

Human ovarian tissue vitrification versus conventional freezing: morphological, endocrinological, and molecular biological evaluation

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Abstract

Cryopreservation as a process can be divided into two methods: conventional freezing and vitrification. The high effectiveness of vitrification in comparison with conventional freezing for human oocytes and embryos is shown, whereas data on human ovarian tissue are limited. The aim of this study was to compare the safety and effectiveness of conventional freezing and vitrification of human ovarian tissue. Ovarian tissue fragments from 15 patients were transported to the laboratory within 22–25 h in a special, isolated transport box that can maintain a stable temperature of between 5 and 8 °C for 36 h. Small pieces of ovarian tissue (0.3–1 × 1–1.5 × 0.7–1 mm) were randomly distributed into three groups: group 1, fresh pieces immediately after receiving transport box (control); group 2, pieces after vitrification; and group 3, pieces after conventional freezing. After thawing, all the pieces were cultured *in vitro*. The viability and proliferative capacity of the tissue by *in vitro* production of hormones, development of follicles, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene expression after culture were evaluated. A difference between freezing and vitrification was not found in respect to hormonal activity and follicle quality. The supernatants showed 17-β estradiol concentrations of 365, 285, and 300 pg/ml respectively, and progesterone concentrations of 3.82, 1.99, and 1.95 ng/ml respectively. It was detected that 95, 80, and 83% follicles respectively were morphologically normal. The molecular biological analysis, however, demonstrated that the *GAPDH* gene expression in ovarian tissue after vitrification was dramatically decreased in contrast to conventional freezing. For cryopreservation of human ovarian tissue, conventional freezing is more promising than vitrification, because of higher developmental potential.

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Introduction

In 2008, ~692 000 women were diagnosed with cancer in the United States of America alone (Jemal *et al.* 2008). Because of the increasing effectiveness of oncological treatments (Grovas *et al.* 1997, Jemal *et al.* 2008), young women seek for a chance to regain their reproductive potential after treatment. This can be achieved by the cryopreservation of ovarian tissue before medical intervention. Therefore, cryopreservation of human ovarian tissue has become one of the main research projects in reproductive medicine. In 1996, the first case of cryopreservation of human ovarian tissue with good survival of follicles after warming was published (Hovatta *et al.* 1996, Hovatta 2005). Childbirth after cryopreservation of ovarian tissue is now a reality (Donnez *et al.* 2004, Meirow *et al.* 2005, 2007a, 2007b, Demeestere *et al.* 2007, Andersen *et al.* 2008).

The use of cryopreserved ovarian tissue is not only limited to reproduction. There are reports of patients

with premature ovarian failure after cancer treatment and restoration of ovarian function after re-implantation of cryopreserved ovarian cortex (Donnez & Bassill 1998, Oktay *et al.* 1998, 2004, Oktay & Karlikaya 2000, Callejo *et al.* 2001, Meirow & Nugent 2001, 2007b, Radford *et al.* 2001, Gosden 2005, Schmidt *et al.* 2005, Silber *et al.* 2005, Wolner-Hanssen *et al.* 2005, Demeestere *et al.* 2006, Donnez *et al.* 2006a, 2006b, 2008, Rosendahl *et al.* 2006, Bedaiwy & Falcone 2007, Meirow *et al.* 2007b, Silber & Gosden 2007). Therefore, cryopreservation of human ovarian tissue cannot be limited only for restoration of reproductive function (pregnancy after oncological treatment), but also can be used for therapeutic purposes (Isachenko *et al.* 2008a).

Cryopreservation of ovarian tissue can be performed using one of two methods: conventional ('slow') freezing and cryopreservation by direct plunging into liquid nitrogen (so called vitrification or rapid 'freezing'). Comparative investigations of vitrification

and conventional freezing performed on mammalian ovarian tissue are limited, and authors present different conclusions. For example, it has been shown that for human ovarian tissue, conventional freezing is more efficient than vitrification (Gandolfi *et al.* 2006). Furthermore, it was established that conventional freezing is a suitable method for cryopreservation of ovarian tissue with better follicle quality and hormonal activity (Isachenko *et al.* 2007). However, it also has been reported that there is no difference in the quality of the follicles and the hormonal activity between conventional and rapid freezing (Li *et al.* 2007).

In addition to former studies, we here tested the proliferative potential of human ovarian tissue at the molecular biological level. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) is a housekeeping gene and plays a crucial role in glycolysis and in several other metabolic and non-metabolic processes (Sirover 1996, 1997). Analysis of *GAPDH* gene expression is a marker for variations in the amount of intact RNA after an experimental treatment of tissues (in our case this is cryopreservation; Barber *et al.* 2005).

The aim of this study was to compare the safety and effectiveness of vitrification and conventional freezing of human ovarian tissue by assessing follicular quality, production of hormones, and *GAPDH* gene expression.

Results

Histological examination

After the *in vitro* culture of ovarian pieces, only primordial as well as primary and secondary follicles were denoted as viable. All the antral follicles after cryopreservation and *in vitro* culture were degenerated, and we have not counted these follicles. The mean primordial follicle density per 1 mm³ for group 1 was 11.1 ± 2.1; for group 2 was 12.0 ± 6.2; and for group 3 was 11.9 ± 4.6 ($P > 0.1$). It was detected that 95, 80, and 83% follicles for groups 1, 2, and 3 respectively were normal ($P_{1-2,3} < 0.05$, $P_{2-3} > 0.1$; Figs 1–3).

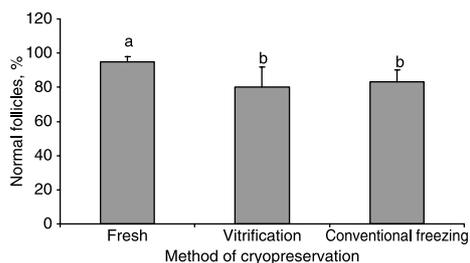


Figure 1 Quality of follicles (expressed as percentage of normal follicles) after vitrification and freezing of ovarian pieces. Different superscripts indicate statistical difference ($P < 0.05$).

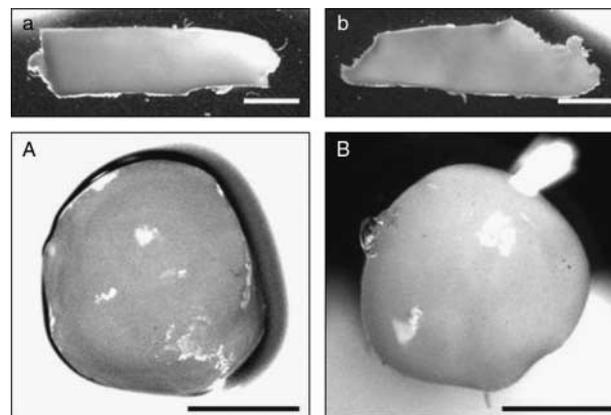


Figure 2 Light micrographs of ovarian pieces (OP) from the same patient: (a) fresh OP, (A) OP after vitrification and culture; (b) fresh OP, (B) OP after conventional freezing and culture by Isachenko *et al.* (2006, 2007, 2008b, 2008c). Bar = 300 µm.

Hormone assays

To exclude the possibility that the medium contains relevant amounts of hormones, we determined the level of hormones in native AIM-V medium: 17-β estradiol (E_2) < 10 pg/ml and progesterone (P_4) < 0.1 ng/ml. E_2 concentrations in the supernatants were 365, 285, and 300 pg/ml respectively, and P_4 concentrations were 3.82, 1.99, and 1.95 ng/ml respectively (Fig. 4).

GAPDH gene expression

After cryopreservation and cultivation, the samples were used for RNA purification, and the expression of *GAPDH* in randomly chosen probes (pairwise) was analyzed by conventional RT-PCR (Fig. 5). Figure 5A demonstrates drastically reduced levels of *GAPDH* gene expression in probes after vitrification (3% agarose gel visualizing the amplified fragments; lines 2–4) in comparison with those after conventional freezing (lines 7–9). For the quantification of this observation, we have chosen some other probes for real-time RT-PCR. Figure 5B presents the typical results of such an analysis: the lines corresponding to the probes after conventional freezing/thawing show more template copies than those corresponding to the probes after vitrification, which reflect great difference in gene expression level. The *GAPDH* gene expression in ovarian tissue after vitrification was dramatically decreased in contrast to conventional freezing.

Discussion

Comparative data about vitrification and conventional freezing of human ovarian tissue are limited. Below, we briefly summarize the results of five investigations with different conclusions.

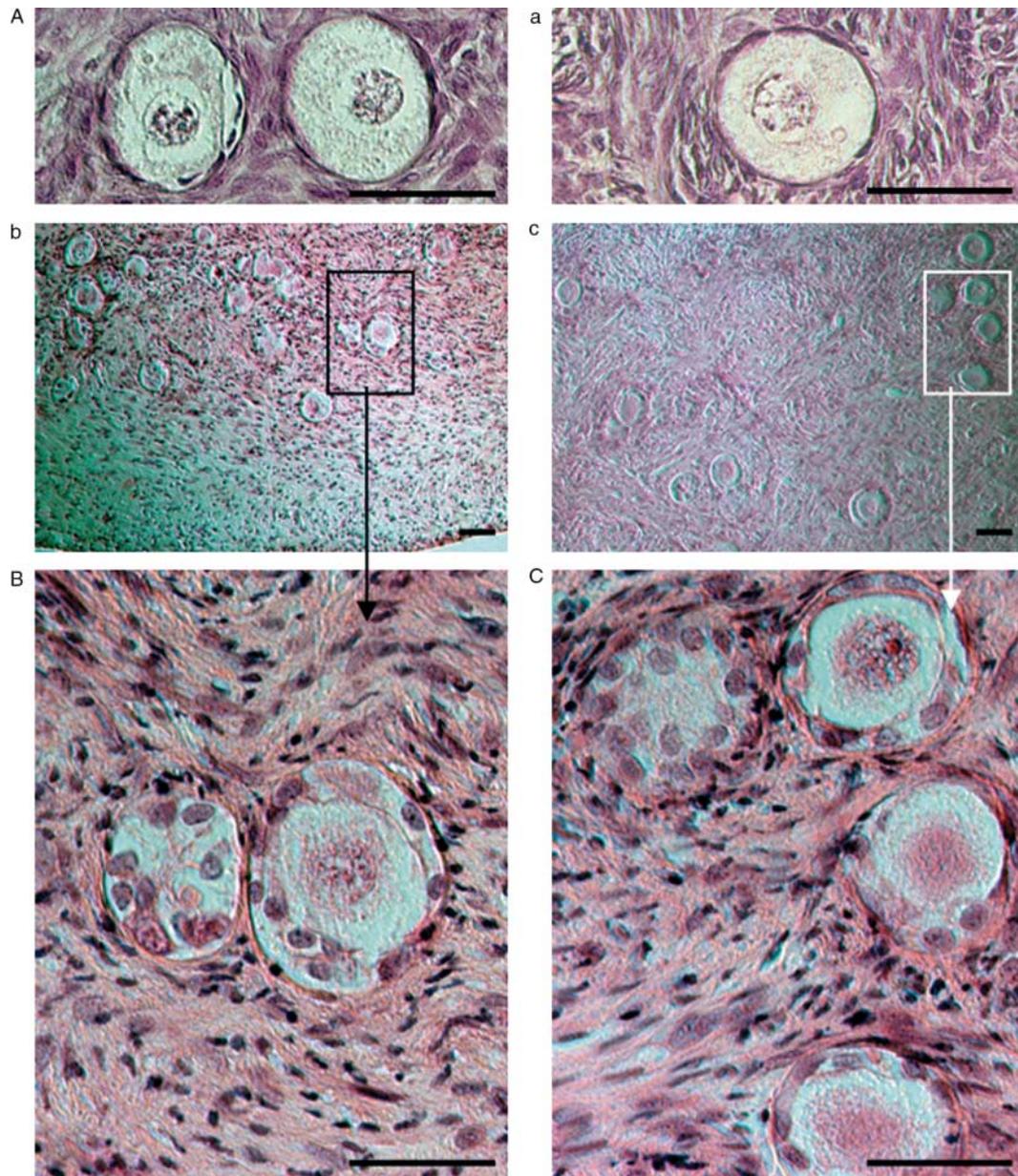


Figure 3 Histological micrographs of follicles from: (A, a) fresh OP; (b) and (B) after vitrification and culture, (c) and (C) after conventional freezing and culture by Isachenko *et al.* (2006, 2007, 2008a). Bar = 35 μ m.

Comparison of conventional freezing and vitrification of human ovarian tissue was performed by Rahimi *et al.* (2004). Examination of the necrotic areas of the pieces after both these methods of cryopreservation and transplantation into SCID mice showed no significant difference between these two methods. Such comparative investigations were also performed on human, bovine, and porcine ovarian tissues (Gandolfi *et al.* 2006). In this study, authors used two vitrification protocols. In the first protocol (Al-Aghbari & Menino 2002), pieces were vitrified in 35% ethylene glycol + 5% polyvinylpyrrolidone + 0.4 M trehalose by

cooling of tissues in small amount of vitrification medium and post-warming (at 37 °C) removal of cryoprotectant in 0.3 M trehalose. According to the second protocol (Silvestre *et al.* 2002), pieces were vitrified in 22% ethylene glycol + 22% DMSO with removal of cryoprotectants in 0.25 M sucrose after warming at 37 °C. Ovarian pieces were also conventionally frozen using 1.5 M DMSO or 1.5 M propylene glycol with post-thawing stepwise exposure of fragments in the respective cryoprotectants (DMSO or propylene glycol) of decreasing concentration (Newton *et al.* 1996). Immediately after warming/thawing, ovarian

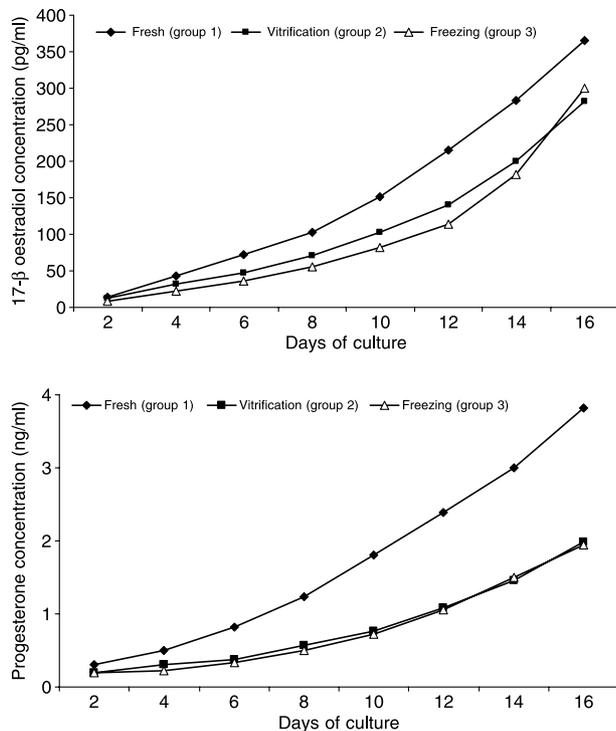


Figure 4 Concentration of 17-β estradiol and progesterone of vitrified and conventionally frozen ovarian pieces cultured *in vitro*.

fragments were fixed and follicles were evaluated. The authors of this study have found some peculiarities of cryostability of follicles dependent on species and type of cryoprotectants. However, the central conclusion was the following: conventional freezing is the method of choice for the cryopreservation of ovarian fragments, resulting in a much better preservation of all types of follicles than vitrification (Gandolfi *et al.* 2006). Our results described in this article suggest the same conclusion about the effectiveness of conventional freezing in comparison with vitrification.

Recently, results from comparative investigations on vitrified and conventionally frozen human ovarian tissue with long term *in vitro* culture of tissue fragments after thawing/warming have been published (Li *et al.* 2007). The ovarian strips were divided into two groups. Tissue strips of the first group were vitrified in 2.5 M (15.6%) DMSO+2.5 M (15.2%) propylene glycol+0.2 M sucrose by direct dropping of the vitrification medium into the liquid nitrogen. Warming of the vitrified strips was carried out by direct plunging of the drops of vitrification medium with ovarian tissue and gentle agitation of 0.5 M sucrose at 38 °C with stepwise rehydration of strips in 0.5, 0.25, and 0.125 M sucrose. Tissue strips of the second group were conventionally frozen in 1.5 M (11.7%) DMSO+0.1 M sucrose with thawing at 37 °C and stepwise rehydration in 0.25 and 0.125 M sucrose. After 2 weeks of culture, it was

established that in tissues of both cryopreservation groups there was no difference in the proportion of normal follicles after conventional freezing and vitrification (Li *et al.* 2007). The authors believe that the original methodology of vitrification by direct dropping of freezing solution into liquid nitrogen is as effective as conventional freezing.

In our present investigation, we also have tested the vitrification methodology with direct dropping of ovarian tissue into liquid nitrogen for cooling and into medium for warming as described in Li *et al.* (2007). Earlier, we have performed comparative investigations of vitrification and conventional freezing for human ovarian tissue. It was established that freezing is a suitable method for cryopreservation because of differences in the quality of follicles: after conventional freezing their quality was higher than after vitrification (Isachenko *et al.* 2007). Later, a new human ovarian tissue vitrification protocol was applied. The use of this vitrification protocol allows to obtain the high-quality follicles after thawing and culture (Isachenko *et al.* 2008b). Different

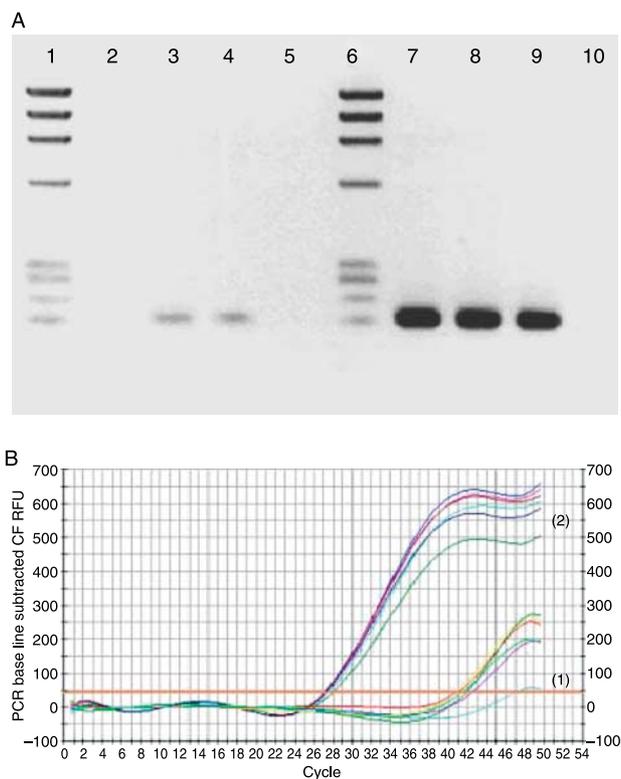


Figure 5 Expression of *GAPDH* in ovarian tissue after vitrification of samples from three patients (lines 2, 3, and 4) and conventional freezing (lines 7, 8, and 9) (lines 5 and 10 represented a negative control). (A) Analysis of amplified cDNAs by electrophoresis in 3% agarose gel and ethidium bromide staining. (B) Validation of the results presented on board A by quantitative real-time PCR analysis. Typical amplification curves. Curves for *GAPDH* gene amplification for the probes after vitrification (1) and conventional freezing (2). CF RFU, curve-fit relative fluorescence units.

cryoprotectant solutions and types of cooling/warming were tested. The best results were observed in the protocol using 2.62 M DMSO+2.6 M acetamide+1.31 M propylene glycol+0.0075 M polyethylene glycol. Just this vitrification protocol was tested in the present experiments.

The hormonal activity of thawed and cultured tissue is a criterion for the effectiveness of the particular cryopreservation protocol. It is known that the steroidogenic activity of ovarian tissue is an additional indicator of tissue viability.

Here, we found an enhanced E_2 production during culture time. The increase in E_2 level by incubation time might be due to the fact that relatively more follicles developed into advanced stages during *in vitro* culture. According to Boland *et al.* (1993), E_2 secretion increases with increasing follicular size. The production of E_2 in *in vitro* culture of ovarian tissue indicates that the steroidogenic pathways were present in the tissue. However, it is not clear whether steroid production is derived only from follicles, since the follicles were still too small and secretion of E_2 might be too sparse (Wotiz *et al.* 1956). The studies in steroid metabolism have shown that hyperplastic stromal cells of ovarian pieces from climacteric patients can produce E_2 *in vivo* and *in vitro* (Wotiz *et al.* 1956). Steroids could be produced by proliferated stromal tissue, which established a cellular monolayer in response to FCS in the culture medium (Isachenko *et al.* 2002, 2003). For example, earlier we have vitrified and *in vitro* cultured human ovarian pieces. Although no follicles were detected in fresh and cultured pieces (Isachenko *et al.* 2002), the level of P_4 was as high as in the control group.

The E_2 and P_4 levels observed in our present studies indicate that the granulosa cells were not luteinized in both control and experimental tissues samples. Here, we have used another type of culture system (Isachenko *et al.* 2006). This system differs from the former (Isachenko *et al.* 2002, 2003) in its permanent moving with 75 osc/min using a rotatory shaker and the big volume of serum-free medium (AIM V) that was not changed during the whole-culture period. A monolayer of stroma of ovarian pieces was not developed. This fact is positive, because by our observation the growing of stroma inhibits the development of follicles (data not shown).

Taking into account that one central aim of ovarian tissue cryopreservation is a post-thawing formation of follicles, the production of steroid hormones is not a highly conclusive test of ovarian tissue viability. Thorough histological examination of the tissue and evidence of undisturbed gene expression are necessary. In fact, a histological test of normality of follicles during *in vitro* culture is an informative method for evaluation of ovarian tissue viability.

It is established that the RNA in cryopreserved tissues does not undergo significant degradation, the specific contributions of different fixatives and cryopreservation conditions to its degradation are not known and the preliminary estimation of the viability of differentially conserved tissues is often evaluated by the measurement of expression level of widely expressed genes like *GAPDH* (Fitzpatrick *et al.* 2002).

Our results obtained in this investigation demonstrate a significant degradation of the large part of intact RNA after vitrification in comparison to conventional freezing. This could be due to inappropriate cryopreservation conditions that allow the mechanical or enzymatic destruction of initially existing RNA. We suppose that our results provide evidence for a difference in the proliferative capacity of frozen and vitrified human ovarian tissues. However, we do not have enough evidence to strongly support this conclusion.

We might speculate that cryopreservation conditions were impaired by microbial contamination. The effective vitrification methodologies are based on the direct cooling of cells in liquid nitrogen. In contrast, conventional freezing completely avoids the direct contact between the liquid nitrogen and the tissue. In fact, any technology in reproductive biology and especially in a medical approach must ensure and guarantee the full protection of biological objects from micro-organisms (Bielanski *et al.* 2000, 2003). Liquid nitrogen, which is used for the storage of frozen material, can be a source of contamination by these micro-organisms (Tedder *et al.* 1995, Bielanski *et al.* 2000). Filtration or u.v. treatment of liquid nitrogen cannot guarantee the absence of contamination of biological material by viruses. For example, contamination of blood probes by hepatitis virus during storage of probes in liquid nitrogen has been reported (Tedder *et al.* 1995). Different types of virus, which are simple and very cryostable structures, may increase their virulence after a direct plunging and storage in liquid nitrogen, such as hepatitis virus (Hawkins *et al.* 1996), papova virus (Charles & Sire 1971), vesicular stomatitis virus (Schaffer *et al.* 1976), and herpes virus (Jones & Darville 1989). The main aim of this investigation was to determine whether vitrification is more or less effective than conventional freezing with the ability of the follicles to develop after thawing. In fact, vitrification is technologically promising, it is simpler and one cryo-cycle is less time consuming and cheaper than the conventional freezing method. However, the central goal of cryo-technology is the preservation of intact follicles. Results of the present investigation have also shown that vitrification can guarantee the storage of viable follicles after warming, but conventional freezing is more effective in terms of gene stability.

We report that there is a high rate of follicular survival as well as an increase in E_2 and P_4 secretion during *in vitro* culture after vitrification and conventional

freezing. These data suggest the viability of the follicle after both procedures and are in contrast with the results of the quantitative PCR. *GAPDH* is a housekeeping gene and should be present in viable tissue. Even if the *GAPDH* mRNA expression may be altered by experimental conditions, conclusions based on this alteration can be evaluated as a paradox.

This discrepancy between *GAPDH* gene expression and hormone secretion is due to two different time points of measurement. This discrepancy can be explained in the following way. For experiments to determine hormonal activity, histological evaluation was performed after post-thawing *in vitro* culture of ovarian tissue for up to 16 days. In contrast, experiments for *GAPDH* gene expression were performed after transport of frozen tissue to another laboratory and tissue after thawing was cultured *in vitro* for up to 5 days only. A decrease in *GAPDH* gene expression in vitrified ovarian tissue, in contrast to this rate in frozen tissue, can be explained by the necessity of the vitrified tissue to take a longer time to adapt for normal development than frozen tissue. Probably, later, the vitrified tissue will 'catch up' with the frozen tissue and also will demonstrate good *GAPDH* gene expression. However, the difference in development during the certain time period of *in vitro* culture allows us to draw the respective conclusion about higher effectiveness of conventional freezing.

It was concluded that for cryopreservation of human ovarian tissue, conventional freezing is more promising than vitrification, because of higher developmental potential.

Materials and Methods

Except where otherwise stated, all chemicals were obtained from Sigma.

Tissue collection, dissection, and distribution into groups

Informed consent was obtained from 15 patients aged between 18 and 33 (23.1 ± 4.9), under a protocol approved by the University Ethics Board. Ovarian tissue fragments were transported to the laboratory within 22–25 h in a special, isolated transport box (DeltaT GmbH, Giessen, Germany) that can maintain a stable temperature between 4 and 8 °C for 36 h, containing a transport medium (Brama I; CryoBioSystem, L'Aigle, France) specially developed for ovarian tissue.

Tweezers and scalpel No 22 were used for dissection of medulla and for further division of ovarian tissue fragments into small pieces. The dissection of ovarian tissue was done in a medium specially developed for this purpose (Brama II, CryoBioSystem). A small sample of ovarian tissue of each patient was removed for routine histology and follicle counts and immediately fixed with Bouin's solution. Small pieces

measuring $\sim 1 \text{ mm}^3$ of experimental ovarian tissue were randomly distributed as follows: non-treated fresh control group 1 (immediately after transport to the laboratory; $n=45$); 'vitrification' group 2 ($n=45$); and freezing group 3 ($n=45$).

Vitrification

Previously, we have chosen from 18 protocols the optimal one (Isachenko *et al.* 2008b). Just this protocol was used for the present research.

The vitrification solution, prepared on Dulbecco's phosphate-buffered solution with serum substitute supplement (SSS, Irvine Sci., St Ana, CA, USA) and antibiotic-antimycotic, contained 2.62 M DMSO, 2.60 M acetamide, 1.31 M propylene glycol, and 0.0075 M polyethylene glycol. The ovarian tissue pieces (OPs) were dehydrated in vitrification solution of increased concentration: 12.5, 25, 50, and 100%. The first two steps were performed at room temperature, which lasted 5 min each, and the next two steps lasted 15 min each at 4 °C. After this last step of saturation by cryoprotectants, the OPs were dropped directly into liquid nitrogen, together with a small volume ($\sim 20 \mu\text{l}$) of 100% vitrification solution.

For warming, the vitrified OPs were directly plunged into 10 ml of 40 °C pre-warmed 50% vitrification solution under gentle shaking with vortex until the ice melted. The dilution of the cryoprotectants was performed in a decreasing concentration of vitrification solution (25 and 12.5%) at room temperature and OPs were finally washed thrice in a culture medium at 37 °C and 5% CO₂. Each step of the dilution protocol lasted 5 min.

Conventional freezing

The freezing protocol was based on the protocols that were used by Gosden *et al.* (1994), Gosden (2000, 2002) with some modifications. Standard 1.8 cryo-vials (Nunc, Roskilde, Denmark) were filled with 1.8 ml freezing medium containing L-15 Medium (Leibovitz) with L-glutamine + 1.5 M DMSO + 0.1 M sucrose + 10% SSS (Irvine Sci.), and were cooled in ice water (0 °C). Then, OPs were transferred to cryo-vials and these cryo-vials were introduced in ice water for 30 min. After that, cryo-vials were placed in the freezer, the freezing chamber of which was previously stabilized to 2 °C for 20–30 min. The freezing program was as follows: 1) the starting temperature was at 2 °C; 2) cooling from 2 °C to –6 °C at a rate of –2 °C/min; 3) auto-seeding initiated at –6 °C; 4) after beginning of crystal formation, the temperature increased to –5.7 °C and remained at this temperature for 10 min; 5) cooling from –5.7 °C to –40 °C at a rate of –0.3 °C/min; 6) cooling to –140 °C at a rate of –10 °C/min and plunging of cryo-vials into liquid nitrogen.

The procedure of thawing was achieved by holding the vials for 30 s at room temperature followed by immersion in a 100 °C (boiling) water bath for 60 s, and expelling the contents of the tubes into the solution for the removal of cryoprotectants. The exposure time in the boiling water was visually controlled by the presence of ice in the medium; as soon as the ice was

~2 mm apex, the tube was expelled from the boiling water. The final temperature of medium after expelling from 10 °C water bath ranged between 4 and 10 °C. After thawing, OPs were transferred within a few seconds (5–7) to a 100 ml specimens container (Sarstedt, Nuemrecht, Germany) with 10 ml solution for removal of cryoprotectants (0.75 M sucrose + 10% SSS + L-15 medium). The stepwise dilution of cryoprotectants was achieved using the same principle as that used for saturation by ethylene glycol (see Fig. 1 in Isachenko *et al.* 2006). The container was placed on the shaker and continuously agitated with 200 osc/min for 15 min at room temperature. Stepwise rehydration of OPs for 30 min at room temperature was also performed using the same dropping methodology (Isachenko *et al.* 2006). For dropping rehydration, we used 50 ml of holding solution (L-15 medium + 10% SSS) in a 50 ml tube (Greiner Bio-One GmbH, Frickenhausen, Germany). This method includes the slow adding (dropping) of holding medium to the solution of sucrose with OPs. The final sucrose concentration was 0.125 M. Finally, the OPs were washed thrice each in DPBS supplemented with 10% SSS and in culture medium for 10 min. After warming and washing, OP was transferred for *in vitro* culture.

Culture

Pieces of control (group 1) immediately after receipt of transport box and experimental pieces after cryopreservation (groups 2 and 3) were translocated to 200 ml dishes for suspension culture (Cellstar, Greiner Bio-One GmbH) with 30 ml AIM-V medium (Gibco) for *in vitro* culture at 37 °C in 5% CO₂ with 75 osc/min agitation using a rotation shaker. From each patient, two pieces were used for determination of hormonal activity during 16 d culture and histological evaluation at the end of this culture and one piece was used for study of *GAPDH* gene expression for up to 5 d culture.

Histological examination

For histological investigation, tissue pieces from up to 16 d *in vitro* culture were fixed in Bouin's solution, embedded in paraffin wax, serially sectioned at 5 µm, stained with hematoxylin/eosin, and analyzed under a microscope (×400). The number of viable and damaged follicles was counted. To avoid over counting of the same follicles, only the section with a visible oocyte nucleus was counted.

Morphology of follicles was evaluated by considering the parameters previously described by Paynter *et al.* (1999). Two types of follicles were evaluated: 1) primordial follicles composed of an oocyte surrounded by a layer of flattened follicular cells and 2) primary follicles that are similar to primordial follicles, but surrounded by one to two layers of spheroid granulosa cells. The qualities of follicles were graded from one to three. A follicle of grade 1 is spherical and is randomly distributed around oocytes' granulosa cells, with homogenous cytoplasm and slightly granulated nucleus, in the center of which condensed chromatin in the form of dense spherical structure is detected. A follicle of grade 2 has the same peculiarities, but the granulosa cells cover irregular oocytes; these cells can be flat and condensed chromatin is not

detected in cytoplasm. A follicle of grade 3 has partly or fully disrupted granulosa or cytoplasm and picnotic nucleus. Follicles of grades 1 and 2 were denoted as normal and those of grade 3 were denoted as degenerated. Examples of the different follicular degenerations can be observed elsewhere (for example, see Isachenko *et al.* (2008b, 2008c)).

Hormone assays

The medium after culturing of cryopreserved and fresh OP was collected every second day (300 µl per collection) during culture and stored at –80 °C for 1–2 months for subsequent hormone assays. The level of E₂, analytical sensitivity 5.0 pg/ml) and P₄, analytical sensitivity 0.1 ng/ml) was measured using heterogeneous competitive magnetic separation immunoassay (Bayer Vital GmbH).

GAPDH-gene expression

After *in vitro* culture of ovarian pieces, total RNA preparation, RT, PCR, and real-time PCR were performed by the following methodology. Fifteen ovarian pieces (one piece from patient) were mechanically disrupted and homogenized, total RNA was extracted using GenElute Total RNA purification kit, and residual genomic DNA was removed by DNase I treatment (Sigma) according to the manufacturer protocols. One microgram of each total RNA was reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen). PCRs contained 1×PCR RedTaq Ready Master Mix, 500 nM of each *GAPDH* primer (forward: 5'-ggatcgtg-gaaggactcatgac-3'; reverse: 5'-atgccagtgagcttcccgttcagc-3'), and template in a total volume of 10 µl. The thermal profile used for amplification was 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 40 s. PCR products were visualized by acryl amide (7.5%) or agarose (3%) gel electrophoresis.

The results were validated by real-time PCR approach used the SYBR green assay in a 96-well plate format using a Bio-Rad iCycler. Reactions contained 1×SYBR MasterMix (Qiagen), 300 nM of each primer, 10 nM of Calibration Dye, and template in a total volume of 15 µl. The thermal profile used for amplification was 95 °C for 8 min followed by 50 cycles of 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 30 s. At the end of the amplification phase, a melting-curve analysis was carried out on the products formed.

Statistical analysis

The effects of cryopreservation on the production of hormones and development of follicles were evaluated by ANOVA. The level of statistical significance was set at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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