Case report

Two successful pregnancies obtained following oocyte vitrification and embryo re-vitrification

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Abstract

Recent clinical reports not only show that cryopreserved embryos can be successfully used for human fertility treatment, but also that cryopreserved oocytes may be used successfully as an adjunct to human assisted reproductive technologies. Vitrification is known to establish a glass-like solid state during the cooling process. The high concentration of cryoprotectants and an extremely rapid rate of cooling are responsible for the formation of the solid state, and also prevent formation of intracellular ice crystals. Hence, in theory, vitrification should minimize cryo-injuries, and therefore has great promise for oocyte and embryo cryopreservation. This article describes two pregnancies from vitrified–warmed blastocysts obtained after intracytoplasmic sperm injection fertilization of vitrified–warmed oocytes. Vitrification was employed to cryopreserve the oocytes and the subsequent blastocysts. The results present the intriguing implication that vitrification may serve as an efficient method for clinical oocyte cryopreservation and embryo re-cryopreservation.

Introduction

In theory, cryopreservation of reproductive cells could be made more efficient and cryo-injury less common if ice crystal formation could be eliminated or reduced during the freezing or cooling process (Koutlaki *et al.*, 2006). Vitrification holds great promise precisely because it avoids ice crystal formation (Rall and Fahy, 1985). By using high concentrations of cryoprotectants and very rapid cooling rates, vitrification causes the transition from liquid state to solid state without crystal formation (Taylor, 1987).

In prior studies using a mouse model, re-vitrification was applied at four successive stages of embryo development without impairing embryo growth. It has also been shown that re-vitrification of mouse embryos has minimal effect on preimplantation embryo development or implantation potential (Sheehan *et al.*, 2006).

It has been over 20 years since the birth of the first human child conceived through the fertilization of a frozen and thawed metaphase II oocyte (Chen, 1986); however, human oocyte cryopreservation has not become efficient enough to apply in clinical situations until recent years. Recent reports suggest that the efficiency of vitrified–warmed oocytes can be comparable to fresh oocytes with regard to embryo development (Kuwayama *et al.*, 2005; Antinori *et al.*, 2007). Similar claims in humans also suggest that the age of routine oocyte cryopreservation has finally arrived. As the use of egg cryopreservation becomes more common, the number of supernumerary embryos arising from previously cryopreserved oocytes will increase in the near future. Therefore, it will become increasingly important to have a cryopreservation method with low rates of cryo-injury and high rates of cellular survival. Ideally, this cryopreservation method would also allow for successive rounds of cryopreservation without severely impairing embryo development. This report documents two pregnancies from embryo transfer of vitrified–warmed blastocysts which were both generated from fertilization of vitrified–warmed oocytes.

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Case reports

First case

A 38-year-old woman and her 42-year-old husband presented for assisted reproduction treatment at the authors' facility in October 2006. Both male and female components of infertility were diagnosed, including diminished ovarian reserve. The couple elected to participate in an oocyte cryopreservation study, where eggs from anonymous donors were cryopreserved by vitrification. The oocyte cryopreservation study was submitted to and approved by a corresponding Institutional Review Board. The couple consented to the procedure in writing after extensive counselling and full disclosure.

Second case

The second couple joined the oocyte cryopreservation study because of primary infertility due to premature ovarian failure (age of woman: 38 years, age of man: 35 years). The couple also consented and signed the corresponding Institutional Review Board protocol.

Ovarian stimulation of oocyte donors

The oocyte donors were young (<35 years old) healthy women who volunteered to participate anonymously in the study and donate their oocytes. They underwent ovarian stimulation using a daily dose of 200 IU of recombinant FSH (Follistim; Organon, Roseland, NJ, USA). Gonadotrophin-releasing hormone (GnRH) antagonist (ganirelix acetate; Organon) was administered, starting on day 6, for LH surge prevention. Recombinant human chorionic gonadotrophin (HCG; Ovidrel; Serono, Rockland, MA, USA) was administered to trigger nuclear maturation of oocytes when two or more follicles reached 18 mm. The mature (metaphase II stage) oocytes were cryopreserved by vitrification.

Oocyte/embryo vitrification

The basal medium used for oocyte/embryo cryopreservation was HEPES-buffered embryo culture medium (Cooper/Sage, Bedminster, NJ, USA) supplemented with 20% (v/v) serum protein substitute (SPS; Cooper/Sage). The denuded oocytes were vitrified by the minimum volume cooling (MVC) method, essentially as described by Kuwayama *et al.* (2005). Briefly, the oocytes/embryos were equilibrated in equilibration medium [basal medium with 7.5% (v/v) ethylene glycol and 7.5% (v/v) dimethylsulphoxide (DMSO)] at room temperature for 15 min. Oocytes/embryos were transferred into the vitrification medium (basal medium with 15% (v/v) ethylene glycol, 15% (v/v) DMSO, and 0.5 mol/l sucrose) at room temperature for 45–60 s. The cryoprotectant-treated oocytes/embryos were placed onto a fine polypropylene strip (Cryotop®; Kitazato Bio Pharma Co., Japan). Then the polypropylene strip carrying the oocytes/embryos was submerged into liquid nitrogen and ready for storage.

Oocyte/embryo warming

The polypropylene strip with vitrified oocytes/embryos was immersed directly into 5.0 ml of warming solution [HEPES-buffered embryo culture medium with 20% (v/v) SPS and 1.0 mol/l sucrose] at 37°C for 1 min. Oocytes/embryos were then picked up and transferred into 1.0 ml of the dilution solution [HEPES-buffered embryo culture medium with 20% (v/v) SPS and 0.5 mol/l sucrose] for 3 min at room temperature. The oocytes/embryos were subsequently washed in 1.0 ml washing solution [HEPES-buffered embryo culture medium with 20% (v/v) SPS] for 10 min at room temperature. Oocytes were incubated in Quinn's advantage fertilization medium (SFM, Cooper/Sage) with 10% SPS for 3 h before intracytoplasmic sperm injection (ICSI).

ICSI fertilization, embryo culture, and embryo replacement

The husbands' spermatozoa were prepared by density gradient centrifugation, and ICSI fertilization was performed as previously described (Nagy *et al.*, 1994). Eighteen hours after ICSI, oocytes were assessed for the presence of pronuclei and switched to Quinn's advantage cleavage medium with 15% SPS for further culture. Embryo cleavage was assessed at 42h post-insemination. At 72 h post-insemination, embryos were transferred to Quinn's advantage blastocyst medium with 15% SPS. At 116 h post-insemination, blastocyst formation was assessed and graded for embryo replacement or embryo re-cryopreservation (Utsunomiya *et al.*, 2006; Sato *et al.*, 2007). Embryo replacements were performed in supplemented menstrual cycles (using Estrace and progesterone i.m.).

Results

First case

Eight vitrified donor oocytes were warmed and all eight survived. These were inseminated by ICSI. Seven out of eight were fertilized, and two out of five blastocysts were transferred on day 5. The remaining three blastocysts were re-cryopreserved. The patient did not become pregnant after the first embryo replacement.

Subsequently, in a second attempt, two re-cryopreserved blastocysts were warmed and both survived. Following 3–4 h of culture, two blastocysts re-expanded and were transferred into the patient's uterus. On day 10 after embryo replacement, the serum β-HCG was 956 IU/l. Two gestational sacs with fetal heartbeats were confirmed by ultrasound 30 days after embryo replacement. A successful pregnancy is still ongoing.

Second case

Ten vitrified donor oocytes were warmed and all 10 survived. After ICSI, nine fertilized, and two out of six blastocysts were transferred on day 5. The remaining four blastocysts were re-cryopreserved. The patient did not become pregnant after the first embryo replacement.

Subsequently, in a second attempt, three re-cryopreserved blastocysts were warmed and all survived. Following 3–4 h of culture, three blastocysts re-expanded and were transferred into the patient's uterus (**Figure 1**). On day 11 after embryo replacement, the serum β-HCG was 929 IU/l. A successful pregnancy is still ongoing.

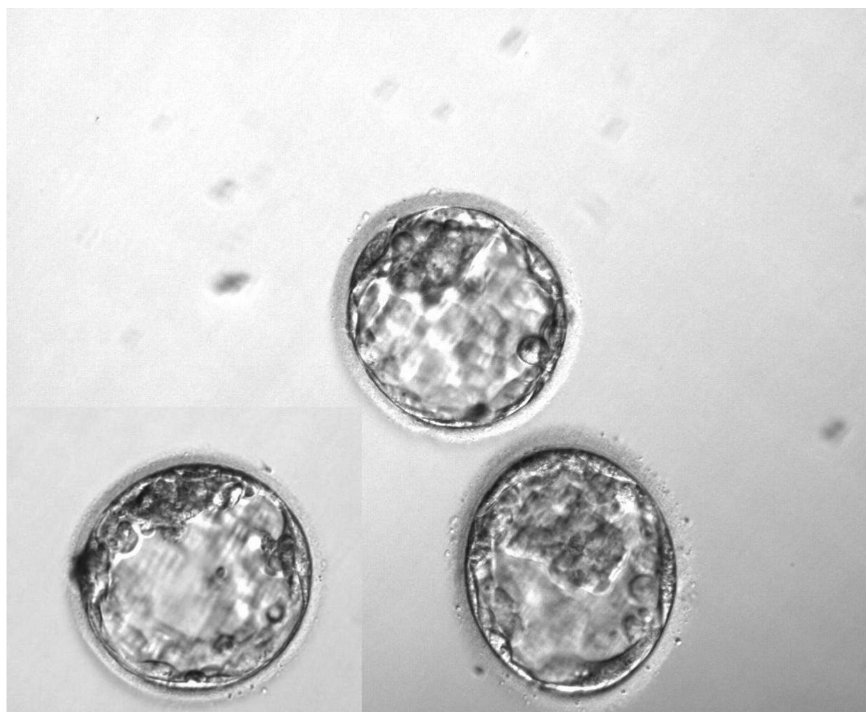

Figure 1. Photomicrograph of re-vitrified and warmed blastocysts derived from vitrified and warmed oocytes

Discussion

These results describe for the first time, ongoing pregnancies from embryo replacements of vitrified–warmed blastocysts, which were themselves generated from fertilization of vitrified–warmed oocytes. The results indicate that vitrification may be an efficient method for both oocyte cryopreservation and subsequent embryo re-cryopreservation. Vitrification is a relatively new approach to oocyte cryopreservation in human IVF; therefore, it is currently impossible to draw any conclusions from a comparative analysis of the clinical outcomes between vitrification and conventional slow freezing (Oktay *et al*., 2006). Recently, clinical outcomes for both slow freezing and vitrification have improved dramatically. In the late 1990s, using slow freezing, approximately 60–100 frozen–thawed oocytes were needed to achieve one pregnancy (Tucker *et al*., 1998; Porcu *et al*., 1999). The efficiency of slow freezing has improved of late: 13–18 frozen–thawed oocytes/implantation (Boldt *et al*., 2003; Fosas *et al*., 2003; Barrit *et al*., 2007; Bianchi *et al*., 2007). Initial reports on vitrification show that about 5–13 vitrified–warmed oocytes are needed to achieve an implantation (Kuwayama *et al*., 2005; Antinori *et al*., 2007). Since the difference between the number of vitrified–warmed oocytes required per implantation (~13) and the number of fresh oocytes required (~10/implantation) is now negligible (Antinori *et al*., 2007), oocyte cryopreservation seems to have come of age and may be used routinely as an adjunct to IVF. Similarly, supernumerary embryo re-cryopreservation from previously frozen or vitrified oocytes can now become routine management.

Oocyte cryopreservation is becoming a mature technology in the field of human assisted reproduction (Kuwayama *et al*., 2005; Borini *et al*., 2006; Oktay *et al*., 2006; Antinori *et al*., 2007; Barrit *et al*., 2007). The present report confirms that human embryos generated from previously vitrified oocytes can be re-vitrified at the blastocyst stage, with the resulting blastocysts initiating ongoing pregnancies. Thus, it is most likely that vitrified and warmed human oocytes retain their developmental competency, and re-cryopreservation during embryo development may not block further potential for implantation.

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