

Ultra-rapid vitrification using cryoloop technique for human blastocyst cryopreservation

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In the cryopreservation of embryos, there is a risk of various types of injuries (Kasai, 1996). Among the injuries, the formation of intracellular ice would be the biggest obstacle to cryopreservation. The first strategy to prevent intracellular ice formation was to adopt a long slow-cooling stage. However, it was difficult to eliminate injuries from ice completely. Furthermore, the slow freezing method required a long time before embryos were stored in liquid nitrogen. Vitrification is an approach in which injuries related to ice are less probable and embryos can be cooled directly in liquid nitrogen (Rall and Fahy, 1985). We have shown that vitrification using an ethylene glycol (EG)-based solution with conventional cryo-straws is effective for human embryos at the 4-8 cell stage (Mukaida et al., 1998).

Recent advances in culture systems have made it possible to develop IVF embryos into blastocysts easily, and blastocyst transfer has proven effective for increasing the pregnancy rate. Therefore, cryopreservation of blastocysts has become an important technique. Using the slow freezing method, Menezo et al. (2000) reported reasonable clinical results (27% pregnancy rate, 17% implantation rate) with supernumerary co-cultured blastocysts. However clinical results reported by other clinics have not been consistent. Menezo et al. speculated that co-cultured blastocysts and the ones from sequential media do not have the same cryoresistance.

Recently, human blastocysts were successfully vitrified in straws (Yokota et al., 2000). However, our attempts to vitrify human blastocysts using straws resulted in disappointing survival rates. This is probably because human blastocysts are much less permeable to cryoprotectant and water, since they shrink more slowly than mouse and bovine blastocysts in the cryoprotectant solution. This suggests that human blastocysts are more likely to be injured by intracellular ice.

An innovative strategy to circumvent the problem of intracellular ice formation in less permeating embryos is ultrarapid vitrification, in which embryos are vitrified with a minimal volume of a vitrification solution using minute tools such as EM grids (Martino et al., 1996) or open pulled straws (Vajta et al., 1998). In this approach, samples are cooled and warmed extremely rapidly, which reduces the chance of ice formation. We attempted ultrarapid vitrification of human blastocysts using cryoloops, and have obtained a high rate of pregnancy (Mukaida et al., 2001).

The cryoloop for vitrification was originally described by Lane et al. (Lane et al., 1999a; 1999b). The cryoloop consisted of a minute nylon loop mounted on a stainless steel pipe inserted into the lid of a cryovial. A metal insert on the lid enables the use of a stainless steel handling rod with a small magnet for manipulation of the loop

at low temperature. Initially, blastocysts were placed in cryoprotectant solution I, which is a base medium containing 7.5% (v/v) DMSO and 7.5% (v/v) EG, at 37°C. While the embryos were suspended in solution I, a cryoloop was dipped into cryoprotectant solution II, which is a base medium containing 15% (v/v) DMSO, 15% (v/v) EG, 10 mg/mL Ficoll 70 and 0.65 M sucrose, at 37°C to create a thin, filmy layer of the solution on the nylon loop. At 2 min after suspending the blastocysts in solution I, the embryos were washed quickly in solution II and transferred onto the filmy layer on the nylon loop using a micropipette. Within 30 sec of suspension in solution II, the cryoloop was plunged into liquid nitrogen. Using the stainless steel rod, the loop containing the blastocysts was sealed in a cryovial, which had been previously submerged in liquid nitrogen. The vials were attached in standard canes and stored in liquid nitrogen. The whole procedure was completed within 5 min.

For recovery, the cryovial was submerged under liquid nitrogen and was opened with the aid of a stainless steel rod. Then the loop containing blastocysts was removed from the liquid nitrogen and placed directly into a well of the base medium containing 0.25 M sucrose at 37°C. Blastocysts immediately fell from the loop into the solution. After 2 min, blastocysts were transferred to the base medium containing 0.125 M sucrose. After an additional 3 min, blastocysts were suspended in base medium for 5 min. Finally they were transferred to G2.2 medium for further culturing until transfer. About 2 h after warming, blastocysts with morphologically intact inner cell mass and trophectoderm and a re-expanding blastocoel were judged to have survived. Lining of endometrium of the patients for warmed blastocyst transfer is prepared by exogenous transdermal estrogen administration. Between one to three surviving blastocyst were transferred into the uterus of the patient 5 days after progesterone administration, regardless of the timing of vitrification.

A total of 444 blastocysts from 137 patients were vitrified. After warming, 351 blastocysts (79%) survived, and 299 of them were transferred into 126 patients; in eleven patients no blastocysts survived after recovery. Of 126 transfers, 45 women (36%) conceived. Of these 45 patients, twelve delivered a total of 25 healthy babies (12 singles, 5 twinnings, and one triplet), thirteen had miscarriages, and 14 remain pregnant.

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