Whole sheep ovary cryopreservation: evaluation of a slow freezing protocol with dimethylsulphoxide

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Abstract

Purpose To evaluate a slow freezing method for whole ovary cryopreservation by evaluating effects of added cryoprotectant.

Methods Sheep ovaries were isolated during surgery, flushed with either Ringer-Acetate or dimethylsulphoxide and cryopreserved by slow freezing. After rapid thawing, viability was assessed by ovarian in vitro perfusion, cell culture, histology and fluorescent live-dead assay.

Results Production of cyclic AMP and progesterone was slightly higher in the dimethylsulphoxide group. Cultured ovarian cells from dimethylsulphoxide-preserved ovaries secreted larger amounts of progesterone than cells from Ringer-Acetate preserved. Light microscopy of ovarian biopsies obtained after perfusion, revealed well-preserved tissue in the dimethylsulphoxide group but not in the Ringer-Acetate group. The density of small follicles and ovarian cell viability were higher in dimethylsulphoxide ovaries compared to Ringer-Acetate ovaries.

Conclusions Equilibrium with its protective effect can be achieved by slow freezing protocol, with an additional protective effect by the presence of dimethylsulphoxide.

Keywords Cryopreservation · Cyclic AMP · Dimethylsulphoxide · Ovary · Perfusion

Introduction

Approximately 8% of all cancer victims are below 40 years of age [1]. The combined 5 years relative survival rate for children with cancer has improved from around 63% for patients diagnosed during the period 1975–1979 to 79% for those diagnosed 1995–1999 [2]. Several chemotherapeutic agents [3] and radiotherapy used in cancer treatment are toxic to the ovaries [4].

Attention is now focused on fertility preservation/restoration methods in young cancer patients and ovarian cortex cryopreservation, followed by avascular autotransplantation, has resulted in a small number of live-births [5]. In general, the function of these transplanted ovarian cortical grafts persist up to 3–4 years [6]. The restricted life span of the grafts may be explained by their relatively small size, suboptimal protocols for cryopreservation/thawing and/or irreversible damage during extended warm ischemia. Whole ovary cryopreservation with transplantation by vascular anastomosis has been suggested as a future method with a reasonable chance of longevity of the transplant [7]. Research on whole ovary transplantation has been performed in a number of animal models [8–12], including the rhesus macaque [13]. The sheep is the species that has been used most extensively in research on whole ovary cryopreservation [14–16].

The major difficulties with the method of whole ovary cryopreservation seems to be the vascular microsurgery [17] and to effectively cryopreserve a large tissue-mass [18]. The ovary would be regarded as an organ of high cryobiological complexity. A slow freezing method reduces the likelihood

Capsule The study evaluates the impact of dimethylsulphoxide as a cryoprotectant for whole ovary cryopreservation.

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of intracellular ice formation by initiation of extracellular ice crystal usually by seeding at subzero temperature, with freezing rate being accountable for the rate of extracellular ice crystal formation [19]. The extracellular ice draws water out of the cell until little amount of free water remains and only small (non-lethal) ice crystals can be formed [20]. The goal is to attain a cooling rate which allows equilibrium between the rate of water loss from the cell (dehydration) and the rate at which this water is integrated into extracellular ice crystals [19]. At the same time, exposure to cryoprotectant (CPA) and adequate permeation must be obtained. Based on the present knowledge, CPAs lower the freezing point and replace some of the bound water molecules in and around intracellular components [19]. The CPAs also stabilize cell proteins and the plasma membrane [21], to reduce the intracellular concentration of electrolytes [22].

Two chemically different cryoprotectants, dimethylsulphoxide (DMSO) [7, 14, 16, 23] and propanediol [24] have mainly been used in whole ovary cryopreservation research. However, there is a lack of systematic studies that have investigated the different facets of whole organ cryopreservation. Complexity of tissue and the multiplicity of interacting factors make it difficult to create the proper design of experiments [25].

The aim of this study was to evaluate the effect of the presence of DMSO in comparison to plain Ringer-Acetate (RA) at whole ovary freezing.

Material and methods

Animals

Female, sexually mature (2–4 years old) ewes ($n=11$) weighing 45–65 kg were used. The study was approved by the Institutional Review Board and the Animal Ethics Committee in Gothenburg. All animals were synchronized to be at follicular phase at ovarian retrieval by application of vaginal sponges with medroxyprogesterone (60 mg; Sigma-Aldrich, St. Louis, MI, USA) 10–12 days before surgery and then administration of 500 IU equine chorion gonadotropin (eCG; Intervet, International, Boxmeer, Netherlands) im 24 h prior to surgery. The animals were, during the same surgical session, also used for experimental studies of ischemia-reperfusion events after uterus auto-transplantation [26].

Surgery

The sheep were given diazepam (0.2 mg/kg) and pentothal (12 mg/kg) iv for anesthesia induction and 2–4% isoflurane was used for maintenance. Through a midline incision the ovarian artery was dissected free from its attachment to the ovarian veins 20 mm to 30 mm from their inlet into the ovary.

The attachments and vascular connections between the uterine horn/oviduct were then cut after ligations. The ovarian pedicle was clamped and then cut at the level of the previous dissection of the ovarian artery. Unilateral oophorectomy was performed as described above and the contra-lateral ovary was kept in situ for further experiments involving uterus transplantation surgery. Two researchers were conducting the whole ovary cryopreservation study and three other researchers then continued with surgery for uterus auto transplantation. The ovaries were randomly allocated to be frozen with a defined CPA (DMSO; $n=6$) or without CPA (RA; $n=5$).

Cryopreservation and thawing

The ovary was submerged into RA (4°C) immediately after retrieval and a cannula (22/24G) was inserted into the open end of the ovarian artery and tied in place with two 4-0 silk sutures. The ovary was then gently flushed by a hand-held syringe with RA (4°C) supplemented with 50 IU/ml heparin and 0.2 mg/ml xylocaine until clear fluid emerged from the vein.

The ovary was flushed with either a solution containing 1.5M of DMSO (Merck AG, Darmstadt, Germany) with supplementation of 0.1M sucrose and 2% human serum albumin (Sigma-Aldrich St. Louis, MI, USA) in Leibovitz L-15 medium (Gibco, Scotland, UK) or an identical solution but with the same volume of RA instead of DMSO. The two different solutions were in infusion bags at 4°C and a pressure-infusion-device (Rudolf Riester GmbH & Co. KG, Jungingen, Germany) was used at 80 mmHg with a resulting flow rate between 1–2 ml/min.

The ovary was then placed in a 60 ml autoclavable straight-side, wide-mouth polypropylene jar (Nalgene Nunc, Rochester, NY, USA) with 5 ml of the same respective solution. This cryovial was placed inside a pre-cooled (4°C) container (Cryo Freezing Container®, Nalgene Nunc International, Rochester, NY, USA), containing isopropanolol, and placed inside a –80°C freezer for 24 h, as described for slow freezing of the human ovary [23]. The container was then placed in liquid nitrogen and stored (>2 weeks) until thawing.

The ovary was thawed for 10 min by positioning the frozen vial in a 37°C water bath. A small (5×6×8 mm) wedge biopsy was excised, to be used for histology, estimation of follicle density and live/dead assay (see below), and the site was sutured with 6-0 suture. Flushing each 10 min; flow rate 1–2 ml/min) was performed at room temperature with three different solutions of 2% HSA in Leibovitz L-15 containing 0.1M, 0.05M sucrose and no sucrose before the ovary was mounted in the perfusion apparatus (see below).

In vitro perfusion

An in vitro perfusion system was used to assess the functionality of the entire ovary, in a setting where the tissue

architecture and cell communications are intact. This technique, developed in our laboratory [27], is a closed circuit system where perfusion medium is pumped through the ovarian vasculature at a determined pressure. The perfusion medium consists of M199 with Earl's salts (Invitrogen, Carlsbad, CA, USA) plus 2% bovine serum albumin (Roche Diagnostic GmbH, Penzberg, Germany) and is continuously equilibrated with 5% CO₂ in 95% O₂. The perfusion pressure was maintained at 80 mm Hg which resulted in a flow rate through the ovary of around 2 ml/min.

The ovaries were pre-perfused for 1 h before stimulation of endogenous production of cyclic adenosine 3'-5'-monophosphate (cAMP) by addition of 30 µM of the adenylate cyclase stimulator forskolin (7β-acetoxy-8, 13-epoxy-1α, 6β, 9α-trihydroxylabd-14en-11one; Sigma-Aldrich). The concentration (30 µM) of forskolin, as used in the present study, leads to a marked increase in cAMP levels in the perfusion medium and induces ovulations during in vitro perfusion of rabbit ovaries [28]. At termination of perfusion a second tissue sample (4×4×4 mm) was taken from the ovary for live/dead assay (see below). About half of the ovary was prepared for cell culture (see below) and the remaining tissue was used for histology (see below).

Histology and follicle density

Biopsies were fixed in formaldehyde, stained with hematoxylin-eosin, and examined independently by two persons blinded to the experimental data.

The density of small follicles (primordial and primary) was estimated by counting the number of these follicles within 8 square grids (0.16 mm²) occupying a total area on the section of 1.28 mm². The grids were placed with one edge on the ovarian surface epithelium and the opposite side inside the cortex, with the area containing primordial/primary follicles within the grid. A total of 4–6 sections per ovary were counted and the mean of this was used as a data point

Cell culture

Dispersed ovarian cells were obtained by a method previously described for cultures of human ovarian cells, with collagenase and DNase to digest the ovarian tissue after initial mechanical digestion [29]. The suspension of cells was passed through a 100 µm mesh cell-strainer (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) and centrifuged (200×g) for 5 min. The pellet was washed two times in RPMI cell-culture medium (Gibco, Invitrogen, Paisley, UK) supplemented with 10% FCS. Cell viability was assessed by Trypan Blue exclusion test and 5–10·10⁵ live cells per well were placed in a 24-well plate. The cells were left for 24 h to attach to the plastic surface and after

washing human chorionic gonadotropin (hCG; Schering-Plough, Stockholm, Sweden) was added. The cells were left for another 24 h after which the cell culture supernatant was collected.

Live/dead viability assay

To evaluate the proportion of cells surviving the freeze-thaw procedure a live/dead assay (Live/Dead® viability/cytotoxicity kit; Molecular Probes, Eugene, OR, USA) was used. The ovarian sample (4×4×4 mm) was cut into small pieces and washed free from DMSO/RA. Enzymatic digestion was performed for 90 min at 37°C with 5 ml of 0.04 mg/ml Liberase Blendzyme 3 (Roche Diagnostics, Indianapolis, USA) according to a protocol previously used for human ovarian tissue [30]. The cell suspension was strained through a 100 µm mesh cell-strainer (Falcon, BD Bioscience, MA, USA) and centrifuged (200×g). The pellet was suspended in 100 µl PBS and incubated for 30 min at 37 °C with 100 µl of live/dead reagent at a concentration of 4 µmol/l calcein acetomethylester and 12.5 µmol/l ethidium homodimer-I in PBS. A small volume of the incubated cell suspension was transferred on to a glass slide and covered with a cover glass. A minimum of 200 stained cells were counted under UV light in a fluorescence microscope and the proportion of live (green) and dead (red) cells was calculated independently by two observers and the mean of these was taken as a data point. The inter-observer variance was <10%.

Steroid assays

Progesterone and estradiol concentrations were analyzed by an immunofluorometric method (DELFI; Wallac, Turku, Finland). The mean inter-/intra-assay coefficients of variations for progesterone were 6.9% /13.0% and for estradiol 6.6% /4.1%.

Cyclic AMP assay

The cAMP content of the perfusion medium was analyzed using a modified DELFIA method and a high sensitivity (detection limit 46 pmol) acetylation protocol (Wallac OY, Turku, Finland). All standard dilutions were performed in perfusion medium. The standard curve was diluted to give 2.8–180 fmol of cAMP in 50 µl of the final volume. Acetylation was accomplished by addition of 5 µl acetylation reagent to 180 µl of perfusion medium sample/standard. The samples were left for 10 min at room temperature and then 27 µl of a mixture of 1 ml H₂O and 3.5 ml of 10× concentrated buffer for standards were added to all samples. The inter/intra-assay variations were 8.5/3.8% for 87.8 fmol (*n*=10), 9.6/8.6% for 9.4 fmol (*n*=9) and 12.2/ 14.4%, % for 3.3 fmol (*n*=10) cAMP.

Statistics

Statistical comparisons were made by Student's t-test. A p-value of <0.05 was considered significant.

Results

In vitro perfusion

The levels of cAMP and progesterone in the perfusion medium were similar in both groups after the 60 min pre-perfusion period. Addition of forskolin resulted in an increase of the levels of cAMP and progesterone in the DMSO group, but the levels were not significantly higher than those of the RA group after 60 min (Figs. 1, 2a). Concentrations of estradiol were not altered by forskolin (Fig. 2b).

Cell culture

Ovarian cells were cultured for 24 h with three different concentrations of hCG (10, 100 and 1000 IU/l) after a 24 h pre-culture period to allow for attachment of cells. Progesterone levels in the cell culture medium (Fig. 3a) were higher in the cultures of ovarian cells of DMSO-frozen ovaries as compared to cultures of ovarian cells of RA-frozen ovaries. The estradiol levels in the medium were not significantly increased in cultures of DMSO-frozen ovaries (Fig. 3b). No stimulatory effect of hCG was seen.

Fig. 1 Concentrations of cyclic adenosine monophosphate (cAMP) in medium of in vitro perfused sheep ovaries. The ovaries were preperfused for 60 min before addition of the adenylate cyclase stimulator forskolin (0 min) and then perfused for another 60 min. Filled bars represent the means of ovaries frozen in DMSO and open bars represent ovaries frozen in Ringer Acetate. Bars indicate SEM. $n=4-5$

Fig. 2 Concentrations of progesterone (a) and estradiol (b) in medium of in vitro perfused sheep ovaries. The ovaries were preperfused for 60 min before addition of the adenylate cyclase stimulator forskolin at 0 min and then perfused for another 60 min. Filled bars represent the means of ovaries frozen in DMSO and open bars represent ovaries frozen in Ringer Acetate. Bars indicate SEM. $n=4-5$

Live-dead assay

The proportion of live cells in the two groups was assessed both before and after perfusion (Fig. 4). There was no difference in the proportion of live cells before and after perfusion within any of the groups but the proportion of live cells after thawing was markedly higher after cryopreservation in DMSO than after cryopreservation in RA (Fig. 4).

Fig. 3 Concentrations of progesterone (**a**) and estradiol (**b**) in conditioned medium of cultures of dispersed ovarian cells. *Filled bars* represent the means of cell cultures from ovaries frozen in DMSO and *open bars* represent cell cultures from ovaries frozen in Ringer-Acetate. * = significantly ($p < 0.05$) higher than Ringer-Acetate frozen ovaries of respective group. Bars indicate SEM. $n = 4-5$

Histology and follicle density

The density of small follicles (primordial and primary) in the ovarian cortex was higher ($p < 0.01$) after thawing in DMSO-preserved (17.5 ± 2.2 ; mean $\pm$ SEM) than in RA-preserved (7.0 ± 1.4) ovaries. Light microscopy examination of ovarian biopsies was performed on frozen-thawed tissue both before (Fig. 5a, c) and after 120 min of in vitro perfusion (Fig. 5b, d). The histology of the ovarian tissue assessed before perfusion of both groups appeared normal with healthy looking immature follicles (Fig. 5a, c). After perfusion, the

Fig. 4 Proportion of live cells in biopsies taken from ovaries before perfusion (striped bars) and after perfusion (open bars). * = significantly ($p < 0.05$) higher than that of Ringer-Acetate frozen ovaries of respective group. Bars indicate SEM. $n = 5$

tissue of the DMSO-frozen ovaries remained morphologically normal (Fig. 5b) but the tissue-architecture of the RA-frozen ovaries became disrupted with signs of edema, disorganized stroma and small follicles that had lost their round shape (Fig. 5c).

Discussion

Whole ovary cryopreservation followed by transplantation with microvascular anastomosis may in the future become an effective oncofertility procedure. There is a need for strict research protocols for whole ovary cryopreservation that test various freezing protocols and CPA, since most cryopreservation research in reproductive biology has been performed on tissue of small volumes such as oocytes [31], embryos [32] and blastocysts [33].

As a research tool for studies towards whole human ovary cryopreservation the sheep ovary model seems to be appropriate although the ovine ovary is only about 1/6th [11] of the size of a human premenopausal ovary [34]. The tissue architecture of ovine and human ovaries is similar with a well-defined tunica albuginea and a collagen-dense cortical stroma containing the primordial follicles.

The most widely used CPA in ovarian cryopreservation research is DMSO, because of encouraging results in early studies of cryopreservation of human ovarian tissue pieces [35, 36]. Thus, favorable result of cryopreservation in 2M DMSO was shown when DMSO and propanediol of various concentrations were compared [37]. Live births

Fig. 5 Light microscopy of ovarian biopsies taken before (a, c) and after perfusion (b, d) from DMSO-frozen ovaries (a, b) and Ringer-Acetate frozen ovaries (c, d). Scale bar represents 100 μ m

after human ovarian cortex cryopreservation and autotransplantation have been reported with either DMSO [38] or ethylene glycol [39] as CPA.

The fertility results of whole ovary cryopreservation and autotransplantation is still unsatisfactory as illustrated by the low pregnancy rate of 1/9 in ewes [16] and 1/7 in rats [9]. A standard procedure for evaluation of cryopreserved whole ovaries after thawing is light microscopy, which has been used in the sheep [14–16, 24] and human [7, 23]. In the present study a higher density of small follicles was seen after cryopreservation in DMSO as compared to RA, further pointing towards the necessity of inclusion of a CPA. The morphology of the frozen ovaries of the present study was evaluated both directly after thawing, but also after *in vitro* perfusion with oxygenated medium. The ovarian morphology was unaltered in the ovaries preserved with DMSO, both before and after perfusion, but a disintegration of the tissue was seen in the RA-frozen ovaries after perfusion. This result indicates that morphological analysis by light microscopy is only sufficient to detect changes after the frozen-thawed tissue has been challenged by normal oxygen levels, as seen after perfusion with oxygenated medium or reinitiated blood flow. However, a caution is that species differences may exist in regards to morphology after cryopreservation [40].

In the present study we assessed the function of the complete ovary with the vasculature and tissue architecture intact, by *in vitro* perfusion. This method has been used by us extensively for studies of ovarian physiology of freshly

isolated ovaries from experimental animals [41] and human ovaries [42]. To assess the function of the ovary, we stimulated the ovaries with the adenylate cyclase stimulator forskolin. The levels of cAMP and the ovarian steroids were comparable in the two groups after the pre-perfusion period but after forskolin stimulation the DMSO-preserved ovary responded with a slight rise in production of cAMP and secondary to that slightly higher progesterone secretion. The differences in cAMP and progesterone levels between the DMSO and RA groups did not reach significance. It can be speculated that cell membrane-related functions such as adenylate cyclase dependent production of cAMP may be damaged during the equilibration process during cryopreservation as previously discussed [43, 44]. However, it is likely that the differences in progesterone secretion would have been more pronounced after a longer perfusion period since this only partly involve the cAMP second messenger system.

An additional method for evaluation of function and viability of ovarian tissue is ovarian cell culture with measurements of steroid secretion [45, 46]. In the present study we cultured a mixture of all ovarian cell types, which is an established method to study ovarian function when cooperation between different cell types is needed [41]. The progesterone levels were considerably higher in the conditioned media of cells from ovaries frozen in DMSO than in RA, demonstrating the protective benefit of DMSO. In our previous study on whole ovary cryopreservation with propanediol [24], a less pronounced effect on progesterone release was seen. Progesterone production after ovarian

cryopreservation has also been studied in human cryopreserved ovarian cortical pieces, which after 14 days culture showed higher progesterone production after slow freezing as compared to rapid freezing [47].

The live/dead assay of the present study showed a higher proportion of viable cells after cryopreservation in DMSO than in RA, with the live-rate being similar to that reported for small follicles of frozen-thawed human ovaries [23]. The results are in line with the demonstration of the present study of higher follicle density after preservation in DMSO.

The primordial follicular viability after cryopreservation of whole ovine ovary using DMSO as assessed with Trypan blue test was 83% [14] and with the same test the viability was 76% after thawing of the whole human frozen ovary [7]. In studies of cryopreserved ovarian cortical biopsies from PCOS patients a similar proportion of live follicles was seen [48]. Collectively, the results on human and ovine cryopreserved ovaries indicate a viability of around 80% of the immature follicles/cells. However, higher survival rates [15] have been reported and this may be due to small modifications of the cryopreservation-thawing protocol as previously discussed [7].

The thawing procedure is also important for the survival and post-thawing condition of the cryopreserved ovary. In our present study we used the common method of placing the frozen vial into a water bath of 37°C [14, 49]. Further studies are needed to define thawing procedures corresponding to adequate freezing protocol.

In spite of the generally accepted opinion that all living cells exposed to low subzero temperature would die, we have shown cell viability after freezing and thawing without CPA. The ovaries were perfused by RA, which is an isotonic solution without influence on osmotic equilibrium. In general, viability of cells after cryoprotection can be attributed to the effectiveness of equilibrium between cell dehydration and extracellular ice crystal formation [19]. Moreover, the protective effect of innocuous intra cellular ice has been reported [50]. High cellular viability after freezing and thawing of fibroblasts and endothelial cells was shown by inducing intracellular ice formation and in the absence of any CPA. In our study, we can only speculate about these phenomena. We applied a freezing method, which originally was established for human ovary freezing and thawing [23]. This is an uncontrolled freezing method without induction of extracellular ice formation by seeding, similar as those used for cryopreservation of peripheral blood progenitor cells [51].

In conclusion, this slow freezing protocol demonstrates adequate rate of cell dehydration as well as benefit of CPA. Further research should be extended to optimize CPA concentration and thermodynamic conditions according to principles of basic cryobiology research.

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