Survival rate of human oocytes and pregnancy outcome after vitrification using slush nitrogen in assisted reproductive technologies

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Objective: To report the survival rate of oocytes and the rate of successful pregnancies obtained from super-rapid cooling of oocytes using slush nitrogen (SN_2).
Design: Prospective clinical research.
Setting: A university-affiliated hospital.
Patient(s): Twenty-eight infertile women who underwent 30 cycles of IVF-ET using previously vitrified oocytes.
Intervention(s): Oocytes were vitrified by super-rapid cooling using SN_2.
Main Outcome Measure(s): Morphological normality of thawed oocytes and clinical outcome.
Result(s): In 30 cycles of ovarian stimulation for IVF, 364 surplus oocytes from 28 patients were vitrified using SN_2. Three hundred two (85.1% ± 2.9%) of the oocytes survived after warming. Fertilization and cleavage rates were 77.4% ± 3.5% (168/218) and 94.3% ± 2.1% (158/168), respectively. Thirteen pregnancies (43.3%) resulted from 30 uterine transfers of 120 embryos with an implantation rate of 14.2% (17/120). There were no differences between the pregnancy rate after vitrification/warming and that obtained from routine noncryopreserved oocytes.
Conclusion(s): The present report suggests that super-rapid cooling may improve the clinical efficacy of human oocyte vitrification and may be a valuable tool for human assisted reproductive technologies. (Fertil Steril® 2007;88:952–6. ©2007 by American Society for Reproductive Medicine.)

Key Words: Oocyte vitrification, super-rapid cooling, slush nitrogen (SN_2), IVF-ET, pregnancy

Since the first reported pregnancy from the use of frozen mature human oocytes (1), numerous studies have been carried out with the goal of developing an ideal method for oocyte cryopreservation. These studies have used surplus mature oocytes from patients undergoing IVF-ET, which were stored for future use by slow cooling or vitrification methods (2–9). When the patients failed to become pregnant in their fresh IVF-ET cycles, the stored oocytes were then used for IVF-ET. In addition, several centers have offered oocyte banking to patients who were at risk of losing gonadal function because of chemotherapy, radiation treatment, or extirpative therapy for pelvic pathology and pain.

Although cryopreservation of human oocytes has been successfully carried out, clinical outcome has been limited because of the decreased viability of thawed oocytes. To improve the viability and quality of oocytes after thawing, several changes have been introduced. Recently, a modified protocol using slow cooling has been suggested to improve survival rates as a result of changes involving an increase in sucrose concentration or the replacement of sodium with choline in the freezing media (10–13). Our group has developed a vitrification method for the cryopreservation of human oocytes, and previous results have been encouraging (6, 7, 14, 15). To optimize our vitrification method, we have recently introduced a new method, super-rapid cooling using slush nitrogen (SN_2). Boiling of liquid nitrogen (LN_2) occurs when a sample is immersed in it and results in gas bubbles around the specimen, which, in turn, results in poor heat transfer. By applying negative pressure with a vacuum, LN_2 will freeze and will be transformed into a slush state (SN_2) with a lower internal temperature of −210°C and without vaporization (16). In 2001, Isachenko and coworkers reported that they obtained a higher maturation rate of ovine immature oocytes using SN_2 rather than LN_2 (17). In this paper, we report a high survival of vitrified human oocytes with SN_2, which resulted in good morphology and an excellent pregnancy rate using this new super-rapid cooling technique.

MATERIALS AND METHODS

The Institutional Review Board of CHA General Hospital, Seoul, Korea, approved this clinical application in January 2003. All of the study participants gave their written informed consents.

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Controlled Ovarian Hyperstimulation for Standard IVF-ET

Starting on the third day of the cycle, controlled ovarian hyperstimulation was carried out with recombinant FSH (rFSH) or rFSH/hMG. To prevent a premature LH surge, we used GnRH agonist (Lucrin; Abbott, Seoul, Korea) in the long protocol or the GnRH antagonist cetrorelix (Cetrotide; Serono, Halle, Germany) after 5–6 days of stimulation. Ovulation was triggered with 10,000 IU of hCG (Profasi; Serono) when at least two follicles were over 18 mm in diameter. Transvaginal oocyte retrieval was carried out 34–36 hours after hCG administration.

Vitrification and Warming of Oocytes

Recovered cumulus-oocytes complexes (COCs) were briefly incubated for 10 seconds with 80 IU/mL of hyaluronidase (Sigma, St. Louis) to remove excess cumulus cells (CCs) and were then pre-equilibrated for 2.5 minutes in 2 mL of Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL, Grand Island, NY) supplemented with 1.5 M of ethylene glycol (EG; E-9129; Sigma) and 10% (vol/vol) fetal bovine serum (FBS; Gibco BRL) at 37°C. COCs with two to three layers of CCs were then placed for the final equilibration in the same volume of DPBS supplemented with 5.5 M EG, 1.0 M sucrose, and 10% FBS for 20 seconds. Two to five COCs that were partially denuded were mounted on an electron microscope gold grid (Gilder, Westchester, PA) using a fine glass pipette. Excess cryoprotectant solution was removed with sterilized paper (Kimwipes, Yuhan-Kimberly, Gunpo, Korea). The gold grids containing COCs were immediately plunged into either LN_2 or SN_2 as described below. For long-term storage, a cryovial cap and goblet were used for placement of the grid. For thawing, the gold grids were sequentially transferred to culture dishes containing 2 mL of DPBS supplemented with 1.0, 0.5, 0.25, 0.125, or 0 M sucrose and 10 % (vol/vol) FBS at intervals of 2.5 minutes at 37°C.

Generation of SN_2

SN_2 was generated in a rapid-cooling LN_2 chamber by applying negative pressure (Vit-Master, IMT, Ness Ziona, Israel). To make SN_2, three-quarters of the chamber of this machine was filled with LN_2 and the vacuum pump was switched on. After approximately 10–20 minutes, the temperature in the chamber declined to a maximum of −210°C, and the pressure was then reduced to approximately 0 bars. LN_2 in the chamber was then transformed into a slush state (SN_2). SN_2 has a lower internal temperature without vaporization (Figs. 1A–1C) and results in high rates of cooling (18).

Oocyte Freezing

From December 2003 to August 2005, 76 patients agreed to participate in an oocyte cryopreservation protocol using the SN_2 vitrification method for surplus oocytes while they were undergoing IVF. Twenty-eight patients who failed to conceive with their fresh IVF-ET cycles returned for ET using vitrified-warmed oocytes (30 cycles). The mean (±SD) age and duration of infertility for the patients were 33.7 ± 4.6 years and 4.5 ± 2.8 years, respectively. Reasons for IVF-ET were as follows: tubal factor (n = 10), male factor (n = 8), unexplained (n = 4), ovum donation (n = 2), polycystic ovary syndrome (n = 3), and endometriosis (n = 1). The average number of IVF attempts was 2.0 ± 1.7.

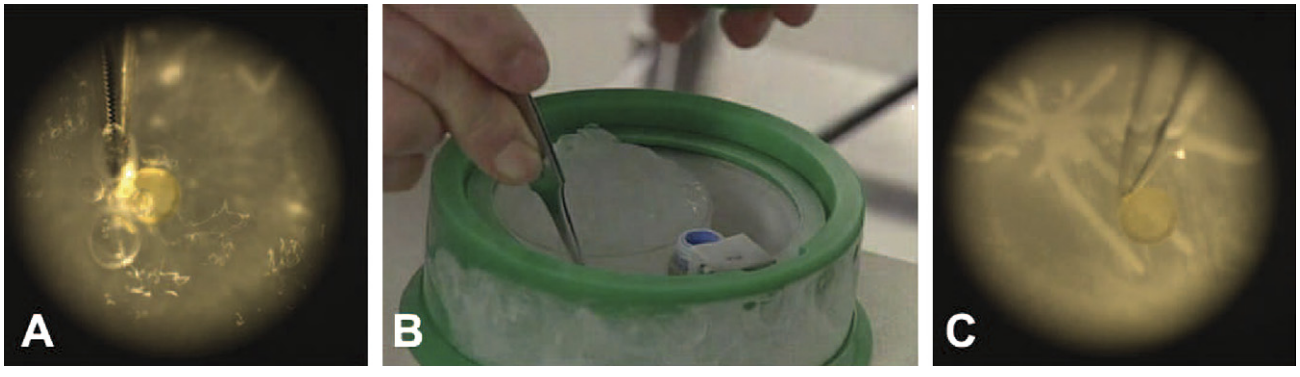
Patients with more than 15 oocytes retrieved were given the option to freeze their supernumerary oocytes for future use. Oocytes were stored for 1–17 months before thawing; the mean interval between the fresh and vitrified cycles was 4.3 months.

Fertilization, Embryo Culture, and ET after Warming

After being washed four to six times, CCs were removed by mechanical pipetting and then transferred into the preimplantation-1 (P-1) medium (Irvine Scientific, Irvine, CA) with

FIGURE 1

Photographs of slush nitrogen (SN_2). (**A**) Gas bubbles were formed when the grid was immersed into LN_2. (**B**) SN_2 was formed in the chamber when negative pressure was applied. (**C**) No bubble formation occurred in SN_2.



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