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Cryopreservation of human ovarian tissue: Comparison of rapid and conventional freezing

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Abstract

Cryopreservation, which is the most important procedure in ovarian tissue banking, can be divided into two methods: conventional freezing and rapid freezing. In previous study, the higher effectiveness of rapid freezing in comparison with the conventional freezing for human oocytes and embryos was shown. Data on comparison of these two methods for human ovarian tissue are limited. The aim of this study was to compare conventional freezing and rapid freezing for human ovarian tissue. Ovarian tissue fragments from 14 patients were transported to the laboratory within 22–25 h in a special, isolated transport box, which can maintain a stable temperature of between 5 and 8 °C for 36 h. Small pieces of ovarian tissue (1×1–1.5×0.7–1 mm) were randomly distributed into four groups: Group 1: control, fresh pieces immediately after receiving transport box, Groups 2 and 3: experimental pieces after rapid freezing/warming, and Group 4: experimental pieces after conventional freezing/thawing. All pieces were cultured *in vitro* for 14 days. The viability of the tissue by *in vitro* production of hormones and development of follicles after culture was evaluated. The level of estradiol 17-β and progesterone was measured using heterogeneous competitive magnetic separation immunoassay. For histological analysis, the number of viable and damaged follicles was counted. After culture of fresh tissue pieces (Group 1), rapidly frozen/warmed pieces (Groups 2 and 3), and conventionally frozen/thawed pieces (Group 4), the supernatants showed estradiol 17-β concentrations of 358, 275, 331, and 345 pg/ml, respectively, and progesterone concentrations of 3.02, 1.77, 1.99, and 2.01 ng/ml, respectively. It was detected that 96%, 36%, 39%, and 84% follicles for Groups 1, 2, 3, and 4, respectively, were normal. For cryopreservation of human ovarian tissue, conventional freezing is more promising than rapid freezing.

Keywords: Human ovary; Conventional freezing; Rapid freezing; Comparison; Hormones; Follicles

In the USA, more then 670,000 women are diagnosed with cancer every year [24]. The chances of restoring reproductive function by cryopreservation of ovarian tissue before oncological treatment can help persuade young women to undergo treatment.

Orthotopic reimplantation of frozen/thawed ovarian cortical strips is a well tolerated technique for restoring ovarian function in women treated with sterilising chemotherapy for cancer [36].

By grafting cortical ovarian tissue after cryopreservation, it was possible to sustain ovulation and pregnancy, and in animals and in humans, live births have also been achieved [32,30,13,7]. Childbirth after cryopreservation of ovarian tissue is now a reality: normal follicular development and ovulation were observed after gonadotrophin stimulation in a woman, who had been grafted with ovarian tissue that had been previously frozen [27].

Cryopreservation of ovarian tissue can be divided into two methods: conventional freezing and cryopreservation by direct plunging into liquid nitrogen (“vitrification”, “rapid freezing”, “ultra-rapid freezing”, “rapid cooling”). Comparative investigations of rapid and conventional

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freezing performed on human ovarian tissue are limited, and authors present diametrically opposite conclusions. For example, it has been shown that for human ovarian tissue, conventional freezing is the method of choice for the cryopreservation of this type of cells compared with rapid freezing, which is less efficient [9]. However, no differences existed between conventional and rapid freezing in the quality of the follicles and the hormonal activity [26].

It is widely accepted that it is possible to successfully cryopreserve human ovarian tissue by direct plunging into liquid nitrogen using only permeable cryoprotectants, without disaccharides [17]. It was reported normal gestation and live births after orthotopic autograft of vitrified hemiovaries into ewes [5]. Just these two protocols [17,5] were used for testing of rapid freezing methodology.

The aim of this study was to compare rapid freezing and conventional freezing for human ovarian tissue by development of follicles and production of hormones.

Materials and methods

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

Tissue collection, dissection, and distribution to groups

Informed consent was given by 14 patients aged between 19 and 38 (32.1 ± 3.6) years, under a protocol approved by the University Ethics Board. Ovarian tissue fragments (1–3 to 2–3 cm) were obtained from 14 patients before oncology treatment and transported to the laboratory within 22–25 h in a special, isolated transport box (DeltaT GmbH, Giessen, Germany), which can maintain a stable temperature of between 5 and 8 °C for 36 h, containing a special medium for transportation of ovarian tissue (Brama I, CryoBioSystem, L'Aigle Cedex, France). Medium for manipulation with ovarian tissue (Brama II, CryoBioSystem, L'Aigle Cedex, France) were used for dissection of biopsies.

Small pieces of ovarian cortex (1 × 1–1.5 × 0.7–1 mm) were randomly distributed into four groups. Group 1: control pieces immediately after receiving the transport box, Groups 2 and 3: experimental pieces after rapid freezing/warming, and Group 4: experimental pieces after conventional freezing/thawing. Total of 280 ovarian pieces (OP) were used for experiments. Twenty OP from every patient were treated, 5 OP in each from 4 groups. Each experiment was performed in five replications by principle "one OP from one patient in one cryovial". All pieces after respective manipulations were cultured *in vitro*.

Rapid freezing and warming

Two protocols of rapid freezing were tested: one using a mixture of permeable cryoprotectants without disaccharides developed by Isachenko et al. [17] (Group 2) and the other using permeable cryoprotectants and acetamide by Bordes et al. [5] (Group 3).

The rapid freezing solution of *Group 2* was composed of Dulbecco phosphate-buffered saline (DPBS), 20% dimethylsulphoxide (DMSO), 20% ethylene glycol (EG), and 10% serum substitute supplement (SSS, Irvine Sci., St. Ana, CA, USA). The OP were placed into a 110-ml specimen container (Falcon, Le Pont de Claix, France) containing 10 ml DPBS + 10% SSS. This container was placed on a rotation shaker, and a stepwise saturation by 40% EG for 40 min at room temperature was carried out using the original dropping method as described earlier [22] and (Fig. 1). During the process of saturation, the container was rotated at 200 oscillations (osc) per minute. For dropping saturation, 40 ml of 50% EG, 10% SSS, and DPBS in 50-ml tube (Greiser Bio-One GmbH, Essen, Germany) was used. After the end of dropping, OP were held in EG for additional 10 min for complete saturation by this cryoprotectant. The final EG concentration in the rapid freezing solution at the end of saturation was 40% (40 ml of 50% EG + 10 ml holding medium).

Then, the OP were placed into standard 1.8-ml cryovials (1 per vial) (Nunc, Roskillde, Denmark) that were partially filled with 1.5 ml of rapid freezing medium, precooled to 4 °C. After 15-min at 4 °C, tubes were closed and plunged into liquid nitrogen. Additionally, the cooling rates of solutions were determined using a Testo 950 electrical thermometer (Testo AG, Lenzkirch, Germany).

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 approximately 60 s and expelling of the contents of tubes into the solution for the removal of cryoprotectants. The strictly exact exposure-

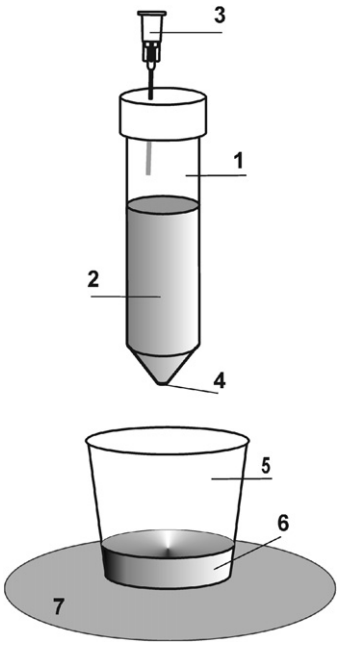

Fig. 1. (1) Scheme of step-wise saturation of ovarian pieces by ethylene glycol hermetically closed tube, (2) ethylene glycol solution, (3) injection narrow with tap for air, (4) opening, (5) container with ovarian pieces, (6) holding medium, and (7) shaker.

time in the boiling water was visually controlled with ice in the medium: as soon as the ice was ~2 mm thick, the tube was taken out from the boiling water. The final temperature of the medium after warming ranged between 4 and 10 °C. Additionally, the warming/thawing rates of solutions were determined using a Testo 950 electrical thermometer (Testo AG, Lenzkirch, Germany).

After warming, OP were transferred within a few seconds (5–7) to a 110-ml specimen container with 10 ml of solution for the removal of cryoprotectants (0.75 M sucrose + 10% SSS + DPBS). The stepwise dilution of the cryoprotectants was achieved using the same principle as that used for the saturation by EG (Fig. 1 and [22]). The container was placed on the shaker and continuously agitated at 200 osc/min for 15 min at room temperature. Stepwise rehydration of OP for 30 min at room temperature was also performed using the same dropping methodology (Fig. 1 and [22]). For dropping rehydration, 50 ml of holding medium was used, which was previously used for the dissection manipulations (DPBS + 10% SSS), in a 50-ml tube (Greiner Bio-One GmbH, Frickenhausen, Germany). The final sucrose concentration was 0.125 M. Finally, the OP were washed three times each in DPBS supplemented with 10% SSS and in culture medium AIM-V® (Gibco, Grand Island, NY, USA) for 10 min, followed by culture. After warming and washing, OP were transferred for *in vitro* culture.

For *Group 3* OP, a rapid freezing protocol proposed by Bordes et al. [5] was used. These authors used this protocol for cryopreservation of ovaries of ewes that resulted in the live birth of lambs, and literature analysis has shown this method to be the most effective method resulting in live births after warming and transplantation of hemiovaries.

The rapid freezing solution comprised: 2.62 M DMSO, 2.60 M acetamide, 1.31 M propylene glycol, 0.0075 M polyethylene glycol in DPBS, and 10% SSS. For dehydration, OP were exposed to increasing concentrations (12.5%, 25%, 50%, and 100%) of rapid freezing solution. The first two steps lasted for 5 min at room temperature and the third step for 15 min at 4 °C. OP were then transferred into cryovials containing 1 ml of 100% rapid freezing solution. Then, after 15 min exposure in 100% freezing solution at 4 °C, OP were cooled by direct plunging of cryovials into liquid nitrogen.

For warming of OP from Group 3, the cryovials were placed onto a plate containing warm water at 37 °C for 10 min. Rehydration was carried out by extracting the cryoprotectants in baths of rapid freezing solution of decreasing concentrations (50%, 25%, and 12.5%) for 15 min in each solution and by final washing in DPBS and 10% SSS and in culture medium AIM-V® for 10 min, followed by *in vitro* culture.

Conventional freezing and thawing

The conventional freezing protocol was based on the protocol that was used by Gosden et al. [13] with some modifications. Cryovials were filled with 1.8 ml of conventional freezing medium containing L-15 Medium (Leibovitz) with L-glutamine, 1.5 M DMSO, and 10% SSS and cooled in icewater (0 °C). Then, OP from dissection medium Brahma II were transferred to cryovials for 30 min and placed in the freezer. The chamber of the freezer was previously stabilized to 2 °C for 20–30 min. The conventional freezing program was as follows. Initial temperature was 2 °C. The temperature was decreased from 2 to −6 °C, at a rate of 2 °C/min. Automatically seed at −6 °C and held after "crystal-formation" at increased temperature (−5.7 °C) for 10 min. Again, the temperature was decreased from −5.7 to −40 °C at a rate of 0.3 °C/min. Thereafter, at a rate of 10 °C/min, the temperature was reduced to −140 °C and vials were plunged into liquid nitrogen. Warming and removal of cryoprotectants was similar as that for rapid freezing, described in detail above. Cryovials were held for 30 s at room temperature in air, followed by immersion in a 100 °C (boiling) water bath for approximately 60 s. Exact time-period of exposure of cryovials in boiling water was detected visually: as soon as the ice was ~2 mm thick, the tube was taken out from the boiling water. 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 (0.5 M sucrose + 10% SSS + DPBS). The container was placed on the shaker and continuously agitated at 200 osc/min for 15 min at room temperature. Stepwise rehydration of OP was also performed using the same dropping methodology [22]. For dropping rehydration, we used 50 ml of holding medium, which was previously used for the dissection manipulations (DPBS + 10% SSS), in a 50-ml tube (Greiner Bio-One GmbH). The final sucrose concentration was 0.08 M. Finally, the OP were washed three times each in DPBS supplemented with 10% SSS and in culture medium AIM-V® for 10 min, followed by *in vitro* culture.

Culture

Ovarian tissue pieces were transferred to 200-ml dishes for suspension culture (Cellstar™, Greiner Bio-One GmbH) in 40 ml of AIM-V® medium for tissue culture (Gibco, Grand Island, NY, USA), and cultured for 14 days *in vitro* at 37 °C in 5% CO_2 in air,with agitation at 75 osc/min using a rotation shaker by Isachenko et al. [22]. AIM-V® medium was not changed during culture. Five OP per one culture dish were cultured by principle "OP of the same patient after the same treatment in the same culture dish". Production of hormones and development of follicles were evaluated.

Histological examination

For histological investigation, tissue pieces were fixed in Bouin solution, imbedded in paraffin wax, serially sectioned at 8 μm, stained with haematoxylin/eosin, and analyzed under a microscope (400×). Follicular density was deter-

mined by counting the number of follicles in every OP. The number of viable and degenerated follicles was counted. Before counting of follicles, sections were coded and scored "blind". To avoid overcounting of the same follicles, only the section with a visible oocyte nucleus was counted.

Normality of follicles was evaluated taking into account the parameters previously described [33]. 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 spheroid granulosa cells. Quality of the follicles was graded from 1 to 3. Follicle of grade 1 is spherical and is randomly distributed around oocytes granulosa cells, with homogenous cytoplasm and slightly granulated nucleus, in center of which condensed chromatin in form of dense spherical structure is detected. Follicle of grade 2 has the same peculiarities, but granulosa cells do not cover the oocyte regularly; these cells can be flat, and condensed chromatin is not detected in cytoplasm. Follicle of grade 3 has partly or fully disrupted granulosa or/and cytoplasm and picnotic nucleus. Follicles of grades 1 and 2 were denoted as normal, and those of grade 3 were denoted as degenerated [33].

Hormones assays

After culturing of cryopreserved and fresh OP, the spent medium was collected every second day (600 μl per collection) during two weeks of culture and stored at −80 °C for 1–2 months for subsequent hormone assays. The level of estradiol 17-β (E_2, analytical sensitivity 5.0 pg/ml) and progesterone (P_4, analytical sensitivity 0.1 ng/ml) was measured using heterogeneous competitive magnetic separation immunoassay (Bayer Vital GmbH, Leverkursen, Germany) eight times during culture, in 56 probes (14 patients in 4 groups).

Statistical analysis

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

Results

Cooling and warming/thawing rates

It was established that cooling rate at rapid freezing in the temperature range from 2 to −140 °C was ~220 °C/min.

At warming/thawing of samples in agitated boiling water the speed of a temperature increasing in the temperature range from −150 to −4 °C was ~150 °C/min.

Histological examination

Only primary and primordial follicles were denoted as viable. All the preantral and the antral follicles after cryopreservation and *in vitro* culture were degenerated, and the authors have not counted these follicles. The mean primordial and primary follicle density per 1 mm^3 for Group 1 (fresh *in vitro* cultured OP) was 14.5 ± 6.1; for Group 2 (post-rapidly frozen by Isachenko et al. [17] *in vitro* cultured OP), 4.1 ± 2.9; for Group 3 (post-rapidly frozen by Bordes et al. [5] *in vitro* cultured OP), 5.7 ± 3.2; and for Group 4 (post-conventionally frozen *in vitro* cultured OP), 15.0 ± 5.3 ($P_{1–4,2–3} > 0.1$). It was detected that 96%, 36%, 39%, and 84% follicles for Groups 1, 2, 3, and 4, respectively, were normal ($P_{1–2,3,4} < 0.05$; $P_{2,3–4} < 0.05$; $P_{2–3} > 0.1$) (Figs. 2 and 3). The remaining follicles were in progressed stages.

Hormone assays

The level of hormones in native AIM-V® medium was determined: $E_2 < 10$ pg/ml and $P_4 < 0.1$ ng/ml. After culture of fresh tissue pieces (Group 1), rapidly frozen/warmed pieces (Groups 2 and 3), and conventionally frozen/thawed pieces (Group 4), E_2 concentrations in the supernatants were 358, 275, 331, and 345 pg/ml, respectively, and P_4 concentrations were 3.02, 1.77, 1.99, and 2.01 ng/ml, respectively (Fig. 4).

Discussion

Data on comparison of the two methodologies of cryopreservation of human ovarian tissue, rapid cooling and conventional freezing with or without following *in vitro* culture are limited.

Such comparative investigations were performed on human, bovine, and porcine ovarian tissues [9]. In this study, authors used two rapid freezing protocols. By first protocol [1], pieces were vitrified in 35% EG + 5% polyvinylpyrrolidone + 0.4 M trehalose by cooling of tissues in small amount of rapid freezing medium and post-warming (at 37 °C) removal of cryoprotectant in 0.3 M trehalose. By second protocol [41], pieces were vitrified in 22% EG + 22% DMSO with removal of cryoprotectants in 0.25 M sucrose after warming at 37 °C. Ovarian pieces

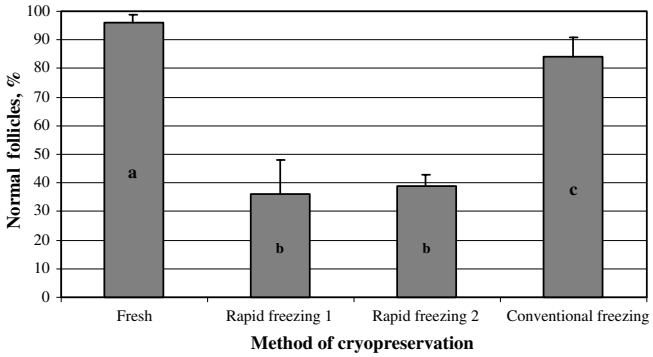

Fig. 2. Quality of follicles after rapid and conventional freezing of ovarian pieces. Different superscripts indicate statistical difference ($P < 0.05$).

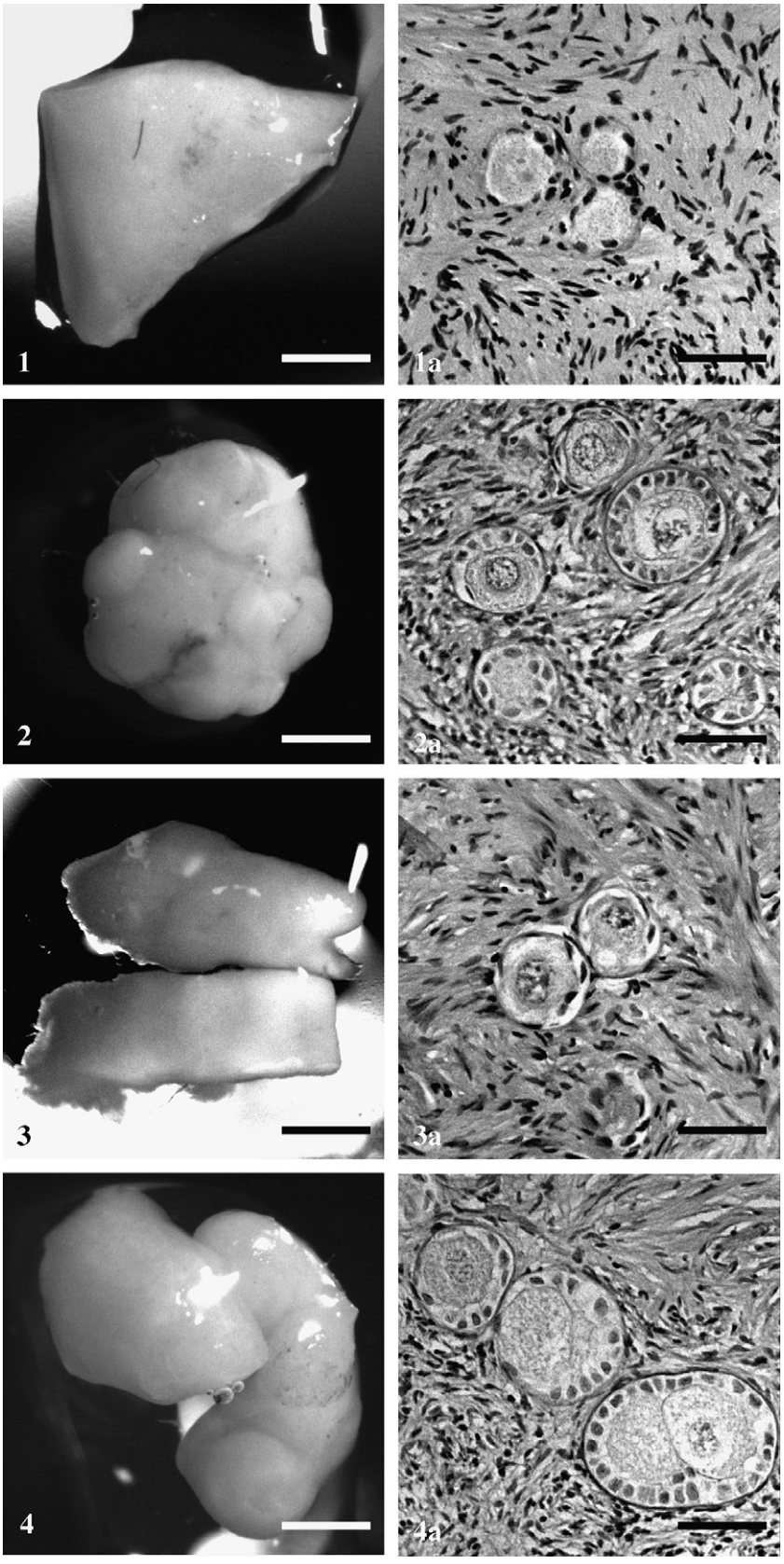

Fig. 3. Micrographs of ovarian pieces (OP) from two patients. Patient 1: (1 and 1a) OP after rapid freezing and culture, (2 and 2a) OP after conventional freezing and culture. Patient 2: (3 and 3a) two OP after rapid freezing and culture (2 and 2a), two accreted together OP after conventional freezing and culture. Bar = 350 μm (light picture) and 35 μm (histology picture).

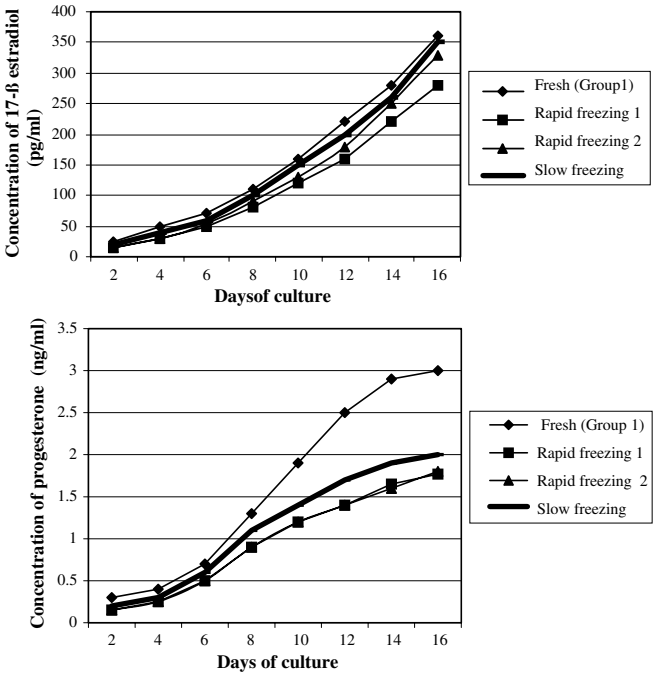

Fig. 4. Concentration of 17-β estradiol and progesterone of rapidly and conventionally frozen ovarian pieces cultured *in vitro*.

were also conventionally frozen using 1.5 M DMSO or 1.5 M propylene glycol with seeding at −9 °C and following cooling rate 0.3 °C/min with post-thawing stepwise exposure of fragments in the respective cryoprotectants (DMSO or propylene glycol) of decreasing concentration [29]. Immediately after warming/thawing, ovarian fragments were fixed and follicles were evaluated. Authors of this study have found some peculiarities of cryostability of follicles dependently from 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 rapid freezing [9]. Our results described in this article allow doing the same conclusion about effectiveness of the conventional freezing in comparison with rapid freezing.

Rahimi et al. [37] compared conventional freezing and rapid freezing of human ovarian tissue. Examination of the necrotic areas of the pieces after both these methods of cryopreservation and transplantation into SCID mice showed no significant rise between these two methods.

In recent years, results from the comparative investigations on rapid and conventional freezing of human ovarian tissue with long term *in vitro* culture of tissue fragments after thawing/warming were published [26]. The 5 mm × 1 mm × 1 mm ovarian strips were divided into two groups. Tissue strips of 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 rapid freezing medium into the liquid nitrogen. Warming of the vitrified strips was carried out by direct plunging of the vitrified drops and gentle agitation of 0.5 M sucrose at 38 °C with following stepwise rehydration of strips in 0.5, 0.25, and 0.125 M sucrose. Tissue strips of 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 two weeks of culture it was established that in both cryopreservation groups there is no difference in the proportion of normal follicles after conventional freezing and rapid freezing [26]. The average concentration of 17-β estradiol and progesterone were not statistically different after rapid and conventional freezing. The authors believe that the original methodology of rapid freezing by direct dropping of freezing solution into liquid nitrogen is more effective method than the conventional freezing.

We, however, believe that with similar effectiveness of conventional freezing and rapid freezing it would be not logical to recommend the described technology of rapid freezing because this technology has the following parameter: direct contact of tissue with liquid nitrogen.

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 microorganisms [3,4,10]. Liquid nitrogen, which is used for the storage of frozen material, can be a source of contamination by these microorganisms [3,42]. Filtration or ultra-violet treatment of liquid nitrogen cannot guarantee the absence of contamination of biological material by viruses. For example, it was reported about the contamination of blood probes by hepatitis virus during the time of storage of probes in liquid nitrogen [42]. Different types of viruses, which are simple and very cryostable structures, may increase their virulence after a direct plunging and storage in liquid nitrogen, like hepatitis virus [14], papova virus [6], vesicular stomatitis virus [39], and herpes virus [25].

The main aim of this investigation was to determine if rapid freezing is more or less effective than the conventional freezing by the ability of the follicles to develop after warming/thawing. In fact, rapid freezing 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 the cryo-technology is the preservation of follicles, and is not the guaranteeing a simplicity and availability of technology for operator to the detriment of a post-warming quality of follicles. Results of this investigation have also shown that rapid freezing can guarantee the storage of viable follicles after warming, but conventional freezing is more effective.

As per a standard medical procedure, part of the ovarian tissue, which was obtained before the oncology treatment, are used for the routine histological observation. This is performed 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* to check for the presence of follicles. The

quality of the follicles present in the cultured tissue indicates whether or not it is possible to restore the reproductive function of a given patient.

The quality of cryopreservation for a given ovarian tissue from a given patient without participation of the patient in the process of this evaluation to her full restoration after medical treatments can be determined by three ways: (1) Histological analysis of follicles immediately after cryopreservation/thawing [35]. (2) Histological analysis of follicles after post-thawing *in vitro* culture [17,18,16,15]. (3) Histological analysis of follicles after post-thawing xeno-transplantation [29,31,11,12]. By these three methods, xeno-grafting is still the most conclusive, being in the same time the most expensive. In this investigations, the authors have used the original, previously described method for *in vitro* culture of the ovarian tissue to obtain the best information about the quality of the tissue [22]. Our data show, that the cryopreservation protocol in combination with long term *in vitro* culture in a large volume of culture medium and under constant agitation supports *in vitro* growth of follicles better compared with the routine culture conditions [22].

The protocols described for the rapid freezing of ovarian tissue by Isachenko et al. [17] (Group 2) and for conventional freezing (Group 4) have the following parameters: (1) The rapid freezing and conventional freezing solutions is sucrose-free and includes only permeable cryoprotectants. (2) Stepwise procedure for the saturation by and removal of permeable cryoprotectants. (3) Saturation by DMSO at 0 °C. (4) Quick warming of tissues at +100 °C.

For the testing protocols the authors have used sucrose-free solutions because it was previously shown that the presence of this disaccharide in rapid freezing solution for ovarian tissue, has a detrimental effect [17]. Migishima et al. [28] reported a method for the cryopreservation of whole mouse ovaries by rapid freezing using permeable cryoprotectants without sucrose (dimethyl sulfoxide, acetamide, and propylene glycol). Stepwise introduction and removal of cryoprotectants can be explained by the sensitivity of human cells to osmotic processes accompanying the saturation by permeable cryoprotectants and their removal [19]. Saturation by DMSO at 0 °C was used because 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 [34] as well as the time of removal of cryoprotectant/osmotic injuries after warming. Toxicity of cryoprotective agents is a key limiting factor in cryobiology [8]. DMSO has a high toxicity [40]. The saturation of cells by DMSO at 0 °C reduces DMSO toxicity.

The elevated speed of warming is used because the factor that potentially can decrease the viability of the cryopreserved tissues is re-crystallization at warming. In our protocols, the authors used a very quick warming of the tissue. The research base for this is the previously established positive effect of high speed warming of pronuclear and GV-oocytes at rapid freezing [20,21,23].

Two protocols of cryopreservation by direct plunging into liquid nitrogen were tested. Authors of both these protocols used terminology "vitrification". However, in the Group 2 experiments, melting ice is actually used to determine when warming is complete, which proves that an ice-free state is not maintained through cooling and warming. At warming of cryovials in the Group 3, the ice formation is observed, too. The glass-formation at cooling and the stability of the amorphous state of solution at warming have been previously reported. It was established, that even 40% DMSO, which is a better glass former than ethylene glycol, has a critical cooling rate to avoid ice formation of 500 °C/min, and a calculated critical warming rate to avoid ice formation (devitrification) of over one billion degrees per minute [2]. In the solution of Group 2, the carrier solution and serum solutes provide additional stability against ice formation, but 40% penetrating cryoprotectants are not able to prevent ice forming during the cooling and especially warming conditions described. It was established that cooling rate at warming of Group 2 samples in the temperature range from −150 to −4 °C was ∼150 °C/min. The solution of Group 3 had more solutes than the solution of Group 2, and was previously used for vitrification of mouse embryos [38]. However this solution did not remain free of ice because was warmed in cryovials, in bigger volumes than in straws. This explains why both Group 2 and Group 3 experiments are not "real" vitrification. By this reason we use terminology "rapid freezing" instead "vitrification".

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