Article

Cryopreservation of human ovarian tissue: effect of spontaneous and initiated ice formation

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Abstract

This investigation compared conventional freezing of human ovarian tissue using either spontaneous or initiated ('seeded') ice formation. Biopsies of ovarian tissue were obtained from women with indications for chemotherapy or radiotherapy. Small pieces of experimental tissue were randomly distributed into three groups that were then subjected to different treatments prior to culture *in vitro* for 16 days: the control group, no treatment, cultured immediately after biopsy (group 1); cryopreservation/thawing with spontaneous ice formation (group 2); and cryopreservation/thawing with initiated ice formation (group 3). Follicle viability and hormonal activity were then evaluated. There was no significant difference between groups regarding the concentration of oestradiol 17-β in the culture supernatant, whereas progesterone concentration was significantly ($P < 0.05$) higher in group 1 compared with group 2 or 3. There was a significant ($P < 0.05$) difference in primordial and primary follicle density between all of the groups (group 1 having the highest and group 2 having the lowest) and group 2 had significantly ($P < 0.05$) fewer normal grade follicles than the other two groups. For optimal cryopreservation of human ovarian tissue, the protocol of conventional freezing should therefore include a step of initiated ice formation.

Keywords: *cryopreservation, follicles, hormones, human ovary, ice formation, seeding*

Introduction

In 2007, in the USA alone, it is possible that 678,000 have been diagnosed with cancer (Jemal *et al.*, 2007). The effectiveness of oncological treatment grows every year (Grovas, 1997), increasing the survival rate. Cryopreservation of ovarian tissue prior to their medical treatment gives young female cancer patients the chance to retain their reproductive potential.

Normal follicular development and ovulation of fertilizable oocytes after gonadotrophin stimulation has been obtained in a woman who was grafted with a biopsy of ovarian tissue that had been previously frozen (Oktay and Karlikaya, 2000). Childbirth after cryopreservation of the ovaries is now a reality; by grafting cortical ovarian tissue to an appropriate site after cryopreservation, it is possible to sustain ovulation and pregnancy. Live birth after grafting of frozen/thawed ovarian tissue has also been achieved in both animals and humans (Gosden *et al.*, 1994; Donnez *et al.*, 2004; Meirow *et al.*, 2005, 2007).

Use of cryopreserved ovarian tissue is not limited to achieving a pregnancy alone; ovarian tissue can also be used for therapeutic purposes. It has been reported that patients with premature ovarian failure after cancer treatment can recover ovarian function after reimplantation of an ovarian cortex biopsy, and thus avoid menopausal symptoms (Oktay and Karlikaya, 2000; Callejo *et al.*, 2001; Radford *et al.*, 2001; Oktay *et al.*, 2004; Schmidt *et al.*, 2005; Wolner-Hanssen *et al.*, 2005; Donnez *et al.*, 2006, 2007).

The procedure of ovarian tissue cryopreservation includes the formation of ice under controlled conditions. Generally, this ice formation is artificially initiated in a super-cooled cryopreservation medium (so-called 'seeding') at a certain temperature (as a rule from –6 to –9°C).

Published data have reported effective and promising cryopreservation protocols for isolated follicles and ovarian tissue with spontaneous ice formation, without automatic or manual initiation of the process (Martinez-Madrid *et al.*, 2004; Jadoul *et al.*, 2007). Cryopreservation of ovine hemi-ovaries was performed with a programmable freezer, with semi-automatic seeding (Salle *et al.*, 2002).

Demirci *et al.* (2001) carried out a study in which cryopreservation with spontaneous ice formation was compared with a protocol where initiation of ice formation was semi-automatic; better results were found following semi-automatic seeding. Published data that compare these two methodologies of ice formation (spontaneous and initiated) for cryopreservation of human ovarian tissue are limited.

The aim of this investigation was to compare conventional cryopreservation of human ovarian tissue, using either spontaneous or initiated ice formation.

Materials and methods

Unless otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, Missouri, USA).

Tissue collection, dissection, and distribution to groups

Informed consent was given by seven patients aged between 21 and 32 (28.1 ± 2.4) years, under a protocol approved by the University Ethics Board. Ovarian tissue was donated by women who were at risk of infertility after oncological treatment: all patients suffered from malignant diseases. Ovarian tissue fragments (1–3 to 2–3 cm) were obtained from seven patients before oncology treatment during diagnostic or operative laparoscopies, via biopsies of the ovarian cortex. These were transported to the laboratory within 22–25 h in a special, isolated transport box (DeltaT GmbH, Giessen, Germany). This box, containing a special medium for transportation of ovarian tissue (Brama I, CryoBioSystem, L'Aigle Cedex, France), can maintain a stable temperature of between 5°C and 8°C for 36 h. The biopsies were dissected in medium for ovarian tissue manipulation (Brama II, CryoBioSystem, L'Aigle Cedex, France).

Small samples of ovarian cortex (1 × 1 to 1.5 × 0.7 to 1 mm) were randomly distributed into three groups: group 1 (control) consisted of fresh tissue immediately after receipt of the transport box; group 2 consisted of samples thawed after cryopreservation with spontaneous ice formation; and group 3 consisted of samples thawed after cryopreservation with initiated ice formation. All three groups of tissue were cultured *in vitro* for 16 days. Six samples from each patient were used for the experiments, with two samples allocated to each of the three groups. Fresh samples from each patient were observed for histology, and the follicles were photographed according to the laboratory's routine protocol. Each sample was cryopreserved in a single cryo-vial.

Cryopreservation with spontaneous ice formation

The cryopreservation protocol was based on that of Gosden *et al.* (1994), with some modifications. Standard 1.8 cryo-vials (Nunc, Roskillde, Denmark) were filled with 1.8 ml of cryopreservation medium containing L-15 Medium (Leibovitz) with L-glutamine +1.5 mmol/l dimethyl sulphoxide (DMSO) + 10% serum substitute supplement (SSS, Irvine Sci., St. Ana, CA, USA) and were cooled in iced water (0°C). Samples were then transferred to the cryo-vials and held in iced water for 30 min. Cryo-vials were subsequently placed in an IceCube 14S freezer (SyLab, Neupurkersdorf, Austria), with the freezing chamber previously stabilized at 2°C for 20–30 min. The cryopreservation programme was as follows: (i) the starting temperature was 2°C because the temperature of the cryo-vials increased from 0°C to 2°C after their manipulation and transfer from ice-water to the auto-seeding block; (ii) samples were cooled from 2°C to –6°C at a rate of –2°C/min; (iii) samples were then held at –6°C for 10 min; (iv) samples were cooled from –6°C to –40°C at a rate of –0.3°C/min; and (v) cooling to –140°C at a rate of –10°C/min followed by plunging of cryo-vials into liquid nitrogen.

Development and testing of auto-seeding equipment

An IceCube 14S freezer was used for conventional cryopreservation of samples, because this freezer incorporates a feature that initiates ice formation (seeding) automatically. The auto-seeding block was specially developed for this study in collaboration with the manufacturers (SyLab), generating a novel piece of equipment (**Figure 1**). The location of this block in the freezer chamber guarantees full contact of the upper part of the cryo-vial wall with the tube that conducts liquid nitrogen (**Figure 1**), so that ice formation is initiated at a certain temperature.

The reliability of the auto-seeding block was tested before starting the cryopreservation experiments. Cryoprotectant solution was cooled from room temperature (22°C) to –40°C, with seeding at –6°C. At this temperature, the liquid nitrogen begins to enter and cool the conducting tubes, and these tubes cool the cryo-vials containing cryopreservation medium (**Figure 1**). Twenty-eight cryo-vials with 1.8 ml of cryopreservation medium were placed in the auto-seeding block (**Figure 1**).

The freezer has two identical control electrodes: one to monitor the chamber temperature, and the other to monitor the probe temperature. The probe electrode was placed in the vials to monitor the temperature of cryopreservation medium. This electrode detected the cryo-vial temperature in four different places (positions 1, 2, 3, and 4; **Figure 1**). Eight experiments were carried out, with cryopreservation of 224 cryo-vials in two replicates: four experiments using 28 cryo-vials in two replicates (with four different positions 1, 2,

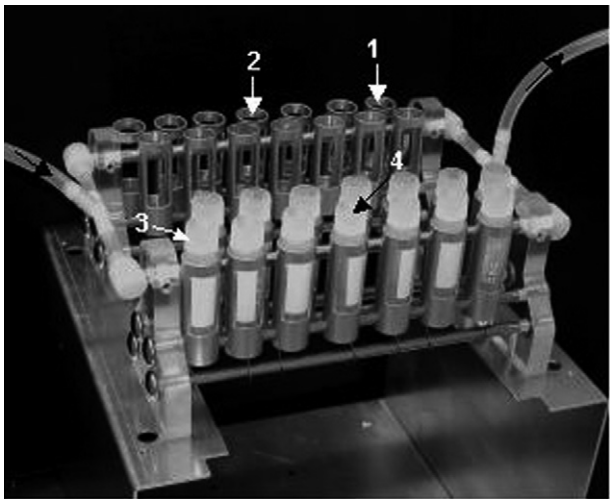

Figure 1. Auto-seeding block of the freezer. Arrows labelled 1, 2, 3, 4 indicate four different positions at which the temperature of the cryo-vials was measured. Black unnumbered arrows show the direction of movement of liquid nitrogen into and out of the cooling elements (cooling conductor) at auto-seeding.

3, and 4 of a probe electrode). The cryo-vial temperatures in positions 1, 2, 3, and 4 were measured as +5, 0, –2, and –5°C, respectively, using a Testo 950 electrical thermometer (Testo AG, Lenzkirch, Germany). Synchrony of the formation and growth of crystals, dependent on the position of the cryo-vial in the seeding block, was monitored visually through special openings in the cylinders containing the cryo-vials (**Figure 1**). Using these openings, it is also possible to initiate seeding manually.

The following three parameters to measure the reliability of auto-seeding were considered: start of seeding relative to the position of the cryo-vial in the seeding block; rate of temperature decrease after the beginning of crystallization (at –6°C) relative to the position of the cryo-vial in the seeding block; rate of temperature increase after the beginning of crystallization relative to the position of the cryo-vial in the seeding block.

Cryopreservation with auto-seeding

This cryopreservation protocol differs from the protocol with spontaneous ice formation only by the initiation of ice formation at –6°C (**Figure 2**) using an auto-seeding block. The cryopreservation programme is as described above, and includes the following stages: (i) starting temperature is 2°C; (ii) samples were cooled from 2°C to –6°C at a rate of –-2°C/min; (iii) samples were then held at –6°C for 10 min; (iv) cooling of samples from –6°C to –40°C at a rate of –0.3°C/min; and (v) cooling to –140°C at a rate of –10°C/min and plunging of cryo-vials into liquid nitrogen.

Thawing and removal of cryoprotectants

Samples from groups 2 and 3 were thawed using an identical procedure: the vials were held for 30 s at room temperature followed by immersion in a 100°C (boiling) water bath for 60 s, and the contents of the tubes were expelled into solutions for the removal of cryoprotectants. The exposure time in boiling water was visually controlled by monitoring the presence of ice in the medium; as soon as the ice was only about 2 mm from the apex, the tube was removed from the boiling water. The final temperature of the medium after warming ranged between 4 and 10°C. After thawing, samples were transferred within 5–7 s to a 100 ml specimen container (Sarstedt, Nuemrecht, Germany) containing 10 ml of solution for removal of cryoprotectants (0.75 mmol/l sucrose + 10% SSS + L-15 medium). The cryoprotectant dilution steps were prepared using the same principle as that used for saturation by ethylene glycol (see Figure 1 in Isachenko *et al.*, 2006b). The container was placed on a shaker and continuously agitated at 200 oscillations/min for 15 min at room temperature. The tissue was rehydrated for 30 min at room temperature using the same stepwise dilutions (Isachenko *et al.*, 2006b) starting in 50 ml of holding solution (L-15 medium + 10% SSS) in a 50 ml tube (Greiner Bio-One GmbH, Frickenhausen, Germany). Drops of holding medium were slowly added to the solution of sucrose with samples. The final sucrose concentration was 0.125 M. Finally, the samples were each washed three times in Dulbecco's phosphate-buffered saline supplemented with 10% SSS (Irvine). After warming and washing, each sample was transferred into the culture system.

Culture

Samples from control group 1 (samples cultured immediately after receipt of transport box) and experimental groups 2 and 3 (samples cultured after cryopreservation/thawing) were placed into 200 ml dishes with 30 ml of AIM-V® medium (Gibco, Grand Island, NY, USA) for suspension culture (CellstarTM, Greiner Bio-One GmbH, Frickenhausen, Germany). These were cultured *in vitro* for 16 days at 37°C in 5% CO_2, with 75 oscillations/min agitation using a rotation shaker (Isachenko *et al.*, 2006b). Replicate cultures were studied by culturing two samples in each dish, each sample in the dish being from the same patient, having had the same treatment.

The viability of the tissue was evaluated by assaying in-vitro production of hormones during culture, and development of follicles after culture.

Hormone assays

The spent medium after culture of cryopreserved and fresh samples was collected every second day (40 µl per collection from two cultured samples from each patient) and stored at –80°C for 1–2 months for subsequent hormone assays. Before measurement of hormones, frozen medium was thawed, and culture media of respective in-vitro culture days from each of the seven patients were pooled together. The level of oestradiol 17-β (analytical sensitivity <10 pg/ml) and progesterone (analytical sensitivity <0.1 ng/ml) was measured in 280 µl medium (40 µl medium from 7 patients) using Architect chemiluminescence microparticle immunoassay (Abbott Diagnostic, Wiesbaden-Delkenheim, Germany).

Histological examination

For histological investigation, tissue pieces were fixed in Bouin solution for 22–24 h at room temperature, embedded in paraffin wax, serially sectioned at 5 µm, stained with haematoxylin/eosin, and analysed under a microscope Zeiss (×400). The number of viable and damaged follicles was counted. To avoid over-counting of the same follicles, only sections in which a complete oocyte nucleus was fully visible were evaluated for follicle counts.

The follicles were evaluated by considering the parameters previously described by Paynter *et al.* (1999), i.e. characteristics of granulosa cells and of oocyte nucleus and cytoplasm. Two types of follicles were evaluated: primordial follicles surrounded by a single layer of flat cumulus cells, and primary follicles, which are similar to primordial follicles but surrounded by 1–2 layers of spheroidal granulosa cells. The quality of the follicles was graded from 1 to 3. A follicle of grade 1 is spherical with granulosa cells randomly distributed around the oocyte, which has homogenous cytoplasm and a slightly granulated nucleus with condensed chromatin in the form of a dense spherical structure detectable in the centre. A follicle of grade 2 has the same features, but granulosa cells do not cover the oocyte regularly; these cells can be flat, and condensed chromatin is not detected in the cytoplasm. A follicle of grade 3 has partially or fully disrupted granulosa and/or cytoplasm, and a pyknotic nucleus. Follicles of grades 1 and 2 were classified as normal, and those of grade 3 were classified as degenerate.

Statistical analysis

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

Results

Cryopreservation with spontaneous ice formation

When the temperature of the cryopreservation medium reached –17 ± 1.5°C, a sharp increase in temperature to –5 ± 1.5°C was noted. After this increase in temperature, the cooling rate was stabilized to the normal –0.3°C/min for 30 min (**Figure 3**). During these 30 min, the rate of temperature decrease was changed from –1°C/min (for a period of 10 min) to the given decreasing rate of –0.3°C/min (**Figure 3**).

Test of auto-seeding equipment

The growth of crystals was observed at –7.7 ± 0.2°C in all four positions of the cryo-vials, in two replicates (224 cryo-vials were used). It was established that the process of crystallization of cryopreservation medium begins and proceeds synchronously independently of the cryo-vial position in the seeding block (**Figure 1**). The difference in temperature in the different cryo-vials was additionally monitored in positions 1, 2, 3, and 4 at +5, 0, –2, and –5°C, respectively, using thermometer Testo 950; the measured temperature difference was not more than 0.2°C.

A decrease in the chamber temperature (**Figure 2**) was noted after the beginning of auto-seeding, due to the cooling of the liquid nitrogen conductor (**Figure 1**). This decrease in temperature had no effect on the temperature of the cryopreservation solution (**Figure 2**). During cooling, the cover of the freezing chamber was also deliberately opened for 7 s, in order to mimic additional manipulations in the chamber, as in the case of manual seeding. This operation produced no increase or decrease in the temperature of the cryopreservation medium (**Figure 2**).

Hormone assays

The level of hormones in native AIM-V medium were determined as oestradiol < 10 pg/ml and progesterone <0.1 ng/ml. After the culture of fresh tissue samples (group 1), samples after cryopreservation/thawing with spontaneous ice formation (group 2) and samples after cryopreservation/thawing with initiated ice formation (group 3) for 16 days the pooled supernatants showed oestradiol 17-β concentrations of 476, 465 and 459 pg/ml, respectively, and progesterone concentrations of 9.68, 5.77, 5.61 ng/ml, respectively. The progesterone concentration as significantly higher ($P < 0.05$) in the supernatant of group 1 compared with that of either group 2 or group 3. There were no other statistically significant differences between the values recorded. Hormone concentrations are graphically presented in **Figure 4**.

Histological examination

The only follicles classified as viable were primordial and primary. All preantral and antral follicles after in-vitro culture were degenerate, and these follicles were not counted. The mean follicle density and number classified as normal (grades 1 and 2), together with morphological appearance, are presented in **Table 1** and **Figure 5**. It was noted that the procedure of initiated ice formation had a positive effect on follicle viability.

Discussion

The protocol for cryopreservation of ovarian tissue is based on an embryo cryopreservation protocol (Whittingham, 1971; Whittingham *et al.*, 1972). Seeding (i.e. initiated ice formation)

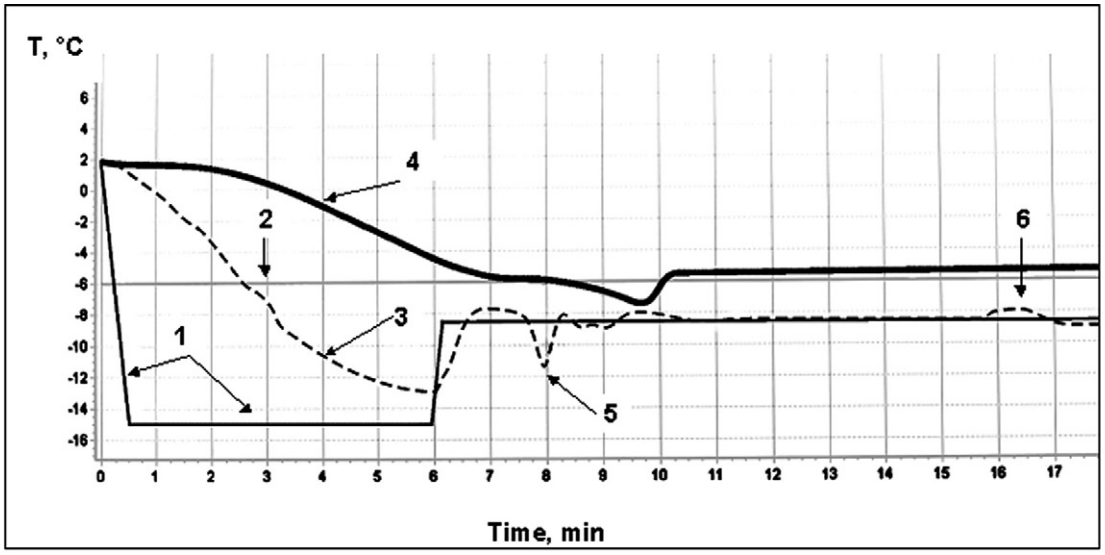

Figure 2. Temperature (T) profile of freezer showing: (1) programme of cooling; (2) auto-seeding temperature (–6°C); (3) temperature of chamber; (4) temperature of freezing medium; (5) decrease in chamber temperature after start of auto-seeding (note: no change in the temperature of the freezing medium); (6) decrease in chamber temperature after 7 s removal of cover (note: no change in the temperature of the freezing medium).

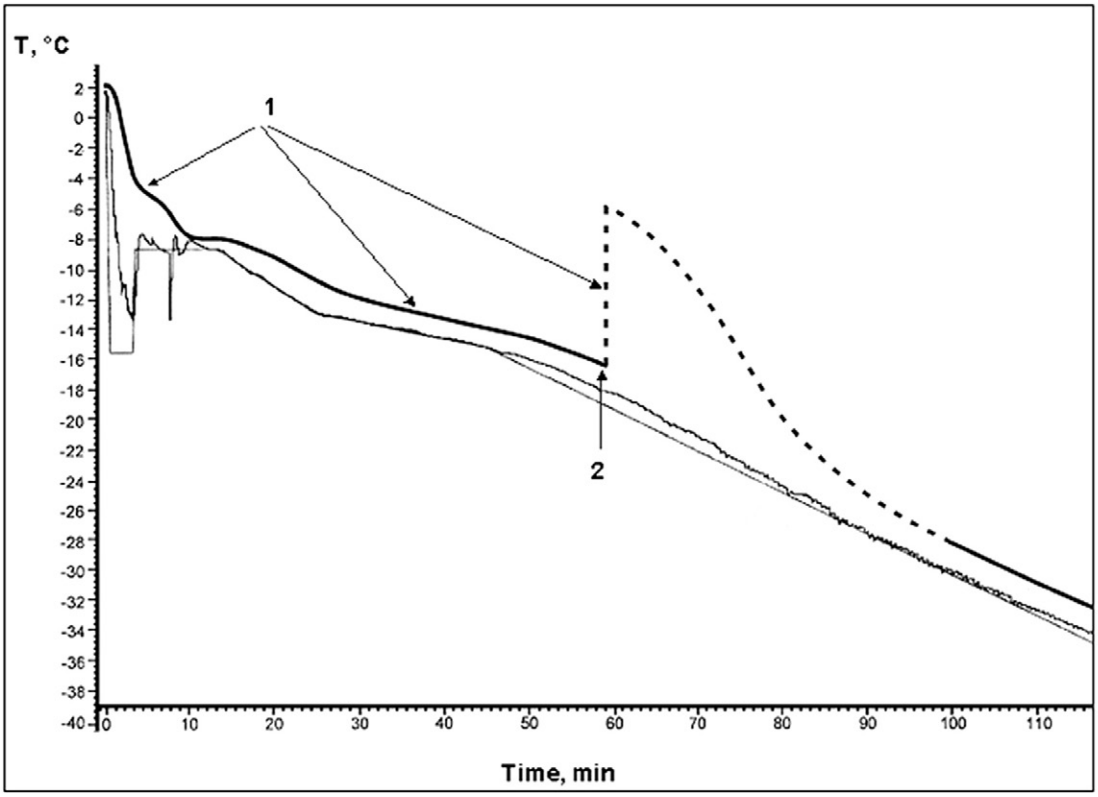

Figure 3. Temperature (T) profile of freezing with spontaneous ice formation. (1) Temperature of freezing medium; (2) beginning of spontaneous ice formation (note sharp increase in temperature). Unbroken line shows zone of programmed (–0.3°C) speed of cooling. Broken line shows zone of elevated (–1 to –0.5°C/min) speed of cooling.

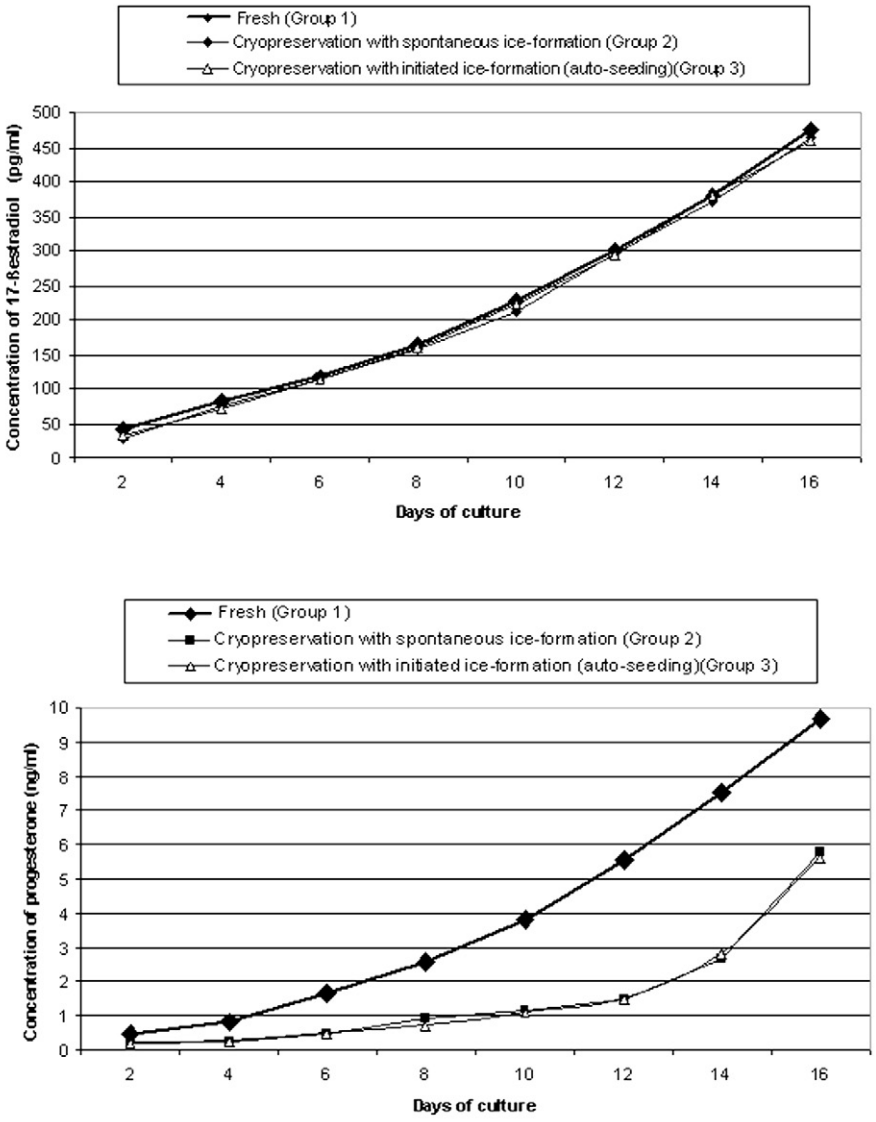

Figure 4. Concentration of oestradiol 17-β and progesterone in pooled supernatants of in-vitro cultured frozen/thawed ovarian samples.

Table 1. Cryopreservation of ovarian tissue with spontaneous and initiated ice formation: effect on follicle density and normality.

Follicle parameter	*Group 1 (Fresh)*	*Group 2 (Spontaneous ice formation)*	*Group 3 (Initiated ice formation)*
Mean follicle density/mm^3	12.1 ± 3.9[a]	3.1 ± 1.4[b]	6.0 ± 2.3[c]
Per cent normal follicles (grades 1 and 2)	91[a]	16[b]	87[a]

[a,b,c]Within each row, values with differing superscript letters are significantly different ($P < 0.05$).

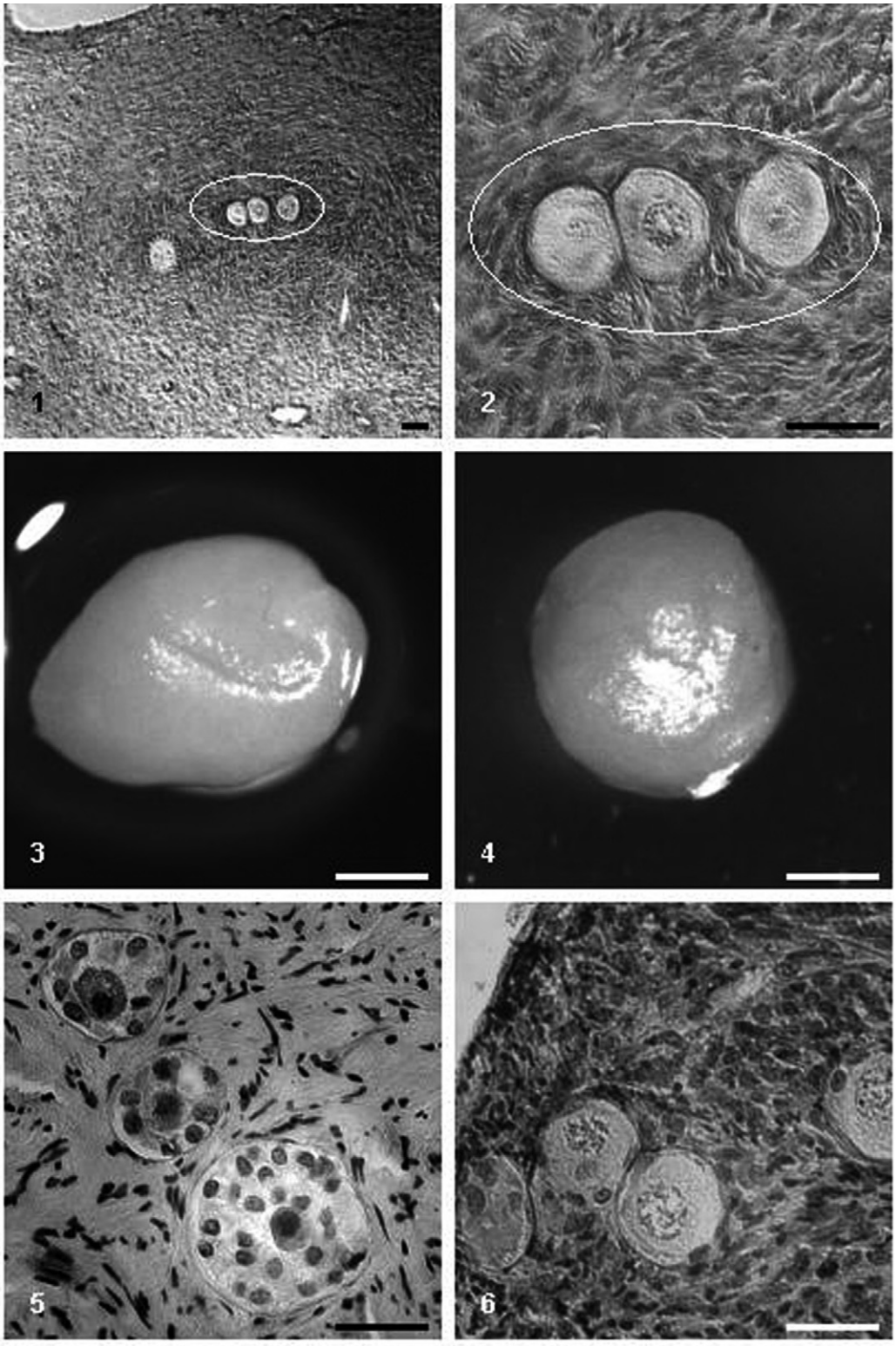

Figure 5. Photographs and micrographs of ovarian samples from the same patient. (1, 2) histology of fresh sample; (3) light micrograph of sample after cryopreservation with spontaneous ice formation and culture; (4) light micrograph of sample after freezing with initiated ice formation and culture; (5) histology of sample after cryopreservation with spontaneous ice formation and culture; (6) histology of sample after cryopreservation with initiated ice formation and culture. Note: after cryopreservation with spontaneous ice formation, samples assume a partially round form (3) and after initiated ice formation, a fully round form (4). No attachment of samples to the bottom of the Petri dish or formation of a stroma cells monolayer was seen during in-vitro culture. Bar = 1 mm (photographs) and 50 μm (micrographs).

usually carried out manually, is a central part of this protocol (Pegg, 1987), and this is followed by very slow cooling at a rate of –0.3°C/min.

Seeding is necessary so that nucleation and propagation of ice crystals can be controlled, minimizing the potential damage caused by the physical changes associated with ice formation, with release of latent heat of fusion. The temperature of seeding varies from –6 to –9°C.

Based on an original protocol of cryopreservation by Demirci *et al*. (2001), at a cooling rate of –2°C/min, ice formation is initiated by the release of negative calories within the freezing chamber (Demirci *et al*., 2001). Two cryoprotectants, DMSO and propylene glycol, were tested at different concentrations of 1, 1.5, and 2 mol/l. Using 2 mol/l DMSO, spontaneous ice formation was observed at the temperature rise at –11°C: mortality rate of follicles was 8.4% (Demirci *et al*., 2001). The same authors later noted that in sheep ovaries the cryopreservation protocol is more effective with initiated ice formation (manual seeding) than with semi-automatic seeding, when ice formation begins without manual initiation (Demirci *et al*., 2002). The results presented here support this point of view.

An entire human ovary with its vascular pedicle was frozen (Martinez-Madrid *et al*., 2004; Jadoul *et al*., 2007) using the following cryopreservation procedure: the ovary was perfused and immersed in 10% DMSO, and placed in a large volume cryo-vial. The cryovial containing the ovary was then placed in a freezer and frozen with a cooling rate of –1°C/min, without initiation of ice formation. The authors observed high survival rates of follicles, small vessels and stromal cells, as well as a normal histological structure in all ovarian components after thawing. The results presented here show a negative effect of spontaneous ice formation in cryopreservation of ovarian tissue. The difference between the present results and those of Martinez-Madrid *et al*. (2004) and Jadoul *et al*. (2007) can be explained by the fact that in this study small fragments of ovary were used, whereas in the other studies whole ovaries were cryopreserved that had been equilibrated with cryoprotectant via perfusion. Based on the authors' observations, it is possible that the use of initiated ice formation might have improved their results further.

For conventional cryopreservation of uterine and ovarian tissue, some groups use an open-freezing system with self-seeding (Dittrich *et al*., 2006, 2007; Maltaris *et al*., 2007a,b). This system has been used for cryopreservation of germ cells since 1986, and the system has been shown to be effective for cryopreservation of oocytes and embryos (Siebzehnruebl *et al*., 1989). In contrast to freezing-chamber equipment, the open-vessel system makes use of the temperature distribution above the surface of liquid nitrogen. Regulation of temperature is achieved by moving the cryo-vessels up and down in the nitrogen vapour (Siebzehrubl *et al*., 1986; van Uem *et al*., 1987). The temperature is measured during this process, and is monitored inside a reference cryo-vessel placed directly at the position of the oocytes or tissue. The damaging effects of supercooling are avoided by self-seeding: the formation of ice crystals begins at the tip of a tail, which is connected to the frozen materials via a straw. The crystals grow upward, and as the temperature reaches the freezing point, the crystals reach the straw and, consequently, act as crystal nuclei. Crystallization occurs at the freezing temperature. In the authors' opinion, this system has two disadvantages: only relatively small volumes of medium can be used for cryopreservation; and it is not possible to use the most popular cryo-tubes (for example, 1.8 ml; **Figure 1**).

Successful cryopreservation of human ovarian tissue was demonstrated by the achievement of live births after autologous transplantation (Donnez *et al*., 2004; Meirow *et al*., 2005, 2007). Part of the ovarian tissue obtained before the oncological treatment is normally used for routine histological observation, in order to minimize the risk of future transplantation of tissue with metastases. After cryopreservation and storage, part of the ovarian tissue can be thawed and cultured *in vitro* in order to check for the presence of follicles. The quality of follicles present in the cultured tissue indicates whether or not it might be possible to restore the reproductive function of a given patient.

The present study used the methodology of in-vitro culture of ovarian tissue. After medical treatment for cancer, the restoration of ovarian tissue function after cryopreservation and thawing can be tested via three approaches: histological analysis of follicles immediately post cryopreservation/thawing (Poirot *et al*., 2002); histological analysis of follicles post-thawing after in-vitro culture (Isachenko *et al*., 2002, 2003; Hreinsson *et al*., 2003; Hovatta, 2004, 2005); and histological analysis of follicles post-thawing and after xenotransplantation. Xenografting remains the most conclusive of the three methods (Newton *et al*., 1996; Oktay *et al*., 1998; Gook *et al*., 2003, 2005), and the most expensive. The present investigations used a previously described method for in-vitro culture of the ovarian tissue in order to optimize the available information regarding the quality of the tissue (Jadoul *et al*., 2007). Previous data demonstrated that long-term in-vitro culture in a large volume of culture medium under constant agitation supports better in-vitro growth of follicles after cryopreservation and thawing than do routine culture conditions (Isachenko *et al*., 2006b).

The present cryopreservation protocol followed three steps: step-wise dilutions for removal of permeable cryoprotectants; saturation with DMSO at 0°C; and rapid warming of tissues at 100°C.

Step-wise removal of cryoprotectants was used because human cells are sensitive to osmotic changes that accompany saturation with permeable cryoprotectants and their removal (Isachenko *et al*., 2004). Saturation with DMSO at 0°C was used because, in contrast to saturation of isolated cells, saturation of tissues by permeable cryoprotectants needs a longer exposure time in cryoprotectant solution. It is therefore important to consider the 'optimal' ratio of equilibration time/negative (toxic) effect of cryoprotectant before cooling (Pegg and Diaper, 1988) as well as the time of removal of cryoprotectants/osmotic injuries after warming. Toxicity of cryoprotectant agents is a key limiting factor in cryobiology (Fahy, 1986). DMSO possesses the quality of high toxicity (Shaw *et al*., 2000) and this toxicity is reduced by saturating the cells with DMSO at a temperature of 0°C. Recrystallization during thawing is a factor that can potentially decrease the viability of cryopreserved tissues, and therefore an elevated temperature for thawing was used to facilitate very rapid warming of the tissue, in order to limit the possibility of recrystallization. This was based on earlier research that established the positive effect of high-speed warming of

pronuclear and germinal vesicle oocytes after vitrification (Isachenko *et al.,* 2005a,b, 2006a).

In conclusion, these results suggest that optimal recovery of viable follicles after freezing and thawing of human ovarian tissue is obtained by using a protocol of conventional cryopreservation that includes a step of initiated ice formation.

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