. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. *Fertil Steril* 2005;84:88–92.
28. Zech NH, Lejeune B, Zech H, Vanderzwalmen P. Vitrification of hatching and hatched human blastocysts: effect of an opening in the zona pellucida before vitrification. *Reprod Biomed Online* 2005;11:355–61.
29. Liebermann J, Tucker MJ. Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertil Steril* 2006;86:20–6.
30. Mukaida T, Wada S, Takahashi K, Pedro PB, An TZ, Kasai M. Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Hum Reprod* 1998;13:2874–9.
31. Saito H, Ishida GM, Kaneko T, et al. Application of vitrification to human embryo freezing. *Gynecol Obstet Invest* 2000;49:145–9.
32. El-Danasouri I, Selman H. Successful pregnancies and deliveries after a simple vitrification protocol for day 3 human embryos. *Fertil Steril* 2001;76:400–2.
33. Balaban B, Urman B, Ata B, et al. A randomized controlled study of human Day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. *Hum Reprod* 2008;23:1976–82.
34. Zheng WT, Zhuang GL, Zhou CQ, et al. Comparison of the survival of human biopsied embryos after cryopreservation with four different methods using non-transferable embryos. *Hum Reprod* 2005;20:1615–8.
35. Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. Potential importance of vitrification in reproductive medicine. *Biol Reprod* 2002;67:1671–80.
36. Arav A, Shehu D, Mattioli M. Osmotic and cytotoxic study of vitrification of immature bovine oocytes. *J Reprod Fertil* 1993;99:353–8.
37. Guerif F, Bidault R, Cadoret V, Couet ML, Lansac J, Royere D. Parameters guiding selection of best embryos for transfer

after cryopreservation: a reappraisal. *Hum Reprod* 2002;17: 1321–6.
38. Edgar DH, Jericho H, Bourne H, McBain JC. The influence of prefreeze growth rate and blastomere number on cryosurvival and subsequent implantation of human embryos. *J Assist Reprod Genet* 2001;18:135–8.
39. El-Toukhy T, Khalaf Y, Al-Darazi K, Andritsos V, Taylor A, Braude P. Effect of blastomere loss on the outcome of frozen embryo replacement cycles. *Fertil Steril* 2003;79:1106–11.
40. Archer J, Gook DA, Edgar DH. Blastocyst formation and cell numbers in human frozen-thawed embryos following extended culture. *Hum Reprod* 2003;18:1669–73.
41. Van den Abbeel E, Camus M, Van Waesberghe L, Devroey P Van Steirteghem AC. Viability of partially damaged human embryos after cryopreservation. *Hum Reprod* 1997;12: 2006–10.
42. Bielanski A, Nadin-Davis S, Sapp T, Lutze-Wallace C. Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology* 2000;40:110–6.
43. Kyuwa S, Nishikawa T, Kaneko T, et al. Experimental evaluation of cross-contamination between cryotubes containing mouse 2-cell embryos and murine pathogens in liquid nitrogen tanks. *Exp Anim* 2003;52:67–70.
44. Bielanski A, Bergeron H, Lau PC. Devenish J. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology* 2003;46:146–52.
45. Morris GJ. The origin, ultrastructure, and microbiology of the sediment accumulating in liquid nitrogen storage vessels. *Cryobiology* 2005;50:231–8.
46. Rama Raju GA, Jaya Prakash G, Murali, Krishna K, Madan K. Neonatal outcome after vitrified day 3 embryo transfers: a preliminary study. *Fertil Steril* 2009;92:143–8.