Article

Highly efficient vitrification method for cryopreservation of human oocytes

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Abstract

Two experiments were performed to develop a method to cryopreserve MII human oocytes. In the first experiment, three vitrification methods were compared using bovine MII oocytes with regard to their developmental competence after cryopreservation: (i) vitrification within 0.25-ml plastic straws followed by in-straw dilution after warming (ISD method); (ii) vitrification in open-pulled straws (OPS method); and (iii) vitrification in <0.1 µl medium droplet on the surface of a specially constructed fine polypropylene strip attached to a plastic handle (Cryotop method). In the second experiment, the Cryotop method, which had yielded the best results, was used to vitrify human oocytes. Out of 64 vitrified oocytes, 58 (91%) exhibited normal morphology after warming. After intracytoplasmic sperm injection, 52 became fertilized, and 32 (50%) developed to the blastocyst stage *in vitro*. Analysis by fluorescence in-situ hybridization of five blastocysts showed that all were normal diploid embryos. Twenty-nine embryo transfers with a mean number of 2.2 embryos per transfer on days 2 and 5 resulted in 12 initial pregnancies, seven healthy babies and three ongoing pregnancies. The results suggest that vitrification using the Cryotop is the most efficient method for human oocyte cryopreservation.

Keywords: *birth, bovine, Cryotop, human, pregnancy, vitrification*

Introduction

The capability to cryopreserve oocytes could be a valuable tool in human assisted reproductive techniques (Oktay *et al*., 1998; Porcu *et al*., 2000; Pool and Leibo, 2004; Paynter, 2005). It would permit patients suffering from various types of malignant diseases to have their oocytes collected prior to the beginning of chemo- or radiotherapy, with the expectation of having their oocytes fertilized after recovery from treatment (Meirow, 2000). As the ability of the oocytes to be fertilized and develop to term dramatically decreases in parallel with age, oocyte cryopreservation would permit women to delay maternity because of career demands. If oocyte cryopreservation were reliable and efficient, a woman's oocytes could be collected when she is young and preserved until she wished to have a child. Women whose husbands need treatment for sterility could avail themselves of this technology.

Recent advances in cryobiology have made it possible to preserve various types of reproductive cells with relatively little loss of viability (reviewed in Karow and Critser, 1997; Fuller and Paynter, 2004). However, other types of reproductive cells, especially oocytes, are sensitive to chilling injury and are damaged when cooled slowly by standard equilibrium methods (Vincent and Johnson, 1990; Bernard and Fuller, 1996; Leibo *et al*., 1996).

Vitrification is an alternative method to cryopreserve those biological specimens that are sensitive to chilling injury. Since the first report of vitrification of mammalian embryos by Rall and Fahy (1985), successful vitrification of reproductive cells of more than 11 species, including humans, have been reported; among the most studied are oocytes and embryos of cattle (reviewed by Rall, 2001). First births of normal calves derived from vitrified blastocysts that had been produced by IVF were reported 13

years ago (Kuwayama *et al.*, 1992). Calves have also been produced by transfer of embryos obtained by IVF and in-vitro culture (IVC) of bovine oocytes that had been vitrified after in-vitro maturation (IVM) to the metaphase II stage (Hamano and Kuwayama, 1992). Similar procedures have also been used to vitrify porcine blastocysts (Kuwayama *et al.*, 1997; Kobayashi *et al.*, 1998) and oocytes (Nagashima *et al.*, 1999), both of which are considered to be especially difficult to cryopreserve because of their extreme sensitivity to chilling injury (Leibo *et al.*, 1996). Several methods have been described for vitrification of bovine and porcine embryos. Among these are: in-straw dilution (ISD; Kuwayama, 1994), open-pulled straw (OPS; Vajta *et al.*, 1998, Isachenko *et al.*, 2003); and the Cryotop method, a modification of the minimum volume cooling (MVC) procedure (Hamawaki *et al.*, 1999).

This paper reports the results of two experiments designed to derive an efficient method of cryopreserving human oocytes by vitrification. In the first experiment, the three methods are compared for vitrification of bovine oocytes. In the second experiment, using the method that yielded the best results in experiment 1, the effect of two concentrations of ethylene glycol and the presence or absence of cumulus cells on survival of human oocytes after vitrification are compared. Another group of oocytes was cryopreserved by standard equilibration cooling. The normality of those human embryos derived from vitrified oocytes was examined by fluorescence in-situ hybridization (FISH). Day 2 and day 5 embryos derived from intracytoplasmic sperm injection (ICSI) after vitrification of human oocytes were transferred to recipients to obtain data regarding their full developmental competence.

Materials and methods

Bovine oocyte recovery and maturation

Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory at 33–35°C in 0.9% saline containing 0.1 mg/ml kanamycin. All visible follicles (2–5 mm in diameter) on the ovarian surface were aspirated with an 18-gauge needle. Cumulus–oocyte complexes were cultured in groups of 20 in 100 µl droplets covered with oil of maturation medium in a culture dish (35 mm × 15 mm; Nunclon #153066; Roskilde, Denmark) in 5% CO_2 in air with maximum humidity at 38.5°C for 22 h The maturation medium was TCM199 medium with Earle's salts, HEPES and bicarbonate (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Sigma Chemical Co., St Louis, MO, USA) and 250 µg/ml gentamycin sulphate.

Bovine IVF

After maturation, bovine oocytes were subjected to IVF and IVC as previously described (Kuwayama *et al.*, 1992). In brief, capacitation was induced by incubating frozen–thawed and washed bovine spermatozoa in 5 mmol/l theophylline and 10 µg/ml heparin. Insemination was performed by introducing the oocytes into a sperm suspension at a concentration of 2 × 10^7 spermatozoa/ml. After co-culture with spermatozoa for 5 h, oocytes were transferred to a 100 µl droplet of Synthetic Oviductal Fluid Medium (SOFM; Fukui *et al.*, 1996)) containing essential and non-essential amino acids and BSA (A-2153; Sigma Chemical Co.; 5 mg/ml), then cultured in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at 38.5°C for 7 days.

Patients

Experiments were conducted with patients who gave informed consent and with IRB approval. They were without spermatozoa at IVF because of the husband's personal reasons, or were testicular sperm extraction cases. Sixty-seven patients (32.3 ± 6.1 years old; mean ± SD) underwent vitrification experiments. The ovarian stimulation was performed by the clomiphene cycle with human menopausal gonadotrophin (HMG). Clomiphene (Clomid; Shionogi Co., Ltd, Osaka, Japan) was initiated on day 3 of the cycle by 50 mg/day and continued until the flare-up of LH, caused by spraying 300 µg of gonadotrophin-releasing hormone agonist. HMG (Humegon; Organon Co., Ltd, Oss, The Netherlands) was initiated on day 8 by 150 IU and continued every other day until the day before LH flare-up.

ICSI and IVC of human oocytes after vitrification

After recovery of the oocytes, they were cultured for 2 h in the basic medium, i.e. TCM199 medium buffered with 11 mmol/l HEPES, 9 mmol/l Na–HEPES, 5 mmol/l $NaHCO_3$ and supplemented with 10% synthetic serum substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) in 5% CO_2 in air at 37°C and maximum humidity. Those oocytes considered to have survived, as judged by morphological criteria (Figure 2, see below), were inseminated by ICSI, as described by Palermo *et al.* (1992). For ICSI, semen specimens, either fresh or frozen–thawed from an anonymous donor or from the patient's husband with >90% motility, were used for insemination. After insemination, oocytes with 2 pronuclei and a second polar body were transferred into 0.5 ml droplets of P1 medium (Irvine Scientific) containing 10% SSS. Embryos that developed beyond the 4-cell stage were then transferred into 100 µl droplets of G2.2 medium (IVF Science, Göteborg, Sweden) and cultured up for 6 days after ICSI.

Vitrification of bovine oocytes

Cumulus cells were partially removed from cumulus–oocyte complexes by gentle pipetting until only a few layers remained. All oocytes were then transferred into 1.6 mol/l ethylene glycol diluted in TCM199 medium and kept for 5–15 min at ~22°C until the oocytes had completely recovered their original isotonic volume, as judged by microscopic observation. Liquid nitrogen, used for cooling oocytes, was filtered through a Teflon membrane filter (0.22 µm pore diameter; Millipore Corp., Bedford, MA, USA).

To prepare straws for the ISD method, 25 µl of a vitrification solution (6.8 mol/l ethylene glycol + 1.0 mol/l sucrose in TCM199 medium) and 150 µl of a diluent solution (0.5 mol/l sucrose in TCM199) were aspirated with syringes into plastic artificial insemination straws (0.25 ml; IMV, l'Aigle, France) without any air bubbles between the two columns of media. After being equilibrated in 1.6 mol/l ethylene glycol, oocytes were washed in 4.5 ml of vitrification solution in a culture dish for 30 s, picked up by pipette and introduced into the vitrification solution in the straw. Each straw was sealed and plunged directly into liquid nitrogen. The time between introducing oocytes into the