SHORT COMMUNICATION

ALBUQUERQUE, NEW MEXICO

Vitrification of Human Blastocysts Using the Cryoloop Method: Successful Clinical Application and Birth of Offspring

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Purpose: The purpose was to investigate vitrification of human blastocysts using the cryoloop.

Methods: Thirteen couples elected to have blastocysts cryopreserved via vitrification ($N = 54$ blastocysts). Embryos were exposed to two vitrification solutions, pipetted onto the cryoloop then plunged into liquid nitrogen. On the day of embryo replacement, blastocysts were warmed by passage through three dilution media, and rinsed into culture medium.

Results: Four couples returned for cryopreserved embryo replacement. Fifteen blastocysts were warmed; all 15 (15/15; 100%) were recovered. Fifteen (15/15; 100%) blastocysts were deemed viable, and 13 were replaced. Two replacements did not result in pregnancy; one resulted in clinical pregnancy with a blighted ovum; one resulted in clinical pregnancy with the live, term birth of a healthy male infant. Two of 13 embryos implanted yielding an implantation rate of 15.4%.

Conclusions: These preliminary data suggest that blastocysts can be successfully vitrified using the cryoloop method.

KEY WORDS: ART; cryopreservation; embryo; IVF.

INTRODUCTION

In our efforts to reduce the number of embryos replaced at transfer, we have utilized commercially available sequential media to extend embryo culture to 5 and 6 days post oocyte retrieval. As a consequence of this strategy, supernumerary blastocysts have become available for cryopreservation on Day 5 and/or Day 6, post oocyte retrieval. Our results with the standard slow cooling methods for blastocyst cryopreservation have been variable, warranting investigation of alternate strategies. Vitrification and

other rapid cooling techniques have been used successfully for cryopreservation of embryos from several species (1). In particular, vitrification has been successful when applied to embryos of species that do not survive slow cooling methods, e.g., the hamster (2), and pig (3). Addition of a microfilament depolymerizing agent prior to vitrification of the pig blastocysts was important for expanded blastocyst survival. Several recent publications support the efficacy of vitrification for cryopreservation of human blastocysts (4–9). In a study comparing slow cooling cryopreservation to vitrification with the cryoloop, nonhuman primate blastocysts demonstrated greater rates of survival and continued development in vitro when cryopreserved by cryoloop vitrification as compared to slow cooling cryopreservation (10). This study also produced live offspring following transfer of vitrified blastocysts to recipient hosts.

The method under clinical investigation in this laboratory is vitrification using the cryoloop method, as described originally by Lane *et al.* (4). Briefly, embryos are exposed to sequential vitrification solutions then placed into a droplet of medium, on a small nylon loop, which effectively reduces the volume of medium that must be cooled. Exposure of the vitrification medium to liquid nitrogen is direct as no barrier, e.g., the wall of a straw or vial, is between the droplet and the liquid nitrogen.

MATERIALS AND METHODS

Oocyte aspiration and fertilization were performed according to established laboratory protocols. Embryo culture was carried out in microdrops of sequential media (G1.2, G2.2; Vitrolife, Goteborg, Sweden) under mineral oil at 37°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. Blastocysts were replaced on Day 5 post oocyte retrieval and supernumerary blastocysts with an identifiable inner cell mass were cryopreserved. The remaining embryos were allowed to develop in vitro for an additional 24 h, and embryos that developed to blastocysts with obvious inner cell masses were cryopreserved.

Following detailed discussions, 13 couples elected to have blastocysts cryopreserved via vitrification ($N = 54$ blastocysts). Institutional Review Board approval was obtained prior to initiation of this study.

Vitrification of blastocysts was performed described by Lane *et al.* (4) with modifications. The basal medium for the cryoprotectant and warming solutions was a modified HEPES-G2.2 lacking essential amino acids and vitamins (4).

Solution A: HEPES-G2.2. Solution B: HEPES-G2.2 supplemented with 0.340 g/mL sucrose (S1888, Sigma Chemical Co., St. Louis, Missouri). Solution C: HEPES-G2.2 supplemented with 0.340 g/mL sucrose and 10 mg/mL ficoll (F4375, Sigma Chemical Co., St. Louis, Missouri). Vitrification Medium 1: 840 μ L of Solution A plus 75 μ L of DMSO (7.6% v/v; D2650, Sigma Chemical Co., St. Louis, Missouri) and 75 μ L of ethylene glycol (7.6% v/v; E9129, Sigma Chemical Co., St. Louis, Missouri). Vitrification Medium 2: 840 μ L of Solution C plus 175 μ L of DMSO and 175 μ L of ethylene glycol (14.7% v/v DMSO, 14.7% v/v ethylene glycol, 0.240 g/mL sucrose ($\approx$ 700 mM) and 7.1 mg/mL ficoll). Warming Medium 1: 840 μ L of Solution A plus 420 μ L of Solution B (0.113 g/mL sucrose, $\approx$ 330 mM). Warming Medium 2: 840 μ L of solution A plus 210 μ L of Solution B (0.068 g/mL sucrose, $\approx$ 200 mM). Warming Medium 3: 840 μ L of Solution A (0 mM sucrose). All cryopreservation and warming steps were performed at 37°C under a dissection microscope. For vitrification, selected embryos (one or two per cryoloop) were placed into Vitrification Medium 1 for 1 min 45 s. While the embryos were in this solution, the cryoloop (attached to the lid of a screw top cryovial) was rinsed in Vitrification Medium 2 to secure a droplet of medium onto the cryoloop. In addition, a 20 μ L drop of Vitrification Medium 2 was placed on an inverted lid of a sterile plastic petri dish. At the end of the time limit, the embryos were moved, in the smallest amount of medium, into the 20 μ L drop of Vitrification Medium 2 using a positive displacement pipette, pipetted twice in the solution and immediately placed into the drop of medium suspended on the cryoloop. The cryoloop with embryos was immediately plunged into liquid nitrogen and secured onto a cryovial that had previously been submerged in a dewar of liquid nitrogen on a labeled cryocane. This last step required that the embryos were plunged after no longer than 30 s from exposure to Vitrification Medium 2.

Warming of the cryopreserved embryos was scheduled to begin approximately 1–2 h prior to the time of embryo replacement. The identified cryocane was transferred into a dewar of liquid nitrogen, making sure that the level of liquid nitrogen was sufficient to completely submerge the vials. The cap of the cryovial, with the attached cryoloop, was unscrewed from the vial under the surface of the liquid nitrogen. Embryos were recovered from the cryoloop by quickly dipping only the nylon loop portion of the cryoloop into Warming Medium 1. The embryos fell off of the loop and settled to the bottom of the well. Time of

exposure to Warming Medium 1 was 2 min. At the end of this time, the embryos were moved to Warming Medium 2 for 3 min, then moved to Warming Medium 3 for 5 min, rinsed into microdrops of equilibrated culture medium under oil and returned to the incubator until evaluation and replacement. Embryonic survival postwarming was estimated by microscopic evaluation following the final warming step, and again before replacement. Observations noted on individual embryos included maintenance of structural integrity, cellular cohesiveness, formation of a blastocoel, and a visible inner cell mass prior to the time of replacement. Subjective quality scores were not assigned, but rather embryos were determined to be transferable or nontransferable per laboratory protocol.

RESULTS

To date, four individuals have returned for cryopreserved embryo replacement cycles. Fifteen blastocysts were warmed according to the above protocol, and all 15 (15/15; 100%) blastocysts were recovered. The embryos were evaluated at 200 $\times$ magnification using an inverted microscope immediately postwarming, and again prior to replacement. Fifteen (15/15; 100%) of the warmed blastocysts were deemed viable by morphological evaluation and were available for replacement. Thirteen of the 15 warmed blastocysts were replaced; Patient #1 received three embryos (of three embryos warmed), Patient #2 received four embryos (of five embryos warmed; the fifth embryo was vitrified again and stored for a future replacement cycle), Patient #3 received four embryos (of five embryos warmed; the fifth embryo was not vitrified again per patient request), and Patient #4 received two embryos (of two embryos warmed). Two replacements did not result in pregnancy (Patients #2 and #4); one replacement resulted in a clinical pregnancy with a blighted ovum (Patient #3); and one replacement resulted in a clinical pregnancy with a live birth of a healthy male infant (Patient #1). Of the 13 embryos replaced, two implanted yielding an implantation rate of 15.4%.

DISCUSSION

Vitrification has achieved wide attention for use with embryos from several mammalian species. Although the list is not comprehensive, there are now several publications (4–9,11–18) where vitrification or ultrarapid cryopreservation has been studied and/or clinically applied to the cryopreservation of human embryos.

Postwarming morphological evaluation of vitrified blastocysts in this study was extremely encouraging. Of 15 embryos warmed and recovered, all appeared to have organized, intact cell-to-cell cohesiveness, light reflective cell surfaces, and most displayed evidence of blastocoel re-formation prior to replacement. There were minor and localized indications of cellular degeneration, e.g., darkening of the cellular cytoplasm, or debris surrounding the organized embryonic mass, but all 15 blastocysts were deemed to be of transferable quality.

The cryoloop technique is simple to perform, it is time efficient, and it required no controlled rate freezing equipment that would be subject to electrical or component failure. Also, the method was mastered with relative ease. Cryopreserved 2-cell mouse embryos, obtained from a commercial vendor, are routinely used in this laboratory for quality control procedures, including evaluation and teaching of cryopreservation techniques. These cryopreserved 2-cell embryos can be cultured in vitro and subsequently re-cryopreserved using the slow cooling protocol for cryopreservation of human embryos, thawed again and allowed to develop further in vitro. In two practice vitrification trials, 38 and 36 expanded mouse blastocysts were vitrified using the cryoloop method, one or two embryos per cryoloop. Of the 74 mouse embryos cryopreserved, all 74 (74/74; 100%) were recovered and 67 (67/74; 90.5%) continued to develop in vitro for an additional 24 h postwarming, at which time observations were halted.

CONCLUSIONS

These preliminary data, including the live birth of a healthy child, are encouraging. The cryoloop technique for blastocyst vitrification might be used as a primary method of cryopreservation, and certainly as a backup method, for routine clinical application.

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