Article

Effective method for in-vitro culture of cryopreserved human ovarian tissue

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Abstract

It is widely accepted that it is possible to successfully cryopreserve human ovarian tissue by direct plunging into liquid nitrogen using permeable cryoprotectants only, without disaccharides. This study aimed to search for and test a new method for in-vitro culture of vitrified tissue. Ovarian biopsies were obtained during operative laparoscopy. Pieces of ovarian tissue were vitrified and warmed. After warming, tissue pieces were randomly distributed into three groups for further culture: in 2 ml of culture medium which was regularly renewed (group 1), in 30 ml of culture medium without agitation (group 2) and in 30 ml of culture medium with agitation (group 3). During the 2-week and 6-week culture, the growth of follicles within the vitrified-warmed ovarian tissue pieces was investigated. After 2 weeks of culture, mean numbers of non-degenerated follicles per mm^2 of tissue were 1.5, 1.7 and 4.5 for groups 1, 2 and 3 respectively (groups 1 and 2 versus group 3, $P < 0.05$). Agitation during culture of ovarian tissue is beneficial, and can be used as a prognostic tool for future warming and autotransplantation of ovarian tissue.

Keywords: *cryopreservation, culture, follicles, human ovarian tissue*

Introduction

In a single country (USA), more than 50,000 reproductive-aged women will be diagnosed with cancer each year (Practical Committee of the ASRM, 2004). The successful results of oncological treatment (Grovas *et al.*, 1997) especially encourage young women that they will have a chance of later motherhood through cryopreservation of ovaries or ovarian tissue prior to the medical treatment.

The possibility of childbirth after cryopreservation of ovarian tissue has been suggested because normal follicular development and ovulation, with the subsequent formation of a corpus luteum was observed after gonadotrophin stimulation in a woman who had been grafted with ovarian tissue that had been previously frozen (Oktay and Karlikaya, 2000). By grafting cortical ovarian tissue after cryopreservation, it was possible to sustain ovulation and pregnancy, and in animals and humans live birth has also been achieved (Gosden *et al.*, 1994; Donnez *et al.*, 2004).

Part of the ovarian tissue obtained before the oncological treatment is used for routine histological observation. This is mandatory in order to minimize the risk of future transplantation of tissue with metastases. Some time after cryopreservation and storage, part of the ovarian tissue can be thawed and cultured *in vitro* in order to check for the presence of metastases as well as follicles. The quality of follicles present in the cultured tissue indicates whether or not it is possible to restore a woman's reproductive function. Autotransplantation (to the same patient) of cryopreserved tissue has been successful (Imhurn *et al.*, 2000; Radford, 2003; Oktay *et al.* 2004).

At present, there are three ways to determine the quality of the cryopreservation procedure for a given piece of ovarian tissue

from a given patient without participation of the patient to her full restoration after medical treatments: (i) evaluation of follicles immediately after cryopreservation/thawing (Poirot *et al*. 2002); (ii) evaluation of follicles after post-thawing culture (Isachenko *et al*. 2002, 2003; Hreinsson *et al*. 2003; Hovatta, 2004); (iii) evaluation of follicles after post-thawing xenotransplantation. Although all three methods give enough information, xenografting is still the most conclusive (Newton *et al*., 1996; Oktay *et al*., 1998; Gook *et al*., 2003, 2005); however, this method is also the most expensive. Therefore, the search for an efficient method for in-vitro culture of ovarian tissue in order to obtain the best information about the quality of the tissue deserves investigation.

The aim of this study was to find and test an effective method for in-vitro culture of vitrified tissue.

Materials and methods

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St Louis, MO, USA.)

Tissue collection and dissection

Informed consent was given by nine patients aged between 36 and 40 (38.4 ± 2.2) years under a protocol approved by the University Ethics Board. Ovarian biopsies were obtained during operative laparoscopy and uniformly smooth areas of cortex fragments were recovered by antimesenteric dissection of ovarian pieces. Ovarian tissue was transported on ice in phosphate-buffered saline (DPBS) medium with 5% fetal calf serum (FCS).

Dissection of the ovarian cortex was performed in DPBS medium with 10% FCS at room temperature and processed within 1 h. Cryopreservation solutions were prepared in DPBS medium supplemented with 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, CA, USA).

The ovarian pieces (OP) were cut into strips of approximately 1 × 1 × 5 mm using a no. 22 scalpel and tweezers in a sterile laminar airflow. Sufficient ovarian cortical fragments were obtained from each biopsy to provide three specimens to be randomly assigned to cryopreservation and experimental cultivations.

Vitrification and warming

A vitrification protocol using a mixture of permeable cryoprotectants without disaccharides (Isachenko *et al*., 2002) was used. The vitrification medium contained DPBS + 20% dimethylsulphoxide (DMSO) + 20% ethylene glycol (EG) + 10% SSS. Prior to transfer of OP to the tubes with vitrification solution and plunging into liquid nitrogen, saturation with EG was performed in the following way (**Figure 1**). OP with the same holding medium in which the ovarian pieces were dissected (10 ml DPBS medium + 10% FCS) were placed in a 110-ml specimens container (Falcon, Le Pont de Claix, France). This container was placed on a rotation shaker and a stepping saturation by 40% EG for 40 min at room temperature was achieved using the original dropping methodology as demonstrated in **Figure 1**. For dropping saturation, 40 ml of 50% EG + 10% SSS + DPBS in a 50-ml tube (Greiser Bio-One GmbH, Essen, Germany) was used. After the end of dropping, OP were held in EG for an additional 10 min for complete saturation. The final

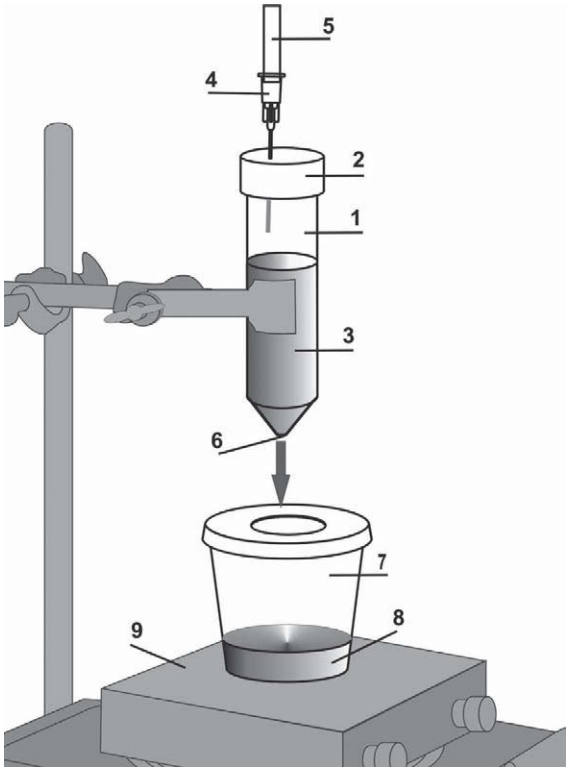

Figure 1. Diagram of step-wise saturation of ovarian pieces by ethylene glycol. (1) tube, (2) lid, (3) ethylene glycol solution, (4) narrow injection tube, (5) tap for air, (6) opening, (7) container with ovarian pieces, (8) holding medium, (9) shaker.

EG concentration at the end of saturation was 40% (40 ml of 50% EG plus 10 ml holding medium). During the process of saturation, the container was rotated at 200 oscillations/min.

OP were then placed into standard 1.8 ml cryovials (5 per vial) (Nunc, Roskilde, Denmark) that were partially filled with 1.5 ml vitrification medium precooled to 4°C. Then, after 15 min exposure of OP in vitrification medium at 4°C, tubes were sealed and plunged into liquid nitrogen.

Quick warming was achieved by holding the vials for 30 s at room temperature in air followed by immersion in a 100°C (boiling) water bath for 60 s and expelling of the contents of tubes into solution for removal of cryoprotectants. The time exposure in the boiling water was visually controlled by the presence of ice in the medium: as soon as the ice was only ~2 mm apex, the tube was expelled from the boiling water. The final temperature of the medium after warming ranged between 4 and 10°C. After warming, OP were transferred within a few seconds (5–7) to a 110 ml specimen container with 10 ml of solution for removal of cryoprotectants (0.75 mol/l sucrose + 10% FCS + DPBS). The stepping dilution of cryoprotectants was achieved using the same principle as that used for saturation by EG (**Figure 1**). The container was placed on the shaker and continuously agitated at 200 oscillations/min for 15 min at room temperature. Stepping rehydration of OP for 30 min at room temperature was also performed using the same dropping methodology (**Figure 1**). For dropping rehydration, 50 ml of the holding medium, which had previously been employed for the dissection manipulations (DPBS + 10% FCS,) was used in a 50 ml tube (Greiner Bio-One GmbH, Frickenhausen, Germany). The final sucrose concentration was 0.125 mol/l. Finally, the OP were washed 3 times each in PBS supplemented with 15% fetal calf serum and in culture medium for 10 min followed by culture. After warming and washing, OP were randomly allocated to three groups: standard culture in small volume with periodical changing of the culture medium (group 1), culture in a large volume without agitation (group 2), culture in a large volume with agitation (group 3). OP were cultured in Iscove's modified Dulbecco's medium supplemented with 10% FCS, insulin–transferrin–selenium (10 and 5.5 μg/ml, and 6.7 ng/ml respectively), 20 ng/ml epidermal growth factor (EGF), 0.3 IU/ml recombinant h-FSH (FSH, Gonal-F; Serono, Unterschleissheim, Germany) and 50 μg/ml of antibiotics/antimycotics (penicillin G, streptomycin sulphate and amphotericin B).

Control culture

The control culture of vitrified group 1 OP was performed for 3 weeks using the method described by Isachenko *et al.* (2002). OP (one piece in one well) were transferred into 24-well culture plates (Nunc) with 2.0 ml of culture medium and incubated under 5% CO_2 in air at 37°C for 21 days. Every day, 500 μl of culture medium was replaced with fresh medium.

Culture in large volume

For group 2, the experimental culture of vitrified OP was performed as follows. Pieces were washed after warming and transferred to 200 ml dishes for suspension culture (Cellstar™; Greiner Bio-One GmbH) with 30 ml of culture medium. OP (20 per 30 ml volume) were incubated under 5% CO_2 in air at 37°C for a period of 2 or 6 weeks.

Culture in large volume with agitation

In group 3, OP were washed after warming and transferred to 200 ml dishes for suspension culture (Cellstar™) with 30 ml of culture medium. OP were incubated for 3 weeks under 5% CO_2 in air at 37°C for a period of 2 or 6 weeks with 75 oscillations/min agitation using a rotation shaker.

Histology

Fresh, as well as vitrified/warmed OP after long-term (2-week and 6-week) culture, were fixed in 3.5% paraformaldehyde for 4–24 h at 4°C. The pieces were embedded in paraffin wax, serially sectioned to 5 µm on a Leica RM 2135 microtome, stained with haematoxylin and eosin and analysed under a Nikon Diaphot 300 microscope (×200 and ×400).

Statistical analysis

Treatment effects on the parameters assessed were evaluated by analysis of variance (ANOVA). The level of statistical significance was set at $P < 0.05$.

Results

Assessment of the quality of the ovarian tissue after culture under different conditions was determined by quantity of morphologically normal follicles. The morphological quality of OP was dependent on the conditions of culture. Standard culture of OP using a decreased volume of culture medium with regular changes of old medium to the new one as well as culture in the large volume of medium without agitation resulted in a low rate of follicular morphological integrity. No microscopic changes of follicles or stroma in groups 1 and 2 were observed. Follicle damage was also similar for both these groups. The majority of follicles in the biopsies were primordial.

The best result was observed when vitrified ovarian tissue was cultured for two weeks in a large volume of culture medium in combination with agitation (**Figures 2**, **3**). In group 3, the proportions of normal follicles were significantly higher than in groups 1 and 2. The mean numbers of non-degenerated follicles per mm^2 of tissue for groups 1, 2 and 3 were 1.5, 1.7 and 4.5 respectively ($P < 0.05$).

The formation of blood vessels after 2-week culture was noted (**Figure 3**). This process was most intensive when cultivation in a large volume of agitated medium was performed. After long-term (6-week) culture the degeneration of all (from primordial to antral) follicles was observed (**Figure 3**).

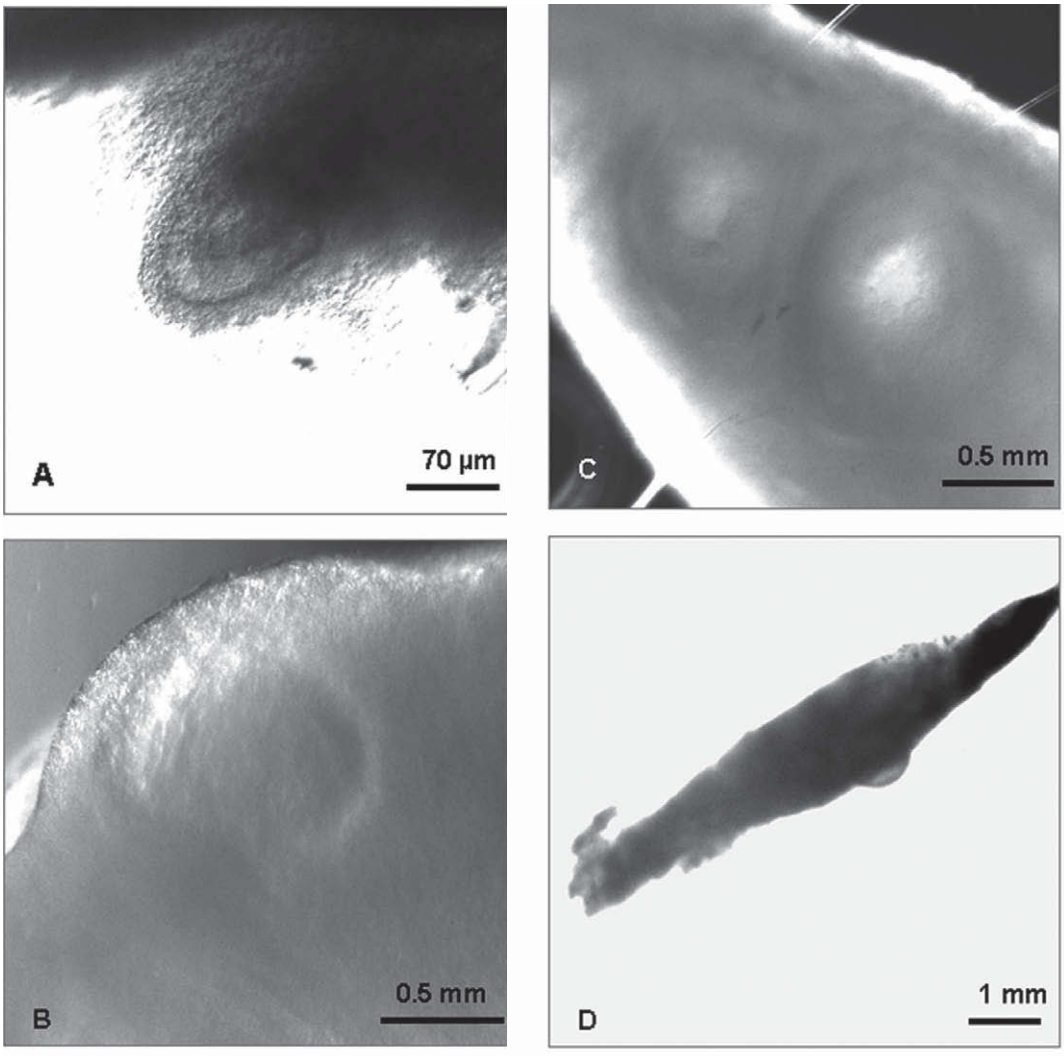

Figure 2. Follicles developed in vitrified/warmed ovarian pieces after 2-week culture: (**A**) premordial, (**B** and **C**) early antral, (**D**) antral.

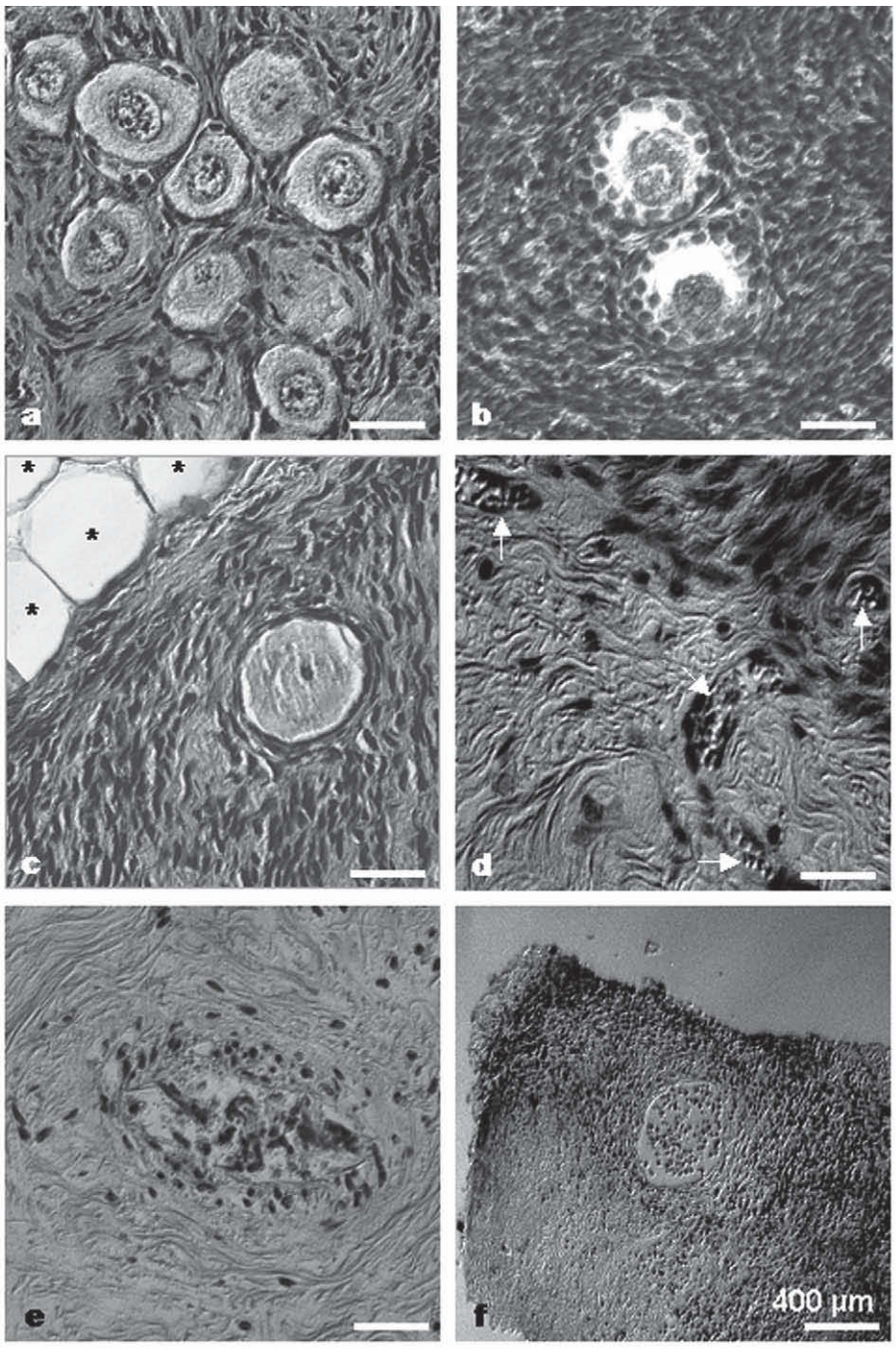

Figure 3. Vitrified/warmed ovarian tissue after 2-week (**a–d**) and 6-week (**e**, **f**) culture in a large volume of medium by agitation. Bar = 50 μm. Note: formation of blood vessels with erythrocytes (arrows in **d**) and adipose tissue (fat cells) (asterisks in **c**).

Discussion

Following biopsy of ovarian tissue prior to oncological therapy, the routine cryopreservation procedure includes histopathological investigation of a sample of the fresh ovarian tissue. In the laboratory, some months after cryopreservation, part of the ovarian tissue is usually warmed and cultured to determine the presence and quality of follicles. This observation allows the determination of the possibility of restoring reproductive function.

Ovarian tissue cryopreservation has several advantages over cryopreservation of oocytes and embryos because the human ovarian cortex contains primordial germ cells, the number of which at birth is about 100,000 in young women and in the range of a few thousand follicles in older women (Faddy *et al.,* 1992). In addition, ovarian tissue can be collected by laparoscopy at any time independent of the stage of the menstrual cycle (Shaw *et al.,* 1996), and a primordial follicle has properties that make it less cryosensitive than mature oocytes. The oocytes and supporting cells are small in size, the tissues have a low metabolic rate, contain only a small quantity of cold-sensitive intracellular lipids, the cells are at prophase of meiosis I and the zona pellucida is absent. Furthermore, primordial follicles do not have species-specific morphological characteristics, making them an especially suitable model for investigation (Shaw *et al.*, 2000). However, cryopreservation of large tissues and organs becomes problematic in contrast to isolated cells (Mazur *et al.,* 1972).

There is a special method of in-vitro culture of ovarian pieces using extracellular matrix (Eppig, 1992). This method is used successfully for the culture of different types of cells of both animal and human. A recent study reported on the effectiveness of this method for in-vitro culture of human ovarian tissue (Scott *et al.,* 2004). Ovarian biopsies were cut into small (1–2 mm^3) or larger (5–30 mm^3) pieces and in-vitro cultured during two time periods, 1 week and 2 weeks. The aim of these studies was to test the different conditions of culture on development of follicles. It was established that culturing ovarian tissue as cubes appeared to be beneficial for sustaining follicle viability and density, at least for culture of 7 days duration, but the increased viability was not significant after 14 days culture (Scott *et al.,* 2004). The new system of in-vitro culture of human ovarian tissue described here allows the culture of relatively large pieces of tissue during at least 3 weeks without decreasing the quality of follicles. Earlier, it was shown that human ovarian tissue shows good viability and growth after long-time culture, but because of the still existing suboptimal culture conditions, they can be cultivated for a limited time only (Rahimi *et al.,* 2001).

Previously, a standard conventional freezing and vitrification of human ovarian tissue has been compared. Examination of the necrotic areas of the ovarian tissue samples after both these methods of cryopreservation and transplantation into SCID mice showed no significant rise in comparison with the control group (Rahimi *et al.,* 2004). The central aim of the present investigations was not shown by the effectiveness of the original vitrification method. The mode of vitrification, described here, can only demonstrate that the objects were human ovarian tissues after cryopreservation. In fact, it is logical to suppose that on fresh tissues as well as on tissues after conventional freezing, the best results can be obtained after cultivation in a large volume of culture medium with agitation, in comparison with culture in a small volume with changing of medium or in a large volume without agitation.

The protocols described for vitrification of ovarian tissue have the following parameters: (i) the vitrification solution is saccharose-free and includes only permeable cryoprotectants; (ii) stepwise procedure for saturation by and removal of permeable cryoprotectants; (iii) saturation by EG at room temperature and by DMSO at 0°C; (iv) rapid warming of tissues at +100°C.

The first parameter of the technology (use of saccharose-free vitrification solution) was chosen because it was previously shown that the presence of disaccharide sucrose in vitrification solution, which is a non-permeable osmotically active cryoprotectant, has a detrimental effect on the developmental rate of cells (Isachenko *et al.,* 2002). Migishima *et al.* (2003) reported a new method of cryopreservation of whole mouse ovaries by vitrification using permeable cryoprotectants without sucrose (dimethyl sulphoxide, acetamide and propylene glycol). It was noted that cryopreservation of the ovary by vitrification seems a promising method to preserve ovarian function, but further studies are required to overcome the possible inhibitory effects of this method on the growth of the ovarian graft.

The second parameter (stepping introduction and removal of EG and removal of both EG and DMSO) can be explained by the sensitivity of human cells to osmotic processes accompanying the saturation by permeable cryoprotectants and their removal (Isachenko *et al.,* 2004).

The third parameter (saturation by DMSO at 0°C) can be explained by the following. Cryopreservation by direct plunging into liquid nitrogen requires pretreatment of the biological object in media containing a high concentration of permeable cryoprotectants. Ethylene glycol was chosen as a basic permeable cryoprotectant, due to its relatively low toxicity (see Hovatta, 2005 for observation). In contrast to saturation of isolated cells, saturation of tissues by permeable cryoprotectants needs a long exposure time in cryoprotective solution. It is therefore important to consider the 'optimal' ratio of equilibration time/negative (toxic) effect of cryoprotectant before cooling (Pegg and Diapper, 1988) as well as the time of removal of cryoprotectant/osmotic injuries after warming. Toxicity of cryoprotective agents is a key limiting factor in cryobiology (Fahy, 1986). DMSO has a high toxicity (Shaw *et al.*, 2000). The saturation of cells by DMSO at 0°C (third parameter of the technology) reduces DMSO toxicity. The thermodynamics of vitrification solution, which was used for vitrification, can detect how cells respond to the cryopreservation process (Fuller and Paynter, 2004). The process of cryopreservation by direct plunging into liquid nitrogen (so-called vitrification) described here includes ice formation at warming: appearance of the rest of thawing ice is a detector for stopping of warming in boiling water. Current concepts of nucleation, ice crystal growth and solute exclusion from the ice lattice are discussed in a review by Fuller and Paynter (2004) which illustrates what cells must negotiate to avoid lethal damage, and the role of cryoprotectants in enhancing recovery.

The fifth parameter of technology is rapid warming. The factor

that potentially can decrease the viability of cryopreserved tissues is re-crystallization at warming. Therefore, in the current protocol, very quick warming of the tissue was used as a fourth parameter. The research base for this is the previously established positive effect of high speed warming of pronuclear and GV oocytes at vitrification (Isachenko *et al*., 2005a,b, 2006).

The data show that the vitrification/warming protocol in combination with long term culture *in vitro* in a large volume of culture medium and under constant agitation supports in-vitro growth of follicles better compared with routine culture conditions. Therefore, this new technology may be beneficial to investigate the potential of vitrified ovarian tissue prior to its use in any therapeutic approach.

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References

Donnez J, Dolmans MM, Demylle D *et al*. 2004 Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* **364**, 1405–1410.

Eppig J 1992 Growth and development of mammalian oocytes in vitro. *Archives of Pathology and Laboratory Medicine* **116**, 379–382.

Faddy M, Gosden R, Gougeon A *et al*. 1992 Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. *Human Reproduction* **7**, 1342–1346.

Fahy GM 1986. The relevance of cryoprotectant 'toxicity' to cryobiology. *Cryobiology* **23**, 1–13.

Fuller B, Paynter S 2004 Fundamentals of cryobiology in reproductive medicine. *Reproductive BioMedicine Online* **9**, 680–691.

Gook DA, Edgar DH, Borg J *et al*. 2005 Diagnostic assessment of the developmental potential of human cryopreserved ovarian tissue from multiple patients using xenografting. *Human Reproduction* **20**, 72–78.

Gook D, Edgar D, Borg J *et al*. 2003 Oocyte maturation, follicle rupture and luteinization in human cryopreserved ovarian tissue following xenografting. *Human Reproduction* **18**, 1772–1781.

Gosden R, Baird D, Wade J *et al*. 1994 Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196°C. *Human Reproduction* **9**, 597–603.

Grovas A, Fremgen A, Rauck A *et al*. 1997 The National Cancer Center Data Base report on patterns of childhood cancers in the United States. *Cancer* **80**, 2321–2332.

Hovatta O 2005 Methods for cryopreservation of human ovarian tissue. *Reproductive BioMedicine Online* **10**, 729–734.

Hovatta O 2004 Cryopreservation and culture of human ovarian cortical tissue containing early follicles. *European Journal of Obstetrics, Gynaecology and Reproductive Biology* **113** (Suppl. 1), S50–54.

Hreinsson J, Zhang P, Swahn ML *et al*. 2003 Cryopreservation of follicles in human ovarian cortical tissue. Comparison of serum and human serum albumin in the cryoprotectant solutions. *Human Reproduction* **18**, 2420–2428.

Imhurn B, Cox SL, Jenkin G *et al*. 2000 Gonadotrophin administration can benefit ovarian tissue grafted to the body wall: implications for human ovarian grafting. *Molecular Cell Endocrinology* **163**, 141–146.

Isachenko V, Montag M, Isachenko E *et al*. 2006 Aseptic vitrification of human germinal vesicle oocytes using dimethyl sulfoxide as a cryoprotectant. *Fertility and Sterility* **85**, 741–747.

Isachenko V, Montag M, Isachenko E *et al*. 2005a Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws. *Human Reproduction* **20**, 492–496.

Isachenko V, Montag M, Isachenko E *et al*. 2005b Vitrification of mouse pronuclear embryos after polar body biopsy without direct contact with liquid nitrogen. *Fertility and Sterility*, **84**, 1011–1016.

Isachenko V, Montag M, Isachenko E *et al*. 2004 Developmental rate and ultrastructure of vitrified human pronuclear oocytes after step-wise versus direct rehydration. *Human Reproduction* **19**, 660–665.

Isachenko E, Isachenko V, Rahimi G *et al*. 2003 Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen. *European Journal of Obstetrics, Gynaecology and Reproductive Biology* **108**, 186–193.

Isachenko V, Isachenko E, Rahimi G *et al*. 2002 Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen: negative effect of disaccharides in vitrification solution. *CryoLetters* **23**, 333–344.

Mazur P, Leibo SP, Chu EHY 1972 A two-factor hypothesis of freezing injury. *Experimental Cell Research* **71**, 345–351.

Migishima F, Suzuki-Migishima R, Song S *et al*. 2003 Successful cryopreservation of mouse ovaries by vitrification. *Biology of Reproduction* **68**, 881–887.

Newton H, Aubard Y, Rutherford A *et al*. 1996 Low temperature storage and grafting of human ovarian tissue. *Human Reproduction* **11**, 1487–1491.

Oktay K, Karlikaya G. 2000 Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *New England Journal of Medicine* **342**, 1919.

Oktay K, Buyk E, Veeck L *et al*. 2004 Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. *Lancet* **363**, 837–840.

Oktay K, Newton H, Mullan J *et al*. 1998 Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone. *Human Reproduction* **13**, 1133–1138

Pegg DE, Diaper MP 1988 On the mechanism of injury to slowly frozen erythrocytes. *Biophysical Journal* **54**, 471–488.

Poirot C, Vacher-Lavenu M-C, Helardot P *et al*. 2002 Human ovarian tissue cryopreservation: indications and feasibility. *Human Reproduction* **17**, 1447–1452.

Practice Committee of the American Society for Reproductive Medicine 2004 Ovarian tissue and oocytes cryopreservation. *Fertility and Sterility* **82**, 993–998.

Radford J 2003 Restoration of fertility after treatment for cancer. *Hormone Research* **59** (Suppl.), 21–23.

Rahimi G, Isachenko E, Isachenko V *et al*. 2004 Comparison of necrosis in human ovarian tissue after conventional slow freezing or vitrification and transplantation in ovariectomized SCID mice. *Reproductive BioMedicine Online* **9**, 187–193.

Rahimi G, Isachenko E, Sauer H *et al*. 2001 Measurement of apoptosis in long-term cultures of human ovarian tissue. *Journal of Reproduction* **122**, 657–663.

Scott J, Carlsson I, Bavister B *et al*.2004 Human ovarian tissue cultures: extracellular matrix composition, coating density and tissue dimensions. *Reproductive BioMedicine Online* **9**, 287–293.

Shaw J, Oranratnachai A, Trounson A 2000 Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology* **53**, 59–72.

Shaw JM, Bowles J, Koopman P *et al*. 1996 Fresh and cryopreserved ovarian tissue samples from donors with lymphoma transmit the cancer to graft recipients. *Human Reproduction* **11**, 1668–1676.

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