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Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue

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BACKGROUND: Controlled-rate freezing of ovarian cortical tissue for preservation of fertility among young women facing chemo- or radio-therapy is a widely accepted procedure. To improve the method for cryopreservation of ovarian tissue, particularly the stroma, we carried out a systematic comparison of vitrification versus slow programmed freezing.

METHODS: Ovarian tissue from 20 women, donated during Caesarean section, was used for parallel comparison of survival and detailed light and electron microscopic (EM) morphology of oocytes, granulosa cells and ovarian stroma after freezing (slow freezing and vitrification), thawing and 24-h culture. Using tissue obtained from the same patient, we compared four cryopreservation protocols and fresh tissue. The cryoprotectants used in slow freezing were 1,2-propanediol (PrOH)-sucrose and ethylene glycol (EG)-sucrose. For vitrification, tissues were incubated for 5 or 10 min in three solutions containing a combination of dimethyl sulphoxide (DMSO), PrOH, EG and polyvinylpyrrolidone (PVP).

RESULTS: Cryopreservation using controlled-rate freezing and vitrification preserved the morphological characteristics of ovarian tissue generally well. As revealed by morphological analysis, particularly EM, the ovarian stroma was significantly better preserved after vitrification than after slow freezing ($P < 0.001$). The follicles were similarly preserved after all freezing methods.

CONCLUSIONS: Vitrification using a combination of PrOH, EG, DMSO and PVP was comparable to slow freezing in terms of preserving follicles in human ovarian tissue. Ovarian stroma had significantly better morphological integrity after vitrification than after controlled-rate freezing.

Key words: cryopreservation / human ovarian tissue / ultrastructure / vitrification

Introduction

Freezing of ovarian tissue for preservation of fertility among young girls and women facing chemo- or radio-therapy has become a widely accepted procedure since the first successful results of cryopreservation of human ovarian tissue were published (Grischenko *et al.*, 1987; Hovatta *et al.*, 1996; Newton *et al.*, 1996; Hovatta, 2005).

Cryopreservation of ovarian tissue has a background of success in rodents and other species (Deanesly, 1954; Green *et al.*, 1956; Parkes, 1958; Parrot, 1960; Gosden *et al.*, 1994; Hovatta, 2005).

The birth of healthy children from frozen-thawed autotransplanted tissue (Donnez *et al.*, 2004; Meirow *et al.*, 2005) has further encouraged clinicians to offer this option to patients who are at risk of undergoing premature ovarian failure as a result of chemotherapy.

Controlled-rate freezing (Hovatta *et al.*, 1996; Newton *et al.*, 1996) was first applied in the preservation of human ovarian tissue. Vitrification has proven to be an effective alternative method in

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cryopreservation of human blastocysts and oocytes (Liebermann *et al*., 2003; Mukaida *et al*., 2003; Yoon *et al*., 2003; Kuwayama *et al*., 2005; Hiraoka *et al*., 2007) and has been further developed in freezing of rat and mouse ovarian tissues, which have looser structures than that in humans (Sugimoto *et al*., 2000; Tokieda *et al*., 2002; Migishima *et al*., 2003; Chen *et al*., 2006). Vitrified murine, rabbit and monkey ovarian tissues survive after transplantation into rat uteri (Kagabu and Umezu, 2000) and live offspring have been born from vitrified mouse ovarian follicles matured *in vitro* (de la Pena *et al*., 2002). Vitrification of sheep ovarian tissue has also been studied: Al-Aghbari and Menino (2002) isolated follicles from vitrified sheep ovarian tissue and obtained metaphase II oocytes after *in vitro* maturation. Courbiere *et al*. (2006) managed to vitrify whole sheep ovaries by perfusing them through their vessels with vitrification solution and bathing them in the solution. Bordes *et al*. (2005) reported the birth of four lambs after autotransplantation of vitrified warmed ovarian cortex into ewes.

Electron microscopic examination is an important method to evaluate the ultrastructural morphology of tissue after cryopreservation. Vitrification has maintained the ultrastructural integrity of canine, goat and monkey ovarian tissue (Yeoman *et al*., 2005; Ishijima *et al*., 2006; Santos *et al*., 2007).

In humans, it is more difficult to study the methodology of cryopreservation because of the limited access to donated ovarian tissue. Gandolfi *et al*. (2006) used human ovarian tissue from three women for comparison of vitrification and two slow freezing protocols, and carried out parallel experiments using pig and bovine ovaries. Human tissue did not behave similarly to the animal tissues, and neither of the animal species proved to be an appropriate model for human tissue.

Different aspects of vitrification of human ovarian tissue (Isachenko *et al*., 2003; Rahimi *et al*., 2004) have been explored, with variable effects on morphology of the tissue described. Li *et al*. (2007) collected and randomly allocated human ovarian tissue to one of three groups: fresh, cryopreserved by means of slow conventional freezing with dimethyl sulphoxide (DMSO) as cryoprotectant, and cryopreserved by vitrification. Morphology and hormone production *in vitro* were similar in the two cryopreserved groups but significantly lower than in the fresh control samples. However, no electron microscopic comparisons were published.

We have carried out the first systematic comparison of vitrification and slow programmed freezing of human ovarian tissue by comparing four cryopreservation protocols using tissue obtained from the same patients. We used two different protocols in slow freezing, and two different incubation times in a mixture of cryoprotectants in vitrification. Morphology was studied using light and electron microscopy, and the viability of the tissue in culture after thawing were evaluated and analysed.

Materials and Methods

Tissue donors

Ovarian tissue was collected as small biopsy pieces (2 × 3–5 × 5–8 mm) from 20 women donors with a mean age of 33.3 ± 4.0 (SD) years (median age 32, range 28–43 years). The biopsies were performed during planned Caesarean sections. The study was approved by the Ethics Committee of the Karolinska Institute, Karolinska University Hospital Huddinge. All women were informed about the ongoing project, and they signed an informed consent form.

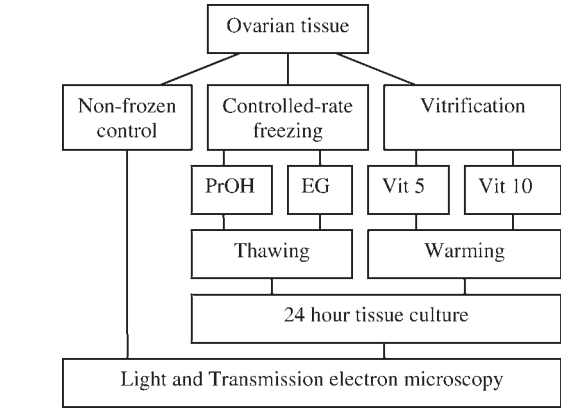

Figure 1 Experimental setup for comparing four cryopreservation protocols using human ovarian tissue.
Each sample ($n = 20$) was divided into five pieces to allow direct comparisons of the four freezing protocols and fresh tissue. PrOH: 1,2-propanediol/sucrose cryoprotectant; EG: ethylene glycol/sucrose cryoprotectant; Vit 5: protocol with 5 min incubation in each vitrification solution; Vit 10: protocol with 10 min incubation in each vitrification solution.

Tissue treatment

Ovarian tissue was placed in sterile 50 ml Falcon tubes (Becton Dickinson, Bedford MA, USA) containing 20 ml of pre-warmed flushing medium (Medicult Jyllinge, Denmark) and immediately transported at room temperature to the laboratory. Within ~5 min after harvesting, the piece of tissue was transferred to a culture dish (Falcon, Becton Dickinson, Bedford MA, USA) containing flushing medium. The ovarian cortical tissue was manually dissected from medullar tissue and divided by scalpel into strips of about 1 × 1–2 × 5–8 mm. Two small pieces of fresh tissue were fixed and subsequently used as non-frozen controls for light microscopy (LM) and transmission electron microscopic (TEM) evaluation.

The remaining pieces of tissue were vitrified and frozen using two slow freezing methods. From every biopsy at least five strips (1 × 1–2 × 5–8 mm), one for each of the cryopreservation procedures and one as non-frozen control (Control), were processed for LM and five smaller pieces (1–2 mm^3) were processed for TEM.

Cortical tissue pieces obtained from any one patient were processed for each cryopreservation procedure at the same time, stored in the same holder in liquid nitrogen for 1–15 weeks, warmed/thawed, cultured and analysed in parallel (Fig. 1).

Cryopreservation

Vitrification

Our vitrification procedure for cortical ovarian tissue samples consisted of three incubation steps in solutions with increasing concentrations of DMSO (Sigma-Aldrich, Sweden), 1,2-propanediol (PrOH) and ethylene glycol (EG) dissolved in Hanks' balanced salt solution (HBSS) (Invitrogen Corporation, Scotland, UK) supplemented with 10 mg/ml human serum albumin (HSA, Vitrolife Inc., Englewood, Colorado, USA). After washing for 5 min in HBSS with 10 mg/ml HSA, pieces of ovarian cortex were transferred sequentially to 1 ml of vitrification incubation solutions VS1 (0.35 M DMSO, 0.38 M PrOH, 0.38 M EG), VS2 (0.7 M DMSO, 0.75 M

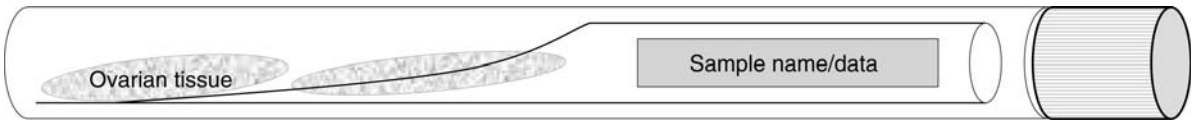

Figure 2 Schematic representation of the device used for vitrification of human ovarian tissue.
Pieces of ovarian cortical tissue are placed on a 0.5 ml hand-cut insemination cryostraw and enclosed in a 5.0 ml Nunc cryotube for storage in liquid nitrogen.

PrOH, 0.75 M EG) and VS3 (1.4 M DMSO, 1.5 M PrOH, 1.5 M EG), at 2.5, 5 and 10% of each cryoprotectant, respectively. At the third step, VS3 was supplemented (10% w/v) with polyvinylpyrrolidone (PVP; Sigma-Aldrich, Sweden). Stepwise incubation was performed by testing two incubation times, 5 min (Vit 5) and 10 min (Vit 10) in each vitrification solution. The first and the second incubation steps were performed at room temperature, while at the higher concentration of cryoprotectants, the samples were incubated at +4°C. Then pieces of ovarian cortical tissue were taken out from VS3 and transferred to 0.5 ml insemination cryostraws (IMV-Technologies, L'Aigle, France), which were hand-cut by scalpel (Fig. 2). Using an open system, the samples were directly plunged into liquid nitrogen (−196°C), preliminary sterilized through 0.2 μm Acrodisc® Sterile Syringe Filters with Supor* Membrane (25 mm diameter) (Pall Life Sciences, Ann Arbor, MI, USA). Afterwards, the straw with vitrified tissue was placed in a pre-cooled 5.0 ml Nunc cryotube (Nunclon, Roskilde, Denmark), closed and stored in liquid nitrogen (Fig. 2).

Controlled-rate freezing

The first slow freezing cryopreservation programme (Hovatta *et al*., 1996) consisted of three steps of incubation in ready-to-use freezing solutions for cryopreservation of cleavage-stage embryos (FREEZE KIT I™, Vitrolife, Gothenburg, Sweden) containing three physiological salt buffers supplemented with 25 mg/ml HSA (Cryo-PBS) with PrOH as a permeating cryoprotectant and sucrose as non-permeating cryoprotectant. The tissue pieces were first incubated for 5 min in 1 ml Cryo-PBS, then for 10 min in the Cryo-PBS solution containing 1.5 M PrOH, and finally for 15 min in the third solution which contained 1.5 M PrOH and 0.1 M sucrose in Cryo-PBS. The third incubation step was performed in 1.8 ml Nunc cryovials (Nunclon, Roskilde, Denmark), which were placed in a programmable freezer (CryoLogic, Australia). Subsequently, the samples were cooled from room temperature to −6.5°C at a rate of −2.0°C/min. Seeding was performed by means of forceps pre-cooled in liquid nitrogen. After a 10-min holding period, the samples were cooled to −35°C at a rate of −0.3°C/min, and after holding for 10 min they were plunged directly into liquid nitrogen (−196°C) and stored until thawing.

In the second slow freezing protocol, the rates were similar to those in the first protocol, but the starting temperature was +4°C. Before cooling, tissue pieces were pre-incubated for 30 min at +4°C in 1.8 ml Nunc cryovials in a solution (1 ml) consisting of 1.5 M EG, 0.1 M sucrose (Sigma-Aldrich, Sweden) and HSA (10 mg/ml) (Vitrolife, Gothenburg, Sweden) in phosphate-buffered saline (PBS) (Invitrogen, Scotland).

Warming/thawing

Three different thawing procedures were performed depending on the freezing protocol.

All the samples were carefully moved from solution to solution using a scalpel and needle. The volume of solutions used for thawing and removal of cryoprotectants in all protocols was 1 ml for each step.

Warming after vitrification procedure

Procedures for warming and removal of cryoprotectants for the vitrified samples consisted of four steps. First, the tube was taken from liquid nitrogen and carefully opened. The open straw was removed from the cryo-tube and directly plunged into the first (pre-warmed, 37°C) warming solution which consisted of HBSS and 10 mg/ml HSA supplemented with 0.5 M sucrose (Sigma-Aldrich, Sweden). Once in the first solution, the sample was out of the straw, and slowly rolled into a dish. The samples then went through two 5-min steps in the second and third warming solutions, which consisted of HBSS/HSA supplemented with 0.25 and 0.125 M sucrose, respectively. The last 5 min step was in the medium composed of HBSS supplemented (10 mg/ml) with HSA. Each piece of tissue was warmed separately.

Thawing after controlled-rate freezing procedure

For PrOH and EG thawing procedures, the cryovials were first taken from liquid nitrogen and exposed for 30 s to room temperature. They were then plunged for 2 min into a warm (37°C) water bath until the ice was melted. Then, medium and pieces of tissue were poured into a dish, and transferred to the respective first thawing solution.

In the PrOH thawing procedure the cortical tissue was thawed with four steps of rehydration using ready-to-use thawing solutions (Cryo-PBS, Thaw Kit 1, Vitrolife). The first step was 5 min in thawing solution 1 (TS1) which contained 1.0 M PrOH and 0.2 M sucrose, then 5 min in TS2 containing 0.5 M PrOH and 0.2 M sucrose, then 10 min in TS3 with 0.2 M sucrose and finally 10 min in TS4 (Cryo-PBS).

The EG thawing and cryoprotectant removal procedure consisted of three steps. The pieces were first placed in a solution consisting of 0.75 M EG and 0.25 M sucrose in PBS for 10 min. They were then kept for 10 min in a solution of 0.25 M sucrose in PBS, and finally for 10 min in Cryo-PBS.

Tissue culture

After thawing, all the samples were cultured in Millicell Cell Culture Inserts, 12 mm (Millipore, Bedford, MA, USA) on a 24-well plate (Nunclon, Roskilde, Denmark) in a humidified incubator (Galaxy, RS Biotech, UK) for 24 h at 37°C and with 5% CO_2 in air. The thawed strips of ovarian tissue were placed into the inserts with 100 μl of pre-equilibrated α-minimum essential medium (Invitrogen Inc.) medium supplemented (10%) with HSA (Pharmacia, Stockholm, Sweden), insulin transferrin selenium (ITS, Invitrogen, 10 μg/ml), 8-bromo-cGMP (Sigma-Aldrich, 1.1 mg/ml), Antibiotic–Antimycotic (Invitrogen Inc., 50 IU/ml) and FSH (Gonal F, Serono Nordic, Solna, Sweden, 0.5 IU/ml). Cyclic GMP was added because in our earlier study we showed that it enhanced the survival and development of human early follicles cultured in ovarian cortical tissue (Scott *et al*., 2004). Into the well outside the insert, 400 μl medium was added dropwise.

Evaluation of the integrity of the ovarian tissue after cryopreservation

Light microscopy

After 24 h of culture, the samples were fixed for LM in Bouin's solution for 2 h. Then the fixative was replaced with 70% alcohol and the samples

stored at +4°C. The fixed samples were embedded in paraffin, sliced and stained by Haematoxylin-eosin. For each case, six 4-μm sections, presenting three pair of neighbouring sections, were placed on the same glass and assessed. The distance between the neighbouring sections was 4-μm; 10 following sections were omitted from the analyses before the next pair of neighbouring sections was mounted on the slide. When no follicles were found, serial sections of the paraffin block were prepared and investigated. To prevent double counting each follicle was followed through neighbouring sections and counted only once.

The number of follicles of different developmental stages was evaluated. Follicles with an oocyte surrounded by a single layer of flat granulosa cells were defined as primordial. Follicles with the oocyte surrounded by flat and one or more cuboidal granulosa cells were regarded as intermediary, and with one layer of only cuboidal granulosa cells, as primary. Follicles with the oocyte surrounded by two or more layers of cuboidal granulosa cells were classified as secondary. The follicles were considered atretic if they had an oocyte with eosinophilic cytoplasm, contraction and clumping of the chromatin material (Gougeon, 1986, Hovatta *et al*., 1997, Hreinsson *et al*., 2003, Gook *et al*., 2008).

For evaluation of cryodamage, intermediary and primary follicles were pooled into one group and mentioned as primary follicles (Gougeon, 1986; Hovatta *et al*., 1997; Hreinsson *et al*., 2003). Atretic follicles were excluded from evaluation.

Evaluation of the follicles

We classified as intact the follicles without any contraction of the cytoplasm or any pyknotic oocyte nuclei. The oocyte had to be in contact with the surrounding granulosa cells, and the granulosa cells did not have any pyknotic nuclei or signs of shrinkage or swelling. The space between neighbouring granulosa cells was not enlarged. The basement membrane of the follicle was intact and attached to the granulosa cells.

The follicle was regarded as influenced if it contained an intact nucleus and nuclear and cytoplasm membranes of the oocyte, but had partial (but less than 50%) detachment of the oocyte from surrounding granulosa cells and/or less than 10% vacuolization of cytoplasm, and had less than 50% of degenerated granulosa cells.

The follicles were regarded as degenerated if more than 50% of any of the structures described above were affected.

The numbers and percentages of primordial, primary and secondary follicles were evaluated separately in each category.

Evaluation of the stroma

To illustrate the changes in the ovarian stromal tissue caused by different cryopreservation protocols, the numbers of undamaged and pyknotic nuclei of the stromal cells were counted using LM on three high power fields (909 μm^2 each) in six sections through five blocks (one per protocol). Calculation was performed using a digital image analysis system (Easy Image Mätning, Bergström Instruments, Stockholm, Sweden) connected to an inverted microscope (Nikon, Bergström Instrument). Images were taken with a digital camera (Nikon, COOLPIX950) at final magnifications of ×400. The results are presented as total number and proportion of intact stromal cells per sample and average number of intact stromal cells per high power field.

Transmission electron microscopic

For TEM, pieces of tissue after thawing and culture were placed in a mixed fixative (pH 7.4) containing 2% glutaraldehyde, 0.5% paraformaldehyde, 0.1 M sodium cacodylate buffer, 0.1 M sucrose and 3 mM $CaCl_2$. The specimens were post-fixed in 1% OsO_4, dehydrated and after embedding in LX-112 (Ladd Research Industries Inc., Burlington, VT, USA), the tissue was cut into 1 μm sections, stained with toluidine blue and observed in LM. Samples containing follicles were selected under LM, cut into 50 nm sections, contrasted with 2% uranyl acetate and lead citrate and examined in a Tecnai 10 (Fei, the Netherlands) electron microscope. Digital images were taken at magnifications of ×1250 to ×30 000 using a Megaview III camera.

The ultrastructural changes in the ovarian tissue after cryopreservation and 24-h organ culture, were evaluated first by assessing the structures of oocytes, granulosa cells and the stroma separately at magnifications higher than ×1650. The investigator was blinded to the experimental background of the specimens. In order to get representative amounts of tissue available for TEM evaluation, each tissue component was analysed separately (Hreinsson *et al*., 2003).

In the oocytes we studied the chromatin structures, the integrity of nuclear membranes, the density and integrity of the mitochondrial cristae, density of the cytoplasm, the size and numbers of vesicles in the cytoplasm, integrity of the cytoplasmic membrane and attachment of the oocyte to tgranulosa cells.

The same parameters were evaluated for assessment of granulosa cells. The attachment between granulosa cells and attachment to the basement membrane, were also evaluated.

If all the structures were undamaged the oocytes and granulosa cells were regarded as intact. Structures with slight non-lethal changes were regarded as influenced. If the cell was collapsed and/or nuclear or cytoplasm membrane disrupted, the cell was considered as degenerated.

The nuclear and cytoplasmic membranes, nuclear chromatin and the structure of mitochondria and other organelles of stromal cells were evaluated separately from integrity of the extracellular matrix. Tissue without any damaged or influenced stromal cells or any changes in the structural appearance of collagen bundles was regarded as intact. Influenced stroma contained less than 50% necrotic cells, and a low number of empty areas in the stromal tissue. Degenerated stroma had increased loss of collagen bundles and the majority of the stromal cells were necrotic. In cases where different areas of the same sample had different structural integrities after cryopreservation, up to five fields were assessed, and the mean normality of the sample was taken into account.

Statistical analysis

Categorical data were summarized using frequency counts and percentages. Continuous data were presented as mean and SD. In order to assess whether the degree of viability (coded as intact, influenced and degenerated) of follicles in LM evaluation was associated with different cryopreservation protocols or non-frozen control, Chi-square test for independence was used with exact *P*-values (StatXact 4, CYTEL Software Corporation, Cambridge, USA). Pairwise comparisons between protocols were then performed by Fisher's exact test. Similar statistical analysis was performed for TEM evaluation of oocytes, granulosa cells and stroma. Difference in viability of stroma between the cryopreservation protocols was analysed using Kruskal–Wallis analysis of variance (Statistica 8.0, StatSoft®, Inc. Tulsa OK, USA) by ranks followed by pairwise comparisons between protocols. The *P*-values were then adjusted according to the Bonferroni procedure for multiple tests of significance. A *P*-value <0.05 was considered statistically significant.

Results

Light microscopy

A total of 354 follicles was analysed by LM to evaluate the viability and the developmental stages in non-frozen, slowly cryopreserved and

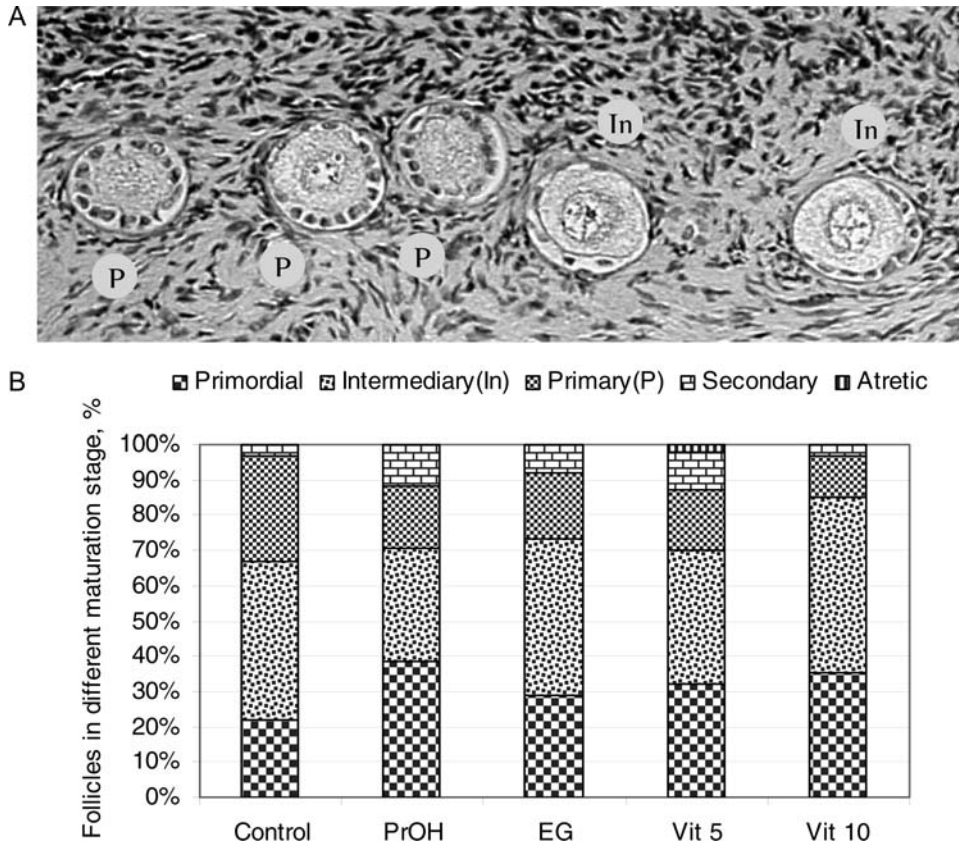

Figure 3 A representative image of human ovarian cortex.
(**A**) A piece of non-frozen control tissue containing intermediary (In) and primary (P) follicles. (**B**) Proportions of primordial, intermediary, primary, secondary and atretic follicles in the groups of human ovarian tissue: non-frozen (Control), cryopreserved using PrOH and EG, and vitrified using Vit 5 and Vit 10 protocols.

vitrified tissue. Of these follicles, 110 (31%) were primordial and 214 (60%) intermediary/primary, comprising 145 (41%) intermediary follicles and 69 (19%) primary follicles. In the analyses, the intermediary/primary follicles were classified in one group of primary follicles. Twenty-eight (8%) secondary follicles were indentified and evaluated. Only 2 (1%) atretic follicles were found. The mean areas and volumes in the assessed sections were 2.1 $\pm$ 1.9 mm^2 (mean $\pm$ SD) and 0.5 $\pm$ 0.4 mm^3, respectively. As the numbers show, most of the follicles were at primordial, intermediary and primary stages in all samples (Fig. 3). There was no significant difference between the protocols in distribution of follicles of different developmental stages (Fig. 3). Large variation in the distribution of the follicles between patients and pieces from the same ovary were observed. Follicles were not found in all sections.

Microscopic visualization of haematoxylin/eosin-stained sections did not reveal any clear differences in the structures of the follicles cryopreserved using the different protocols. Most primordial, intermediary and primary follicles showed intact morphology (Fig. 4). Varying morphology was found between different follicles within the same sample of tissue. The quality of the follicles in the treatment groups is described in Table I. When the overall test was performed, there was a significant association between the protocols and quality of follicles after thawing/warming in the groups of primordial follicles ($P = 0.02$) and intermediary/primary follicles ($P = 0.01$), but not for the secondary follicles (Table I). Pairwise comparisons showed significant difference in the survival of primordial follicles cryopreserved using EG versus control ($P = 0.04$) and intermediary/primary follicles vitrified using Vit 5 protocol versus control ($P = 0.02$).

The architecture of the compact matrix of ovarian stromal tissue, which consists of stromal cells and bundles of collagen fibres (Fig. 4), was changed most in the specimens frozen using the slow protocols. Compared with control tissue, increased numbers of necrotic stromal cells with pyknotic nuclei and empty spaces in the extracellular matrix with disorganized collagen material were found. Quality of the stroma in the vitrified samples was comparable with that in non-frozen tissue (Fig. 4).

To illustrate changes in the ovarian stromal tissue caused by different cryopreservation protocols, a total of 18 high power fields from one block were examined for each treatment protocol (Fig. 4). The proportion of intact stromal cells to the overall number of the cells and an average number of intact cells per high power field is shown in the Table II. Stromal tissue in the non-frozen control tissue was compact, and 98% of stromal cells showed intact nuclei (Table II). The preservation of stromal cells was significantly lower in all

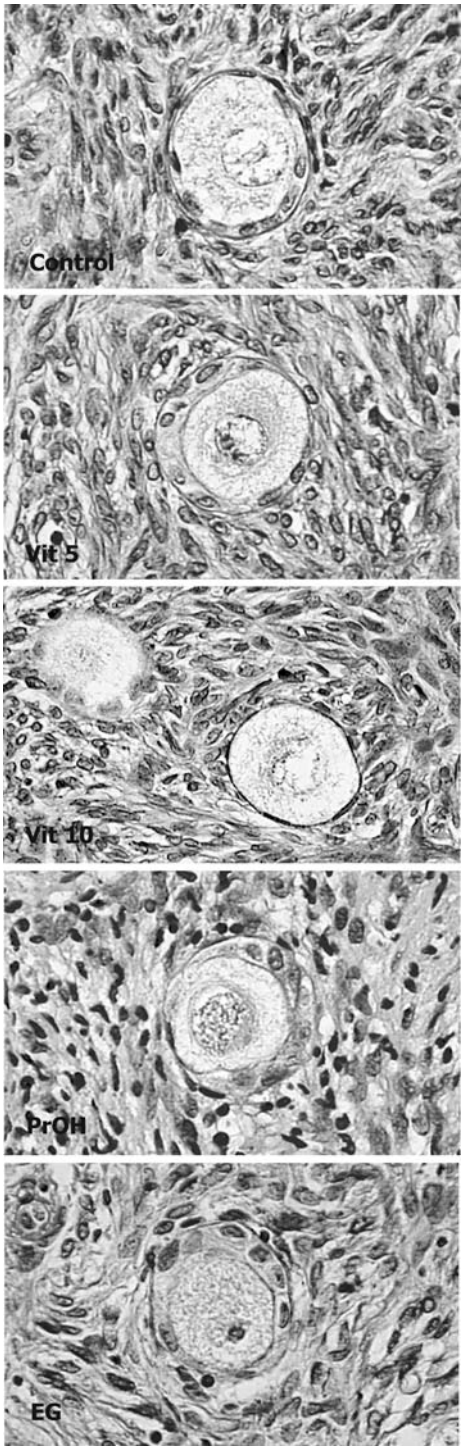

Figure 4 Light microscopic images of non-frozen (Control), vitrified (Vit 5 and Vit 10) and cryopreserved human ovarian cortex after using slow freezing programmes with PrOH and EG cryoprotectants.Well preserved intermediary (Control, Vit 5, PrOH, EG) and primordial (Vit 10, right placed) follicles displayed intact nuclear and cellular membranes, uniform oocyte cytoplasm and a prominent nucleus. Oocytes are surrounded by compact ovarian stroma in non-frozen tissue (control) and slightly influenced (see text for description) stromal tissue in vitrified samples (Vit 5 and Vit 10). Note increased numbers of necrotic cells and empty areas in the stromal tissue after slow programmed freezing (PrOH and EG).

cryopreserved samples when compared with that in the non-frozen control tissue. However, the proportion of intact cells in all the vitrified samples was significantly higher than it was in the samples frozen slowly using PrOH or EG (Table II).

Transmission electron microscopy

Ultrastructural evaluation was performed among 62 follicles, in which 55 oocytes (14 in unfrozen, 10 in PrOH, 6 in EG, 19 in Vit 5 and 6 in Vit 10 groups), and 633 granulosa cells were assessed. Thin sections of seven follicles had been cut near their surfaces and the oocytes could not be visualized. Hence, only the granulosa cells of these follicles were analysed. The integrity of the ovarian stroma was analysed in 85 high-power ultrastructural fields.

When the overall test was performed, there was a significant association between the protocols and the survival of the granulosa cells, and the quality of the stroma after thawing and warming. The differences in the survival of the oocytes were not significant (Table III).

Evaluation of the oocytes

The ultrastructure of the oocytes did not vary between groups (Fig. 5A–E) at the lower EM magnification. The nuclei of the oocytes of different developmental stages in almost all samples displayed a prominent nucleolus, normally distributed euchromatin and groups of intensely stained heterochromatin. Both the inner and outer membranes of the nuclear envelope were clearly visible in the majority of follicles of samples subjected to the different cryopreservation methods (Fig. $5A_1$–D_1).

In the samples cryopreserved using the slow protocol with EG as a cryoprotectant (Fig. $5E_1$) the nuclear membranes of some oocytes were not clearly distinguishable at high magnification in spite of the normal looking structure of the follicles at low magnification. In vitrified and slowly frozen PROH groups, no such changes were seen.

At a higher magnification (Fig. $5E_1$), the nuclei and cytoplasm of the oocytes in EG-cryopreserved tissue appeared blurred, to a similar extent in both structures, reflecting morphological changes caused by cryopreservation. Details of the endoplasmic reticulum could not be distinguished (Fig. $5E_1$) when compared with the well-defined endoplasmic reticulum in the unfrozen and vitrified samples (Fig. $5A_1$–C_1). The mitochondrial matrix was dense and well preserved but the mitochondrial cristae were not always clearly visible (Fig. $5E_1$). Increased vacuolization in the cytoplasm and disruption of cell-to-cell contacts between the oocytes and granulosa cells were the main signs of cryodamage (Fig. $5D_2$, E_2).

Mitochondria in oocytes of the vitrified samples (Fig. $5B_1$, C_1) exhibited highly organized structures with well-preserved outer membranes and, to some extent, a lighter matrix than that in the non-frozen control tissue (Fig. $5A_1$). The mitochondrial cristae were clearly visible (Fig. $5B_1$, C_1). Also after the use of the slow protocol with PrOH, well-preserved follicles contained mitochondria with a dense matrix and intact cristae (Fig. $5D_1$), but some mitochondria were swollen with dilated membrane and pale matrix. There were no clear differences in the EM morphology of the mitochondria between any of the studied groups.

No significant differences were found in the viability of the oocytes between the five treatment groups (Table III).

Table I Numbers and proportions (%) of intact (Int), influenced (Inf) and degenerated (Deg, see text for full descriptions) primordial, intermediary/primary and secondary follicles evaluated in human ovarian tissues which were not frozen (Control), cryopreserved using PrOH and EG, or vitrified using Vit 5 and Vit 10 protocols

Protocol	Total number of follicles	Number of primordial follicles (%)					Number of intermediary/primary follicles (%)					Number of secondary follicles (%)				
		Total	Int	Inf	Deg	Stat	Total	Int	Inf	Deg	Stat	Total	Int	Inf	Deg	Stat
Control	73	16	12 (75)	4 (25)	0	–	55	44 (80)	8 (15)	3 (5)	–	2	2 (100)	0	0	–
PrOH	91	35	18 (51)	7 (20)	10 (29)	–	45	33 (73)	11 (24)	1 (2)	–	11	8 (73)	2 (27)	1 (10)	–
EG	62	18	5 (28)	5 (28)	8 (44)	a	39	22 (56)	14 (36)	3 (8)	–	5	4 (80%)	1 (20)	0	–
Vit 5	68	22	9 (41%)	10 (45)	3 (14)	–	38	17 (45)	15 (39)	6 (16)	a	8	3 (38)	4 (50)	1 (12)	–
Vit 10	58	19	10 (53)	7 (37)	2 (10)	–	37	20 (54)	10 (27)	7 (19)	–	2	1 (50)	0	1 (50)	–

Each sample ($n = 20$) was divided into five pieces to allow direct comparisons of the four freezing protocols and fresh tissue, assessed by LM.
PrOH: 1,2-propanediol/sucrose cryoprotectant; EG: ethylene glycol/sucrose cryoprotectant; Vit 5: protocol with 5 min incubation in each vitrification solution; Vit 10: protocol with 10 min incubation in each vitrification solution.
Pairwise comparisons between treatment groups using Fisher's exact test.
[a]$P < 0.05$ versus non-frozen control tissue.

Evaluation of the granulosa cells

Granulosa cells were well preserved in all groups. They had contact with a uniform basement membrane, neighbouring granulosa cells and oocytes (Fig. $6A_2$–E_2). Well-preserved cells contained intact nucleus with nucleolus, homogeneous euchromatin and peripheral patches of condensed heterochromatin. The cytoplasm of the intact granulosa cells contained undamaged organelles and clearly distinguishable details of endoplasmic reticulum. The thickness of the follicular basement membrane depended on the developmental stage of the follicle (Figs. $5A_2$–E_2 and 6Vit 5). Numerous vesicles, loss of mitochondrial content and cristae, clumping of the cytoplasm and disruption of cell-to-cell contacts between the neighbouring granulosa cells and oocytes were observed in the granulosa cells which were damaged.

Pairwise comparisons between the protocols showed that the viability of the granulosa cells was significantly poorer after both slow freezing and vitrification ($P < 0.001$) than in the fresh control tissue (Table III). The number of morphologically normal granulosa cells was significantly lower in the tissue which had been cryopreserved with slow freezing using PrOH ($P < 0.001$) or after vitrification in Vit 10 group ($P = 0.001$) than after slow freezing using EG. The viability of the granulosa cells was significantly better in vitrified ovarian tissue in Vit 5 group than after slow freezing using PrOH ($P = 0.01$), or vitrification in Vit 10 group ($P = 0.002$) (Table III).

Evaluation of the stromal tissue

Clearly visible differences were observed in preservation of the ovarian stromal tissue. The stroma was better preserved in the vitrified samples (Fig. 7B, B_1 and Vit10) than after slow freezing (Fig. 7C and C_1). The best morphology of the stroma, composed of collagen fibres and spindle-shaped fibroblast-like cells, was found in samples in the Vit 10 group (Fig. 7 Vit10 inset). At a higher magnification, it was clearly seen that neighbouring stromal cells had intact cell contacts (Fig. 7 Vit10). The nuclei had normal morphology with intact nuclear membranes. The heterochromatin was homogenously distributed in the nuclear envelope, as was also seen in the non-frozen control cells (Fig. 7A, A_1) and in the Vit 5 group (Fig. 7B, B_1). Most of the stromal cells contained intact mitochondria and organelles (Fig. $7B_1$ and Vit10). The centrioles were usually found close to the nucleus or the Golgi apparatus (Fig. 7 Vit10). The cells were surrounded by an intact cellular membrane.

The stroma after slow programmed freezing using EG and PrOH showed signs of cryodamage. We found that many of the stromal cells were shrunken, contained pyknotic nuclei, ruptured cell membranes and vacuolization in the endoplasmic reticulum indicating an early sign of necrosis (Fig. 7C and C_1 inset). Most of the cells were at the late stage of necrosis and characterized by disruption of nuclear and cellular membranes, organelle breakdown and cell lysis (Fig. 7C and C_1). Collagen fibres were damaged and the extracellular matrix disrupted. Necrosis of the stromal cells and destruction and loss of collagen bundles has resulted in empty areas in the stromal tissue (Fig. 7C and C_1).

At higher magnification, damaged collagen fibres and empty spaces in the disorganized stroma in slowly frozen samples were seen (Fig. $7C_2$). Collagen fibres in vitrified samples were uniform and intact (Fig. $7B_2$), similar to the non-frozen control tissue (Fig. $7A_2$).

Table II The effect of different cryopreservation protocols using slow freezing (PrOH and EG) and vitrifcation on the integrity of ovarian stromal cells, as assessed by LM

Protocol	Total number of stromal cells	Total number of intact stromal cells (%)	Number of intact stromal cells per high power field (Mean ± SD%)
Non-frozen control	8160	7999 (98)	444 ± 62 (98 ± 1)
PrOH	10 019	2069 (21)[a,b,c]	115 ± 34 (22 ± 7)
EG	9125	4461 (49)[a,b,c]	248 ± 61 (51 ± 15)
Vit 5	7708	6971 (90)[a]	387 ± 40 (90 ± 3)
Vit 10	7619	6983 (92)[a]	388 ± 49 (92 ± 4)

The results are presented as total number and proportion (%) of intact stromal cells assessed within the groups; the average number (Mean ± SD) and proportion (%) of intact stromal cells per 909 μm^2 high power field.
Vit 5/10: protocol with 5 or 10 min incubation in each vitrification solution.
[a]$P < 0.05$ versus non-frozen control tissue.
[b]$P < 0.05$ versus vitrified samples in group Vit 5.
[c]$P < 0.05$ versus vitrified samples in group Vit 10.

Clearly poor preservation of the ovarian stroma was found in the slowly frozen tissue after the use of PrOH or EG. The difference was significant when compared with the non-frozen tissue ($P < 0.001$). The viability of the stroma in the Vit 5 group was significantly better than that seen after slow programmed freezing using EG ($P < 0.001$). However, it was poorer when compared with the non-frozen control tissue ($P = 0.02$). The number of high power fields with a good quality stroma was significantly higher in the Vit 10 group than it was in slowly frozen tissue ($P < 0.01$ versus PrOH and versus EG). The viability of the stromal cells and the extracellular matrix in specimens in the Vit 10 group was slightly poorer than that in the non-frozen control tissue but this difference was not significant. No significant differences were found in the viability of the stroma between the two vitrification protocols (Table III).

Discussion

Vitrification of human ovarian tissue proved to be effective in cryopreservation of follicles within pieces of ovarian tissue. We found that the ovarian stroma was much better preserved by vitrification than it was when using slow freezing.

Slow programmed freezing of ovarian tissue with PrOH and sucrose as cryoprotectants was earlier developed by our team as a relevant procedure for preservation of human ovarian tissue (Hovatta *et al.*, 1996; Hreinsson *et al.*, 2003). Cryopreservation media containing DMSO and EG have also been used for slow freezing of ovarian tissue (Hovatta *et al.*, 1996; Newton *et al.*, 1996; Oktay *et al.*, 1997; Kim *et al.*, 2002; Schmidt *et al.*, 2003a, b). Live births have been reported after transplantation of thawed tissue (Donnez *et al.*, 2004; Meirow *et al.*, 2005). In spite of successful preservation of oocytes and fairly good viability of granulosa cells, low survival of

Table III Ultrastructural evaluation (by transmission electron microscopy) results of intact, influenced and degenerated oocytes, granulosa cells and ovarian stroma in non-frozen (Control) ovarian tissue, in tissue cryopreserved using slow programmed freezing with PrOH and EG, and vitrified tissue using Vit 5 and Vit 10 protocols.

Protocol	Number of oocytes (%)					Number of granulosa cells (%)					Number of stromal cells high power fields (%)				
	Total	Int	Inf	Deg	Stat	Total	Int	Inf	Deg	Stat	Total	Int	Inf	Deg	Stat
Control	14	4 (29)	10 (71)	0	–	166	165 (>99)	1 (<1)	0	–	14	7 (50)	7 (50)	0	–
PrOH	10	0	7 (70)	3 (30)	–	131	105 (80)	7 (5)	19 (15)	a, b, d	13	1 (8)	1 (8)	11 (84)	a, b, c
EG	6	0	6 (100)	0	–	50	33 (66)	16 (32)	1 (2)	a, c	17	0	2 (12)	15 (88)	a, b, c
Vit 5	19	4 (21)	15 (79)	0	–	188	147 (78)	29 (16)	12 (6)	a, c	27	3 (11)	15 (56)	9 (33)	a
Vit 10	6	2 (33)	2 (33)	2 (33)	–	98	65 (66)	10 (10)	23 (24)	a	14	8 (57)	4 (29)	2 (14)	–

Data presented as total number and percentage (parentheses).
Vit 5/10: protocol with 5 or 10 min incubation in each vitrification solution.
For pairwise comparisons between protocols:
[a]$P < 0.05$ versus non-frozen control tissue.
[b]$P < 0.05$ versus vitrified tissue in group Vit 5.
[c]$P < 0.05$ versus vitrified tissue in group Vit 10.
[d]$P < 0.05$ versus tissue cryopreserved using EG.

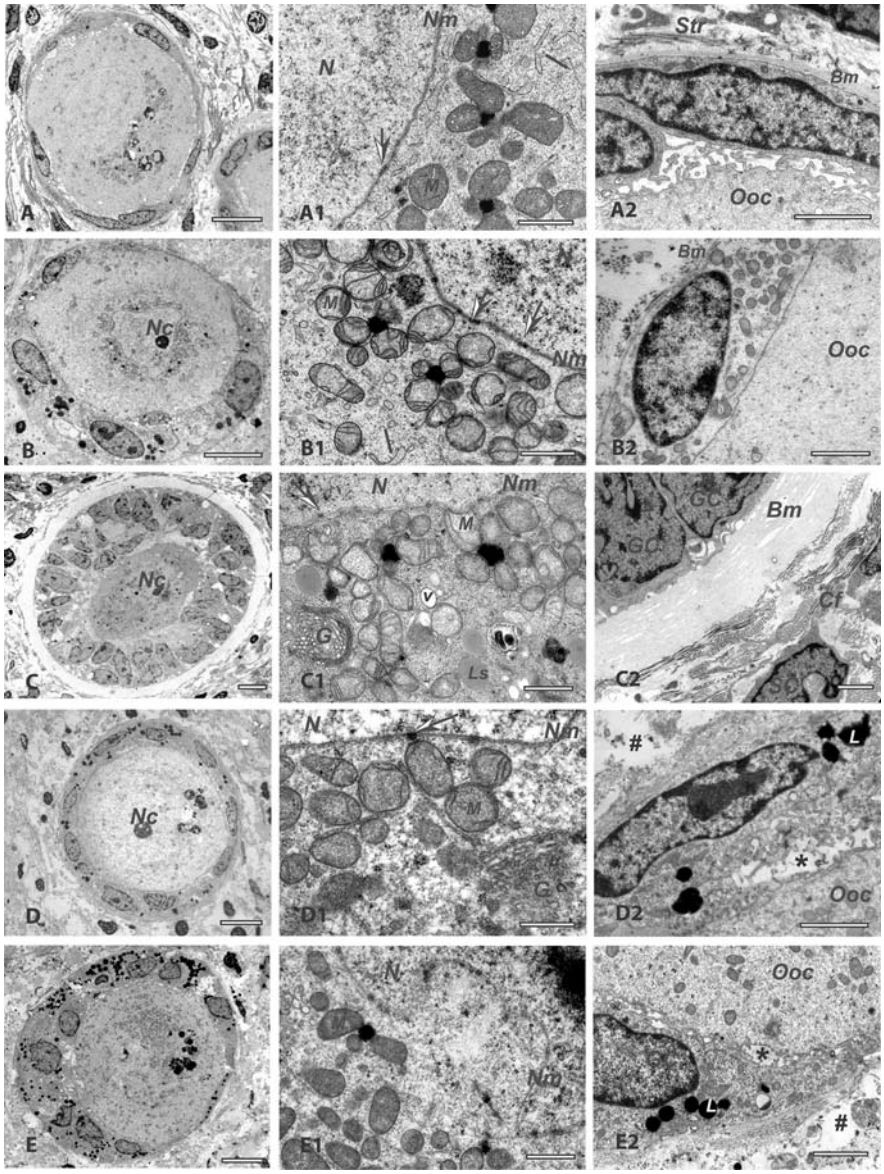

Figure 5 Transmission electron microscopy images of follicles within pieces of non-frozen (control) human ovarian tissue (**A, A_1** and **A_2**), vitrified tissue using Vit 5 (**B, B_1** and **B_2**) and Vit 10 (**C, C_1** and **C_2**) protocols and tissue cryopreserved using slow freezing protocols with PrOH (**D, D_1** and **D_2**) and EG (**E, E_1** and **E_2**).

(A) A primordial follicle within compact ovarian stroma (Str) in the control tissue. The oocyte (Ooc) is surrounded by one layer of flattened granulosa cells. (A_1) A high magnification shows the oocyte nuclei with homogenous euchromatin (N) surrounded by the prominent nuclear membrane (Nm) with visible nuclear pores (arrow). Cytoplasm is organized and contains well defined microtubules and cisternae of endoplasmic reticulum (line) and dark crista-type mitochondria (M). (A_2) Flattened granulosa cells are attached to the thin basement membrane (Bm) and keep contacts with the oocyte microvilli (mv). The nuclei appear as homogenous structures with partial densities and dense aggregation of heterochromatin attached to the nuclear membrane. (B) Well preserved intermediary/primary follicle from Vit 5 with visible nucleolus (Nc). Indented nuclear membrane was seen in all groups. (B_1). Abundant mitochondria with uniform cristae and lighter matrix than those in the control tissue are grouped around non-damaged nucleus. The cytoskeleton in the cytoplasm is similar to that in the control. (C) A secondary follicle from the Vit 10 group with an oocyte surrounded by cuboidal cells. (C_1) The nucleus and the cytoplasmic organelles, Golgi apparatus (G), mitochondria and lysosomes (Ls) are well preserved. Small vacuoles (v) appear in the cytoplasm of some of the oocytes. The same small vacuoles are seen in the control samples (A). (C_2) Well preserved stroma consists of compact collagen fibres (Cf) and stromal cells (SC). Cuboidal granulosa cells are well attached to thick Bm. (D and E) Healthy looking intermediary follicles after slow controlled rate freezing are surrounded by the damaged stoma. (D_1) Undamaged nuclear membrane with nuclear pores is well defined. Mitochondria and Golgi apparatus are well preserved but the details of endoplasmic reticulum are not distinguished. (E_1) The cytoplasm is disaggregated and nuclear membrane is not visible at a high magnification. Mitochondria are dense but cristae are not distinguished. (D_2 and E_2) The follicles surrounded by disrupted collagen fibres, forming empty spaces (#) in the ovarian stroma. Intact granulosa cells contain undamaged nuclei and lipid droplets (L) in the cytoplasm and partially detached from oocyte surface (*). Scale bars = 10 μm (A–E), 1 μm (A_1–E_1) and 2 μm (A_2–E_2).

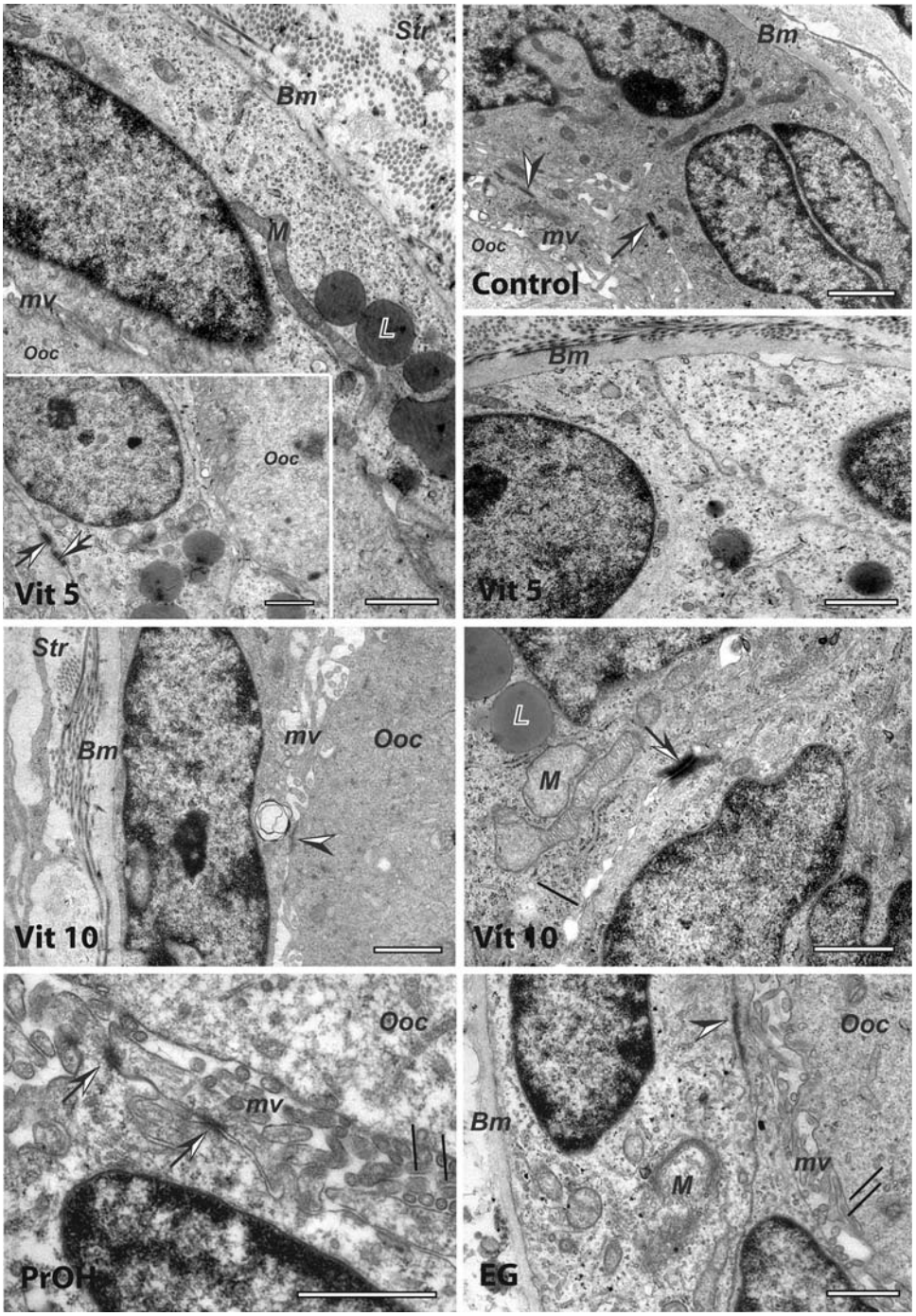

Figure 6 Transmission electron microscopy images of granulosa cells, cryopreserved within pieces of human ovarian tissue by vitrification (Vit 10 and Vit 5), or by a slow freezing protocol with PrOH or EG.
Granulosa cells were well preserved and displayed tight contact with a uniform Bm within all groups. Clearly visible desmosomes (arrows) and tight junctions (arrowheads) connect neighbouring granulosa cells. Mv, forming gap junctions (lines) between the Ooc and granulosa cells are clearly seen. The thickness of the follicular Bm depended on the developmental stage of the follicle (Vit 5). Granulosa cells contain L in the cytoplasm. Well preserved M with dense matrix and defined cristae in vitrified tissue (Vit5 and Vit 10). Some of the mitochondria have lighter matrix without distinguished cristae (EG). The cytoplasm is slightly disaggregated and shows changes in the microtubule network (PrOH) but the contacts between granulosa cells and oocytes are well preserved (mv). Scale bar = 1 μm.

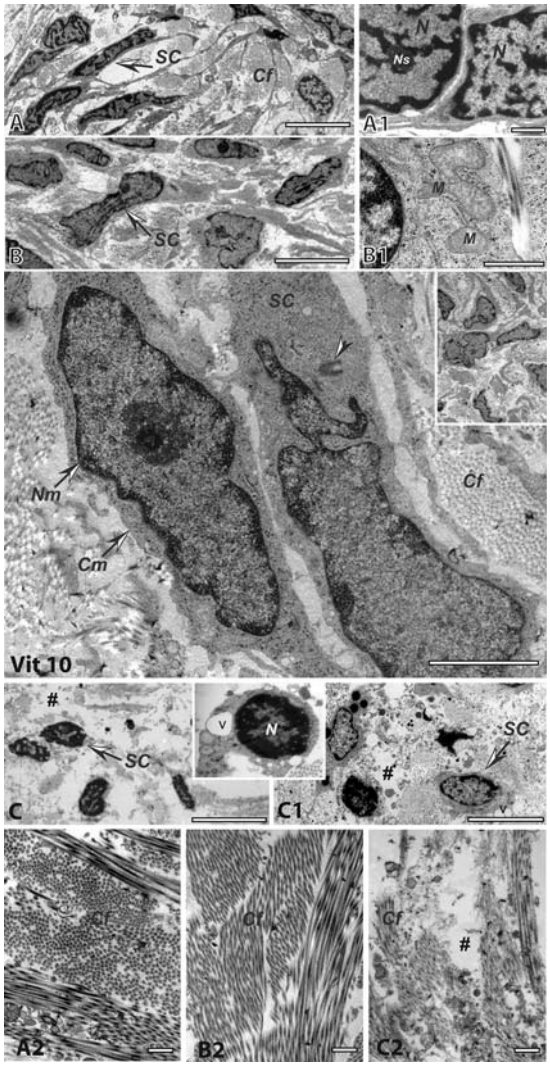

Figure 7 Transmission electron microscopy of the stroma in non-frozen ovarian tissue (**A**–**A_2**), vitrified using Vit 5 protocol (**B**–**B_2**) and Vit 10 (**Vit 10**) protocols and in tissue cryopreserved by a slow programme using PrOH (**C** and **C_2**) or EG (C_1 and inset). (A) Cortical stroma in non-frozen ovarian tissue is composed of closely packed, spindle-shaped fibroblast-like cells (SC) and bundles of Cf. The sample from group Vit 5 (B) shows a structure similar to those in non-frozen (A) and vitrified tissue in Vit 10 group (Vit 10 inset). At a higher magnification (B_1), the nuclei displayed normal morphology with distinct nuclear membranes and clearly distinguished mitochondria. High magnification of SC with intact Nm and cellular membranes (Cm), keep contact with each other (Vit 10). A centriole is present close to the nucleus (arrowhead). Ovarian stroma after slow programmed freezing with PrOH contains collapsed SCs with pyknotic nuclei (N) and vacuolated cytoplasm (v) (C–C_1, inset). Ovarian stroma after slow programmed freezing with EG (C_1) contain influenced SCs (arrow) and collapsed SCs with ruptured cellular membrane, as well as N and v. No disintegration of the stroma is seen in cross-sections of Cf or in longitudinally sectioned Cf in non-frozen (A_2) and vitrified tissue (B_2). Disrupted bundles of Cf and empty spaces (#) in the disorganized stroma are typical signs of cryodamage in slowly frozen sample (C_2). Scale bars = 5 μm (A, A_1, B, B_1, C, C_1 and Vit 10) and 2 μm (A_2, B_2 and C_2).

ovarian stroma is a limitation of slow programmed freezing (Hreinsson *et al*., 2003).

We have now carried out the first systematic comparison between vitrification and slow-programmed freezing of human ovarian tissue. The integrity of the tissue after cryopreservation depends on the condition of non-frozen tissue used in the experiment. There appeared to be clear patient to patient variation. In the present study, we compared the results of cryopreservation using different protocols performed on tissue obtained from the same patient. Tissue samples from each donor were processed in parallel in all protocols. This gave us an opportunity to minimize the effect of patient variation.

In the tissue which we received for our study the proportion of primordial follicles was relatively low, and intermediary/primary follicles represented the major population of follicles. Our data are in agreement with previous findings showing that the proportion of primordial follicles decreased and the proportion of growing follicles increased with age (Gougeon and Chainy, 1987; Lass *et al*., 1997). The mean age of our patients was 33.3 years. Significant relationship between age and follicular density as well as a wide variation in the number and distribution of ovarian follicles in different patients, even between each fragment of the same ovary, was also shown by Schmidt *et al*. (2003a, b). There was also a large variation in morphology of the tissue from different patients and from different fields of the ovary. We found only two atretic follicles, but the oocytes may have disappeared and the structure may have not been recognized as a follicle. The 24-h culture which was used as a viability assay, may have caused some atresia, which is a common feature *in vitro* (Hovatta *et al*., 1997, 1999).

We pooled into one group both intermediary follicles, which already have at least one cuboidal cell, and primary follicles with a single layer of cuboidal granulosa cells. This classification has also been used in previous studies (Gougeon, 1986; Hovatta *et al*., 1997; Hreinsson *et al*., 2003). In accordance with our earlier results (Hovatta *et al*., 1996; Hreinsson *et al*., 2003), the viability of the follicles was well maintained by slow programmed freezing using either PrOH or EG as permeating cryoprotectants. This is also in agreement with the results of other investigators (Newton *et al*., 1996; Schmidt *et al*., 2003a, b). At the same time, the stroma was not as well preserved. However, after vitrification, the viability of the stroma was much better.

Vitrification results in good morphology because ice crystal formation in the cryopreserved sample can be avoided. Formation of a solidified amorphous state can be achieved by high-speed cooling and by use of high concentrations of penetrating cryoprotectants (Fahy *et al*., 1984; Taylor *et al*., 2004). Addition of non-penetrating cryoprotectants, such as polymers, also prevents ice crystal formation in vitrification solutions. Overall toxicity can be reduced by using a combination of cryoprotectants (Fahy *et al*., 2004), as in the present study.

Recently, Isachenko *et al*. (2007) showed that for cryopreservation of human ovarian tissue, conventional freezing is more successful than rapid freezing. While concentration of cryoprotectants in solutions used for rapid freezing in their study was similar to vitrification solution, the conditions of their protocol were different from ours. Our vitrification procedure was improved at many points in comparison with those used earlier: a mixture of PrOH, EG and DMSO, diluted in serum-free medium, was used for pre-incubation.

The concentration of each cryoprotectant was increased stepwise from 2.5% (total cryoprotectant concentration 7.5%) at the first

incubation step to 15% at the second exposure. At the final step the concentration of each cryoprotectant was increased to 10% (total 30%), and PVP was then added. Hence, the final overall concentration of cryoprotective agents in the vitrification solution was 40%.

To reduce toxicity, we equilibrated the samples at the third pre-vitrification step at +4°C. Trying to reduce the chemical toxicity of vitrification media, we compared the efficacy of using two durations for exposure, 5 or 10 min at each step.

To speed up cooling of the samples, we used an open system which comprised 0.5 ml insemination cryostraws, cut by scalpel. Using these gave us an opportunity to reduce the volume of solution that surrounded the sample. It also enabled us to vitrify several pieces of tissue at the same time by direct plunging into liquid nitrogen when placed on the same straw. To be successful, the procedure requires fast handling of the tissue and the straw, and this has to be practiced well before clinical application of the method.

We did not carry out the procedure in a clinical setting, but our method is transferable to a good manufacturing practice laboratory (European Parliament, 2004). Sterile liquid nitrogen has to be used for clinical applications, and the straws with vitrified tissue have to be closed in pre-cooled cryo-tubes.

To avoid the effect of devitrification during thawing, the samples were immediately placed in pre-warmed solution, which enabled a sufficiently high warming rate. Stepwise removal of cryoprotectants in decreasing concentrations of sucrose in the thawing media enabled avoidance of osmotic damage to the cells.

We used tissue culture to evaluate the viability of the frozen-thawed tissue, because the integrity of the tissue immediately after thawing may not reflect its true state. After 24 h of culture, well-preserved cells are shown to recover. However, this culture procedure may have changed slightly the proportions of primary follicles in the analysed tissues. As we have shown in our earlier studies, initiation of growth of the follicles is a fast event *in vitro* (Hovatta *et al*., 1997).

Ultrastructural assessment of tissue samples gives much more detailed information about structural cryodamage of the cell than LM (Hreinsson *et al*., 2003; Keros *et al*., 2005, 2007). This has also been shown by others (Gook *et al*., 1999; Nisolle *et al*., 2000; Eyden *et al*., 2004; Martinez-Madrid *et al*., 2007). Information regarding ultrastructural changes in the morphology of ovarian tissue after cryopreservation is limited. Chen *et al*. (2006) showed that after direct cover vitrification (DCV) of mouse ovarian tissue, primordial follicles have normal ultrastructure, whereas conventional vitrification, as reported by Salehnia *et al*. (2002), and slow programmed freezing using PrOH cause increased swelling of mitochondria and disappearance of cristae. These data were confirmed with a higher pregnancy rate in mice after transplantation of ovarian tissue after DCV vitrification (Chen *et al*., 2006).

While cryopreservation of ovarian cortex (Gook *et al*., 1999; Eyden *et al*., 2004) and whole human ovaries with their vascular pedicle (Martinez-Madrid *et al*., 2007) resulted in consistently good preservation of oocytes, slow programmed freezing may still have caused damage in the tissue. Structural preservation of ovarian tissue including oocytes, granulosa cells and surrounding stromal tissue depends on the composition and time of exposure to cryopreservation media, and cooling and thawing rates (Gook *et al*., 1999, 2004). After transplantation of human ovarian tissue cryopreserved with 10% DMSO to nude mice, the morphology of the xenografts was assessed by both LM and TEM, revealing the presence of apparently normal follicles at different developmental stages (Nottola *et al*., 2007).

Preservation of granulosa cells has usually been more variable than that of the oocytes (Gook *et al*., 1999; Hreinsson *et al*., 2003; Eyden *et al*., 2004). As regards retaining intact cell and mitochondrial membranes, the structure of the granulosa cells can differ within a given cell and between cells within a follicle (Eyden *et al*., 2004). Loss of plasma membrane, increased chromatin condensation, lysis of stromal cells, vacuolization and disintegration of ovarian stroma has reflected poor preservation after slow programmed freezing (Hreinsson *et al*., 2003; Eyden *et al*., 2004). These observations are in agreement with our results.

Visualization of the nucleus and nucleolus in every oocyte in electron microscopic sections is not possible. However, electron microscopy is the best method to evaluate the cellular membrane. The integrity of the nuclear membranes, which is not always observed on low magnification (×970 to ×1650), was confirmed on the images at magnification ×8900 and higher. We confirmed our findings in the present study by judging many compartments at a high magnification (×8900 up to ×30 000).

Vitrification is a fast method, which saves time and costs. No special equipment, such as the expensive apparatus required for slow programmed freezing, is needed. Only 15–30 min is required to get the samples into storage. Vitrification is particularly useful when a whole ovary or two ovaries are removed and a large amount of tissue has to be cryopreserved. The final proof of the feasibility of vitrification can only be obtained by clinical transplantation of vitrified-thawed tissue, and by the birth of a child. As shown in our study, there was no difference in the survival of oocytes but there was a slight difference in the survival of granulosa cells. Preservation of the cells and matrix of the stroma were clearly better after vitrification than after slow conventional freezing. This indicates that vitrification is an advanced alternative method for cryopreservation of ovarian tissue in patients facing gonadotoxic therapy and post-treatment ovarian failure.

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