Preservation of human ovarian follicles within tissue frozen by vitrification in a xeno-free closed system using only ethylene glycol as a permeating cryoprotectant

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Objective: To study the preservation of follicles within ovarian tissue vitrified using only one or a combination of three permeating cryoprotectants.
Design: Experimental study.
Setting: University hospital.
Donor(s): Ovarian tissue was donated by consenting women undergoing elective cesarean section.
Intervention(s): Ovarian tissue was vitrified in closed sealed vials using either a combination of dimethyl sulfoxide, 1,2-propanediol, and ethylene glycol (EG), or only EG as permeating cryoprotectants.
Main Outcome Measure(s): Ovarian tissue was vitrified with the use of two vitrification methods. Tissue from the same donor was used for comparison of two different solutions. The morphology of the follicles was evaluated after vitrification, warming, and culture by light microscopy and transmission electron microscopy. Apoptosis was assessed by immunohistochemistry for active caspase-3 in fresh and vitrified tissue.
Result(s): Light and electron microscopic analysis showed equally well preserved morphology of oocytes, granulosa cells, and ovarian stroma when either of the vitrification solutions was used. No apoptosis was observed in primordial and primary follicles.
Conclusion(s): Using only EG as a permeating cryoprotectant in a closed tube gives as good ultrastructural preservation of ovarian follicles as a more complicated system using several cryoprotectants. (Fertil Steril® 2013;100:170–7. ©2013 by American Society for Reproductive Medicine.)
Key Words: Ovary, vitrification, ultrastructure, preantral follicle, cryoprotectant

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Cryostorage of human ovarian tissue to preserve fertility before starting cancer therapy has been developed since 1996 for prepubertal girls as well as for adult women who do not have time for oocyte collections (1). After retransplantation, functional recovery of cryopreserved ovarian tissue has been described (2–6). Since 2004, when the first infant (7) was born from frozen-thawed transplanted ovarian tissue, 20 healthy babies have been born after transplantation (8, 9). Also, maturation of ovarian follicles from frozen-thawed tissue is under development, and we regularly obtain secondary and early antral follicles (10).

A problem in the traditional slow-rate freezing is the formation of ice crystals causing cellular damage.

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Vitrification has been suggested to overcome this problem (11–13). Vitrification requires a high cooling rate and high concentrations of cryoprotectants with short time exposure before immersion of the tissue into liquid nitrogen. During very rapid cooling, ice crystal formation is avoided in vitrification owing to the high-viscosity environment (14, 15). Ovarian tissue in large mammalian species, such as the bovine and human, consists of heterogeneous cellular compartments and fibrous stroma. This makes the penetration of cryoprotectants throughout the tissue challenging. We have developed a vitrification procedure for human ovarian tissue and showed that it is equally good for the follicles but maintains the structure of the stromal tissue better than slow freezing (16). To date, several vitrification procedures with successful results have been reported in mice, rats, ewes, monkeys, and humans (17–22).

We recently used a closed vitrification system that can be carried out in a clinical setting without direct contact of the tissue with liquid nitrogen (23). However, the tissue was vitrified in a complex vitrification solution containing a combination of dimethyl sulphoxide (DMSO), 1,2-propanediol (PrOH), ethylene glycol (EG), and polyvinylpyrrolidone (PVP) as cryoprotectants. The combination was used to avoid the toxicity of each of the cryoprotectants at higher concentrations.

The type and concentration of the cryoprotectant, the exposure time, and the size of the tissue fragments play important roles in the success of ovarian tissue cryopreservation. Cryoprotectants may be toxic (24) when high concentrations are used. Addition of a sugar, polymers, or other nonpermeable cryoprotectants into the vitrification solution decreases the toxicity of the cryoprotectants, allowing a lower concentration of the cryoprotectants to be used (15, 24).

The aim of the present study was to improve and simplify the cryopreservation procedure by using a solution that contains only one permeating cryoprotectant, EG, in addition to the nonpermeable ones, Ficoll and sucrose. EG has been shown to give good preservation in vitrification of mouse ovarian follicles (17). In the present study, we compared follicles in tissues vitrified with the use of solutions containing either a combination of DMSO, PrOH, and EG or only EG. The morphology was evaluated with the use of light microscopy (LM) and transmission electron microscopy (TEM). TEM is the best known method to evaluate cryoinjury of follicles during the cooling and warming process (25, 26). The viability of the follicles within the tissue after warming was evaluated in tissue culture.

MATERIALS AND METHODS

All reagents were purchased from Gibco Invitrogen unless otherwise stated. The study was approved by the Regional Ethics Board in Stockholm, Sweden.

Ovarian Tissue Collection

Ovarian cortical tissue (2 mm × 2 mm × 5 mm) was obtained by biopsy from nine consenting women (mean age 33.7 ± 2.7 years, range 29–36 years) undergoing elective cesarean section.

Tissue Preparation

Ovarian tissue was collected into cold flushing medium (Medicult) and transported directly to the laboratory. The tissue immersed in collection medium was cut into thin elongated slices approximately 1 mm^3 to 1.5 mm^3 with the use of a scalpel under a stereomicroscope.

Two pieces of each biopsied sample were taken as fresh control samples and fixed for LM and TEM evaluation. The remaining pieces were frozen with the use of the two different methods, as described below. The tissue was stored in liquid nitrogen for at least 1 week before it was warmed. After warming, the pieces were fixed for LM and TEM analysis either directly or after 24-hour culture.

Vitrification Procedure: Solution Containing Combination of DMSO, PrOH, and EG

The vitrification procedure was performed as described in our previous article (23). The cortical tissue pieces were first washed with a solution consisting of Hank's balanced salt solution (HBSS) containing10 mg/mL human serum albumin (HSA; Pharmacia) for 5 minutes at room temperature (RT). Tissue pieces were then transferred into three vitrification solutions (VSs) with increasing concentrations of cryoprotectants DMSO, PrOH, and EG (Sigma-Aldrich) supplemented with 10 mg/mL HSA, VS1 2.5%, VS2 5%, and VS3 10%.

The incubation time in VS1 was 5 minutes and in both VS2 and VS3 was 10 minutes. The first two steps were performed at RT and the third step at 4°C. After equilibration in VS3 supplemented with (10% w/v) PVP, the pieces were vitrified with the use of a 1.8-mL cryovial (Nunclon) with a minimum volume of the medium. The tissue pieces were transported into the vial with the use of a small spoon to avoid mechanical pressure. The internal thread cap preventing leakage was closed and sealed and immersed in liquid nitrogen. The tissues were stored in a vapor-phase nitrogen storage freezer (Air Liquid–DMC, Espace 151-331-661) (27).

Warming. For warming, the cryovial was kept at RT for 30 seconds and then immersed in a 37°C water bath until the ice melted. The cortical tissue was transferred into the first prewarmed 37°C solution, which consisted of HBSS and 10 mg/mL HSA supplemented with 0.5 mol/L sucrose for ~2 minutes and at RT. Then the piece was incubated in the warming solutions that consisted of HBSS/HSA supplemented with 0.25 mol/L and 0.125 mol/L sucrose. The fourth solution consisted of HBSS supplemented with HSA (10 mg/mL). The incubation time for the last three solutions was 5 minutes in each at RT.

Vitrification Procedure: Solution Containing 40% EG Only

The procedure we used was similar to that described for mouse ovarian tissue by Salehnia (17). The cortical tissue pieces were transferred into the vitrification solution containing 40% EG (v/v), 30% Ficoll 70 (w/v), and 1 mol/L sucrose supplemented 10 mg/mL HSA (EGFS40) for 5 minutes at RT. The pieces were placed in cryovials with minimum volume of the vitrification solution with the use of a small spoon, and the vial was sealed and immersed in liquid nitrogen as described above.