The CryoLoop facilitates re-vitrification of embryos at four successive stages of development without impairing embryo growth

Courtney B. Sheehan, Michelle Lane and David K. Gardner

Research Department, Colorado Center for Reproductive Medicine, Englewood, CO, USA

BACKGROUND: Vitrification has been shown to be an effective method of cryopreservation, but little is known about re-vitrification of embryos. This study investigated the effect of re-vitrification on mouse embryo preimplantation development and viability post-transfer. METHODS: Mouse embryos at the 1-cell stage were vitrified using the CryoLoop technique. Embryos were warmed and then re-vitrified successively at the 2-, 8-cell and blastocyst stages. The effects of multiple rounds of vitrification on development, differentiation and viability were assessed and compared with non-vitrified embryos. RESULTS: Development to the 8-cell stage on day 3 and blastocyst on day 5 were not affected by re-vitrification. However, better hatching rates were observed in the non-vitrified control group. Total cell number and the number of cells allocated to the inner cell mass (ICM) were not different between treatments. The percentage of ICM development was also not different between treatments. Implantation rate and fetal weights were the same between treatments. However, overall there were fewer fetuses per embryo transferred in the re-vitrified group. CONCLUSION: Re-vitrification of mouse embryos has minimal effect on preimplantation embryo development or implantation potential.

Key words: blastocyst/cryopreservation/embryo culture/vitrification

Introduction

Since the inception of embryo cryopreservation in 1972 (Whittingham et al., 1972; Wilmut, 1972), there have been significant improvements in our ability to cryopreserve gametes and preimplantation stage mammalian embryos. Slow freezing remains the common method of cryopreservation in most IVF laboratories (Fabbri et al., 2001; Veeck, 2003; Anderson et al., 2004); however, vitrification is a technique with which many laboratories have had success (Mukaida et al., 2003; Kuwayama et al., 2005a). Slow freezing uses a combination of permeant (typically propanediol or glycerol) and non-permeant (sucrose) cryoprotectants to initiate dehydration in preparation for slow cooling. After seeding, whereby extracellular ice formation further draws water outside the cell, the embryo is cooled to between −32 and −36°C before plunging into liquid nitrogen (LN2). Vitrification commonly uses a combination of permeant cryoprotectants at higher concentrations [typically dimethyl sulphoxide (DMSO) and ethylene glycol (EG)], as well as sucrose to dehydrate the embryo in preparation for cryopreservation. In contrast to slow freezing, embryos are immediately plunged into LN2 so that both intracellular and extracellular ice crystal formation are prevented.

In assisted reproduction technology (ART), cryopreservation of embryos maximizes the potential of a given IVF cycle from an ethical, financial and medical perspective. Criticism of IVF often stems from the disposal of supernumerary embryos, which is circumvented by cryopreserving these embryos for use by the couple in the future or for embryo donation. Furthermore, hormonal ovarian stimulation is not without cost and may be associated with certain health risks, therefore, patients are cautioned to limit their exposure to these drugs. Performing a frozen embryo transfer increases the overall efficacy of ART, by facilitating subsequent embryo transfers in natural cycles. Storage of supernumerary embryos can also be performed for a variety of medical reasons, including ovarian hyperstimulation syndrome (OHSS) (Van den Abbeel et al., 1988; Ferraretti et al., 1999) and for fertility preservation for cancer patients. In addition, the movement towards single embryo transfer, to limit multiple gestations, will call for an increased number of embryos to be cryopreserved (Gerris et al., 2004).

There is, therefore, an immediate need to assess which cryopreservation technique is preferential in human IVF. When comparing methods of cryopreserving mouse and bovine oocytes and embryos, vitrification has demonstrated advantages over conventional slow rate cooling (Vajta et al., 1998;
Lane et al., 1999). Furthermore, it has been shown that ultra-rapid vitrification procedures can be used to vitrify mouse embryos at individual stages of development without any loss of viability (Lane et al., 1999). In specific studies, vitrification has been shown to be more successful for human embryos compared to slow freezing with regard to survival, ongoing pregnancy and implantation rates (Kuleshova and Lopata, 2002; Stehlik et al., 2005).

Ultra-rapid vitrification employs the use of minute tools that allow the loading of the embryos in sub-microlitre volumes. There are now several methods available for this, such as the electron microscope grid (Martino et al., 1996), the open pulled straw (OPS; Vajta et al. 1998), solid surface vitrification (Dinnyes et al., 2000), microdrops (Papis et al., 2000), cryotop (Hochi et al., 2001), nylon mesh (Matsumoto et al., 2001) and cryotop (Kuwayama et al., 2005a). All these approaches work on the same basic premise, that ultra-rapid vitrification is a quick procedure in which embryos are out of the incubator for less than 5 min, compared with slow freezing where equilibration alone may take more than 20 min.

One method of ultra-rapid vitrification employed in our laboratory is the CryoLoop (Lane et al., 1999). A distinct advantage of this technique is the ability to visualize the gamete or embryo at every step of the procedure. Furthermore, it is less technically demanding than other methods of ultra-rapid vitrification. Additionally, supplementing the cryopreservation media with an antioxidant such as ascorbate has been shown to reduce oxidative stress, increase survival and stimulate inner cell mass (ICM) development (Lane et al., 2002).

To assess the merits of either slow freezing or vitrification, it was decided to use both techniques of cryopreservation on successive stages of embryonic development. Using this approach of re-cryopreservation, one can determine cumulative damage to the embryo and therefore select the procedure that is the least stressful to the conceptus. Although it is not envisaged that re-cryopreservation will become a routine procedure in human IVF, there are situations where such a technique can be of value. For example, the availability of a safe and effective method of re-cryopreservation may allow all pronucleate (PN)-stage embryos from a patient who underwent embryo cryopreservation because of OHSS to be warmed, cultured together to benefit from group culture and re-cryopreserved at the blastocyst stage for future transfers. Although this has been successfully performed with slow freezing (Farhat et al., 2001) and vitrification (Smith et al., 2005), one study showed a drop in cell number following re-freezing of mouse embryos (Vitale et al., 1997).

The aim of this study was therefore to compare the efficacy of vitrification and slow freezing for repeatedly cryopreserving mouse embryos at successive stages of development.

Materials and methods

Media

The medium for embryo collection and the base medium for vitrification solutions were a 4-4-Morpholinepropanesulfonic acid (MOPS)-buffered version of medium G1 designated G-MOPS (Lane and Gardner, 2004) (Vitrolife, Gothenburg, Sweden) and were subsequently supplemented with 5 mg/ml of human serum albumin (HSA). Sequential media for embryo culture were G1 and G2 (series 3) supplemented with 5 mg/ml of HSA (Vitrolife).

Embryo collection

All procedures were approved by the Institutional Animal Care and Users Committee. Four-week-old F1 hybrid (C57BL/6 × CBA) females (Jackson Laboratory, Bar Harbor, ME, USA) were superovulated by administering 5 IU pregnant mare’s serum gonadotrophin (PMSG; Sigma Chemical), 48 h later 5 IU of hCG (Pregnyl; Organon Inc., West Orange, NJ, USA) was administered, and females were mated with males of the same strain. Zygotes were collected at 21 h post-hCG in G-MOPS and denuded by incubation with 1 mg/ml hyaluronidase (0.5 mg/ml bovine testes, Type IV; Sigma Chemical) for 1 min. Zygotes with normal morphology were washed three times in G-MOPS and allocated to either culture or cryopreservation.

Embryo culture

Embryos were cultured in groups of 10 in 20 μl drops of medium under paraffin oil (G-OIL, Vitrolife). At the 1-cell stage, embryos were allocated to the vitrification or the control group. All embryos were cultured in medium G1 for 48 h to the four- to 8-cell stage and then transferred to G2 for a further 48 h. All cultures were performed at 37°C in 6% CO2, 5% O2 and 89% N2 (Gardner and Lane, 2004). Embryo development was assessed after 24, 48, 78 and 96 h of culture for on-time development to the 2-, 8-cell, blastocyst and expanded and hatching blastocyst stages, respectively.

Vitrification and warming of 1-, 2-, 8-cell- and blastocyst-stage embryos

Embryos were vitrified using the CryoLoop procedure (Lane et al., 1999). All vitrification and warming procedures were performed at 37°C. Embryos were vitrified in a two-step procedure in G-MOPS supplemented with 5 mg/ml of HSA with cryoprotectants DMSO and EG. In addition, 0.1 mmol/l ascorbate was added to all vitrification and warming solutions (Lane et al., 2002).

For vitrification, embryos were placed in G-MOPS containing 8% DMSO and 8% EG for 1 min for 1-, 2- and 8-cell-stage embryos and for 1 min and 45 s for blastocyst-stage embryos. Embryos were subsequently moved into G-MOPS containing 16% DMSO, 16% EG, 10 mg/ml of Ficoll 400 and 0.65 mol/l sucrose for less than 30 s. The CryoLoop consists of a 2-mm nylon loop at the end of a stainless steel rod (Hampton Research, Laguna Niguel, CA, USA). By dipping the loop in the second vitrification solution, a film was created. The surface tension, due to the low volume and Ficoll, kept this film intact for the vial and dipped into G-MOPS containing 0.25 mol/l sucrose at 37°C. After 1 min in the first solution, the embryos were moved into G-MOPS containing 0.125 mol/l sucrose for 2 min. Embryos were transferred to G-MOPS at 37°C for 5 min before being moved to wash drops of G1 or G2, depending on developmental stage, and placed into culture.

Slow freezing procedure for 1-, 2- and 8-cell embryos

All slow freezing procedures were performed at room temperature. The base medium for all freezing solutions was G-MOPS with 5 mg/ml of HSA. Regardless of stage, embryos were first exposed to G-MOPS containing 0.75 mol/l propanediol (Sigma Chemical) for 10 min, then moved to G-MOPS with 1.5 mol/l propanediol solution for 10 min and then to G-MOPS with 1.5 mol/l propanediol and 0.1 mol/l sucrose solution for 7 min (Emiliani et al., 2000). Embryos were...
loaded into 25-cc straws (Institute Medicine Vetinaire, Bicef, L’Aigle, France) in groups of 15 to 20 and placed into a freezing machine (Freeze Control CL2000; CryoLogic, Napa, CA, USA). The starting temperature of the freezing procedure was 20°C. Embryos were cooled at the rate of 1.0°C/min to −6°C, seeded at −6°C and held for 10 min, then were cooled at the rate of 0.3°C/min to −37°C before plunging into LN₂. Embryos were stored in LN₂ for a minimum of 24 h before thawing.

Embryos were thawed in air for 15 s and placed in a 30°C water bath for 30 s. The base medium for thawing was G-MOPS supplemented with 5 mg/ml of HSA. Embryos were thawed in a four-step procedure at 20°C to remove the cryoprotectants. Embryos were expelled into 1.5 mol/l propanediol with 0.2 mol/l sucrose for 10 min, then moved to 0.75 mol/l propanediol with 0.2 mol/l sucrose for 10 min and to 0.2 mol/l sucrose for 10 min in G-MOPS. Finally, embryos were washed in G-MOPS at 37°C for 10 min and placed into culture.

**Allocation of cells to the ICM and trophectoderm**

Allocation of cells of the blastocyst was determined using previously published techniques (Hardy et al., 1989), with a further minor modification using G-MOPS.

**Assessment of embryo viability**

To assess the viability of blastocysts, embryos were transferred to day 4 asynchronous (day minus 1) (C57BL/6 × CBA/Ca) (Jackson Laboratory) pseudopregnant female recipients. Six blastocysts were transferred to either uterine horn from each treatment. Non-vitrified controls and vitrified blastocysts were alternately transferred to the left and right horn of recipients. On day 15 of pregnancy, implantation, fetal development and fetal weights were assessed.

**Experimental outline**

Following collection from the oviduct, half of the zygotes were immediately placed in culture (control) and the other half allocated to the vitrification procedure. Those embryos allocated to the vitrification procedure were vitrified and stored for 24 h in LN₂. Embryos were then warmed, washed in G1 and cultured to the 2-cell stage. At the 2-cell stage, embryos were vitrified, stored for 24 h, warmed, washed in G1 and grown to the 8-cell stage. The embryos were once again vitrified, stored for 24 h, warmed, washed and then cultured in G2 for a further 48 h to the blastocyst stage. Blastocysts were then vitrified and warmed before embryo transfer.

The experimental design was subsequently repeated using slow freezing rather than vitrification.

**Statistics**

Data for embryo morphology and results from embryo transfer experiments were analysed by linear logistic regression using the Software package GLIM. The day of experiment was fitted as a factor. Between treatment, differences were assessed using the log likelihood statistic. Data for cell numbers and fetal weights were assessed by analysis of variance (ANOVA) using Bonferroni post-comparison. No between-replicate differences were detected, and so, data from all experiments were subsequently pooled for analysis. Between treatment, differences were determined using Bonferroni’s multiple comparison procedures. A P value of <0.05 was considered significant.

**Results**

**Vitrification**

The survival rates of embryos vitrified at successive stages of development (n = 113) was 100% for the zygote and 2-cell stages and 99% for the 8-cell stage. Of those embryos surviving, vitrification had no effect on cleavage rates or rates of development to the 8-cell or blastocyst stages. The rate of compaction for non-vitrified controls (n = 136) and embryos vitrified a total of two times was 64.7 and 70.8%, respectively. Blastocyst development was not different between non-vitrified controls and embryos vitrified a total of three times (88.2 and 87.6%, respectively). However, blastocyst hatching was significantly improved in the non-vitrified control group compared to re-vitrified embryos (81.6 versus 41.6%, respectively; P < 0.01) (Figure 1).

There were no significant differences between control and re-vitrified embryos when comparing blastocyst cell numbers (86.1 ± 2.4 versus 81.5 ± 2.4), ICM cell numbers (18.2 ± 0.5 versus 17.3 ± 0.7), trophectoderm (TE) cell number (67.9 ± 1.3 versus 64.2 ± 2.0) (Figure 2) and percentage ICM development (21.1 ± 0.5 versus 21.3 ± 0.6%). There was no difference in blastocyst morphology between the control and those blastocysts that had been vitrified, a total of four times during preimplantation development (Figure 3).

**Embryo viability**

Blastocysts which developed from embryos that underwent three rounds of vitrification were subsequently vitrified one more time before transfer. Implantation rate was not different between non-vitrified blastocysts and those that had been vitrified a total of four successive times. Fetal development per implantation site and resultant fetal weight were not statistically different (Table 1). However, overall there were significantly fewer fetuses from re-vitrified blastocysts (P < 0.05).

**Slow freezing**

Multiple rounds of slow freezing resulted in relatively poor embryo survival immediately following thawing (n = 164).
Survival of 1-cell-stage embryos was 52%, 2-cell-stage embryos following a second slow freezing procedure had a survival rate of 20.7% and only 1.8% developed to the 8-cell stage with 0% compaction. Following a third slow freezing procedure, 8-cell-stage embryo survival was 0%. No blastocysts developed from re-freezing of 164 original embryos.

It was determined that multiple rounds of vitrification resulted in little loss in developmental potential, whereas slow freezing was detrimental to embryo survival and development.

**Discussion**

Since the first human pregnancy from the transfer of frozen embryos in 1983 (Trounson and Mohr, 1983), cryopreservation has become a routine technology in IVF. A goal of any cryopreservation procedure is to minimize injury to the cells, for example ice crystal formation. To facilitate this, research in the field of embryo cryobiology has primarily focused on varying cryoprotectant concentrations and changing exposure times to reduce the toxicity of the cryoprotectant, while maintaining adequate levels to protect the embryo (Leibo and Loskutoff, 1993). Slow cooling has been the primary method of cryopreservation in IVF clinics (Lassalle et al., 1985; Veeck, 2003; Anderson et al., 2004); but vitrification has also been shown to be an effective method (Rall and Fahy, 1985; Mukaida et al., 2003; Vajta and Kuwayama, 2006). Vitrification circumvents the problems associated with ice crystal formation, while maximizing rates of cooling and warming (Mazur et al., 1984; Van Wagendonk-De Leeuw et al., 1995; Lane et al., 1999).

According to the American Society for Reproductive Medicine (ASRM), OHSS is one of the most severe complications during ART, and the risk for this syndrome increases with each exposure to hCG (Klemetti et al., 2005). Cryopreservation of all embryos is a good strategy for prevention of complications associated with secondary OHSS. This allows the patients more recovery time before embryo transfer, as a pregnancy could exacerbate the syndrome and waiting until the syndrome has resolved may also provide a healthier uterine environment for the embryo (Delvigne and Rozenberg, 2002). When patients are healthy enough to have a subsequent frozen embryo transfer, those who have embryos cryopreserved at the PN stage may choose between a day 3 or day 5 transfer, depending on the number and quality of the surviving embryos. Having the option to re-freeze embryos gives the patient additional opportunities for an un-stimulated frozen embryo transfer if more embryos survive the thawing procedure than are required at transfer.

The move to single embryo transfer may also increase the number of supernumerary embryos from each cycle. The purpose of single embryo transfer is to reduce rates of multiple gestations, which can otherwise result in increased morbidity and mortality (Norwitz et al., 2005). The financial burden of multiple gestations can be considered significant when compared to a singleton birth because of the increased cost resulting from premature delivery and longer periods in the neonatal intensive care unit (Callahan et al., 1994; Adashi et al., 2003; Gerris et al., 2004). Single embryo transfer may be more attractive to physicians and patients if an effective method for re-cryopreserving of embryos is developed. Providing the option to re-cryopreserve a patient’s previously stored embryos may encourage more patients to take a cautious approach with regard to number of embryos transferred.

A few cases have been reported on successful pregnancy after re-slow freezing embryos at the PN and blastocyst stage.

**Table 1.** Implantation, fetal development and fetal weights of non-vitrified and re-vitrified embryos

<table>
<thead>
<tr>
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<th>Implantation/Blastocyst (%)</th>
<th>Fetal development/Blastocyst transferred (%)</th>
<th>Fetal weights (mg) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63.3</td>
<td>51.7</td>
<td>81.6 (188 ± 11)</td>
</tr>
<tr>
<td>Re-vitrified four times</td>
<td>48 (not significant)</td>
<td>31.5*</td>
<td>65 (not significant) (199 ± 11)</td>
</tr>
</tbody>
</table>

*P < 0.05; n = 54 embryos transferred per treatment.
therefore, progress has already been made in this aspect of clinical treatment (Farhat et al., 2001; Estes et al., 2003). In addition, the refreezing of blastocysts has also resulted in a successful birth (Smith et al., 2005). However, these cases relied solely or partially on slow freezing, which has been shown in some cases to result in less-favourable survival and embryo development when compared to vitrification (Lane and Gardner, 2000; Nedambale et al., 2004; Rama Raju et al., 2005).

With high survival rates and low rates of cooling injury, vitrification has proven to be a safe and inexpensive alternative to slow freezing (Kuleshova and Lopata, 2002; Nedambale et al., 2004; Stehlik et al., 2005). Attention must also be given to studies that have indicated the potential for microtubule damage is high during cryopreservation, particularly during slow freezing because of the long dehydration and rehydration processes (Valojerdi and Salehnia, 2005). This damage, paired with the stress of cooling, has the potential to cause abnormal cell division and chromosome missegregation. When slow freezing mouse metaphase II (MII) oocytes, Valojerdi and Salehnia (2005) found deteriorated mitochondria and destroyed intermediate filaments following thawing. In contrast, those MII oocytes which had been vitrified had minimal damage; only the intermediate filaments were affected and these further reorganized by the time of the first cleavage division. Rates of embryo cleavage were significantly reduced from oocytes that were slow frozen. Only 1.2% compared to 85% in the vitrified oocyte group cleaved to 2 cells, and no blastocysts developed from the slow-frozen group (Valojerdi and Salehnia, 2005).

Previously, no study has determined the effect of multiple rounds of vitrification using the CryoLoop. Vitrifying mouse embryos at the 1-cell, 2-cell, 8-cell- and blastocyst-stages appears to cause minimal damage, and the loss of embryos from cooling injury is negligible. Cleavage rates were not reduced and were similar to rates of development for non-vitrified controls as were cell numbers and differentiation. Hatching rates were decreased, which may indicate the cryoprotectants themselves or that the vitrification process induces zona pellucida hardening. However, this did not impair implantation because rates were comparable to non-vitrified controls. Assisted hatching is often used in IVF laboratories to alleviate the problem of zona pellucida hardening when transferring cryopreserved embryos (Tucker et al., 1991). This procedure could also be performed for re-cryopreserved embryos. Embryo growth after transfer was not retarded, and there were no visible birth defects associated with vitrifying mouse embryos a total of four times. However, fewer fetuses per implantation site developed compared to non-vitrified controls. This may be indicative of embryos are experiencing some stress during successive vitrification procedures. Research to increase the robustness of vitrification solutions and decrease stress to the embryos is currently underway.

In this study, ascorbate was added to the cryopreservation solutions to act as an antioxidant, as previous work indicated that there was an added benefit to the embryo when it was present during freezing (Lane et al., 2002). Ascorbate is hypothesized to scavenge free radicals, which have the potential to damage DNA and the phospholipid membrane of the embryo (Meister, 1992; Halliwell and Gutteridge, 1999). Even a small increase in the level of free radicals may damage the embryo and interfere with normal cell function. The addition of ascorbate has been shown to also benefit embryonic development and cell differentiation (Lane et al., 2002).

A potential concern about some of the ultra-rapid vitrification approaches is the direct exposure of the sample to LN2 (Kuwayama et al., 2005b). There have been reports of contamination to bovine embryos after storage in LN2 (Bielanski et al., 2000) in addition to the potential for cross-contamination between samples (Bielanski et al., 2003). However, the work of Kuywa et al. (2003) on mouse embryos contradicts such findings. Significantly, even though the CryoLoop uses direct LN2 to vitrify the samples, the actual Loop and sample are subsequently stored in a closed vial. If the initial vitrification is performed using sterile LN2, such an argument against the technique is greatly diminished. In addition, the CryoLoop can also be successfully used without direct LN2 contact to eliminate the risk of such contamination (Larman et al., 2006).

This article shows that re-vitrifying mouse embryos at successive stages of development using the CryoLoop with the addition of ascorbate is not discernibly detrimental to embryo development. Regardless of the stage of the embryo, similar survival rates resulted. Compaction rates, blastocyst development, cell numbers or ICM development were not affected by multiple vitrification cycles. A previous study (Vitale et al., 1997) had shown a drop in cell number following re-freezing embryos, but this study failed to see any loss in cell number or differentiation after re-vitrification using the CryoLoop. Survival following multiple rounds of slow freezing was poor compared to re-vitrified embryos, and following three successive slow-freezing cycles, no embryos developed to the blastocyst stage. Although higher survival rates following the slow freezing of human PN oocytes and cleavage-stage embryos have been reported compared to the data presented in this study (Kattera and Chen, 2005; Kuwayama et al., 2005b; Stachecki et al., 2005), vitrification was also found to support higher survival (Kuwayama et al., 2005b).

The data from this work indicate that ultra-rapid vitrification imparts less stress on the embryo than slow freezing, enabling it to be used for successive rounds of cryopreservation without impairing embryo development. As such, vitrification should be considered as the primary method of cryopreservation for human ART.

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References


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