Viability and function of the cryopreserved whole ovary: in vitro studies in the sheep

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BACKGROUND: Cryopreservation of whole ovaries followed by vascular transplantation may improve long-term function in comparison to conventional cryopreservation of ovarian cortex and avascular transplantation. The aim of this study was to assess methods for the evaluation of viability and function of frozen–thawed whole ovaries.

METHODS: Ewe ovaries were flushed with either cryoprotectant (propandiol: FROZEN-PROH) or Ringer Acetate (FROZEN-RA) followed by slow freezing. Some ovaries were assessed fresh after flushing with Ringer Acetate (FRESH-RA). Assessment was done by light microscopy, biochemical response (cyclic adenosine 3',5'-monophosphate (cAMP) and steroids) during in vitro perfusion with forskolin, viability assay and cell culture.

RESULTS: Microscopy showed well-preserved morphology with the presence of small follicles in all groups before perfusion. Stromal oedema was seen after in vitro perfusion of FROZEN ovaries, and shrunken small follicles were seen only in FROZEN-RA at the end of perfusion. During in vitro perfusion, FRESH-RA ovaries responded with large increase in levels of cAMP after stimulation with forskolin. FROZEN-PROH and FROZEN-RA ovaries exhibited lower production of cAMP. Progesterone concentrations in cell cultures of dispersed ovarian cells were higher in FRESH-RA when compared with FROZEN groups. Addition of hCG to cell cultures resulted in higher progesterone levels in the FROZEN-PROH compared with FROZEN-RA. Cell viability assay showed overall viability of 60–75% with no significant difference between groups.

CONCLUSION: In vitro perfusion may prove to be a suitable method to test viability and function of frozen–thawed whole ovaries contributing to the optimization of current cryopreservation protocols.

Key words: cryopreservation / sheep ovary / viability / perfusion / progesterone

Introduction

The proportion of survivors of childhood cancer and of cancer during fertile life is steadily rising, and it is estimated that within 5 years 1 in every 250 women of fertile age will be a cancer survivor. Thus, there is an increasing interest in research that aims to preserve and restore fertility in prepubertal girls and females of reproductive age following gonadotoxic cancer treatments (Donnez et al., 2006). Low doses of radiation (Wallace et al., 2003, 2005) or certain types of cytotoxic drugs, such as alkylating agents (Shalet, 1980), may damage the follicle pool of the ovary and thereby induce premature ovarian failure. Surgical removal of ovarian tissue, followed by cryopreservation storage and later autotransplantation, offers the possibility to circumvent gonadotoxic injury.

Human ovarian cortical tissue strips have been cryopreserved, thawed and autotransplanted to several sites with successful reproductive function (Oktay and Karlikaya, 2000; Radford et al., 2001; Kim et al., 2004; Oktay and Buyuk, 2004; Tryde Schmidt et al., 2004). However, in spite of the high activity in this field and the establishment of several fertility preserving centres, only five pregnancies with live births have been reported after transplantation of cryopreserved human tissue into an orthotopic location (Donnez et al., 2004; Meirion et al., 2005; Demeestere et al., 2007; Andersen et al., 2008).

In these experimental human fertility preserving procedures (Oktay and Karlikaya, 2000; Radford et al., 2001; Donnez et al., 2004; Kim et al., 2004; Oktay et al., 2004; Tryde Schmidt et al., 2004; Meirion et al., 2005; Wolner-Hanssen et al., 2005; Demeestere et al., 2007),...
the retransplanted ovarian cortical tissues typically start to show the signs of spontaneous estradiol production after 2–8 months, but a cessation of the function is seen in most cases within 6–9 months after retransplantation though in some cases the ovarian function persisted for more than 2 years (Schmidt et al., 2005; Donnez et al., 2006). In the above experiments, only a portion of the cortex of a normal ovary is transplanted. The limited amount of tissue in combination with the extended ischemic period may explain the short lifespan of the graft. In a study of sheep ovarian tissue, it was found that 60–70% of follicles were lost after transplantation but only 7% of the loss was dependent on the cryopreservation procedure itself (Baird et al., 1999). Thus, the major loss seems to occur during the warm ischemic period, probably extending over several days until neovascularization has restored the blood flow to the grafted tissue.

Cryopreservation of a whole ovary followed by retransplantation with vascular anastomoses has been suggested as a method to decrease ischemic injury and thereby to increase survival time and gain long-term ovarian cyclicity (Wang et al., 2002; Bedaiwy et al., 2003; Martinez-Madrid et al., 2004; Revel et al., 2004). Successful cryopreservation and autotransplantation of whole ovaries have been achieved in a number of experimental animal species such as the mouse (Migishima et al., 2003), the rat (Yin et al., 2003) and the rabbit (Chen et al., 2006). The sizes of the ovaries of these species are, however, just fractions of a human ovary, and the results would not necessarily be valid for a larger organ. The sheep ovary, with a size more comparable to the human ovary, has been evaluated for whole ovary cryopreservation, thawing and retransplantation with vascular anastomosis (Imhof et al., 2006; Bedaiwy and Falcone, 2007). Approximately, half of the animals showed ovarian cyclicity (Bedaiwy et al., 2003; Arav et al., 2005; Imhof et al., 2006), and a single live birth was registered (Imhof et al., 2006). The difficulty with whole ovary cryopreservation and transplantation in the sheep is also illustrated in a recent study where only one out of five cryopreserved ovaries resumed endocrine function, and the follicle survival in this ovary was only 6% with no pregnancy achieved (Courbiere et al., 2008). Taken together, these studies of whole ovary cryopreservation in the sheep illustrate the difficulties with this method and indicate a need for improvements in cryopreservation, thawing and surgical techniques. In this respect, it is of interest to note that three alternative cryopreservation protocols have been used for whole ovary cryopreservation. Human whole ovaries have been cryopreserved by slow freezing using a passive cooling device (Martinez-Madrid et al., 2004), whereas whole sheep ovaries have been cryopreserved either by slow computerized freezing (Bedaiwy et al., 2003; Arav et al., 2005) or by vitrification (Courbiere et al., 2005). Initial in vitro studies of cryopreservation of the whole human ovary have recently been published (Martinez-Madrid et al., 2004, 2007; Bedaiwy et al., 2006).

The major technical problems with whole ovary freezing is the larger volume of tissue that causes slow penetration of the cryoprotectant and increases the risk of breaking the structure and organization of cells and tissue components. In particular, the capillary vessels seem to be sensitive to the freezing and thawing procedure (Courbiere et al., 2005). Conventional approaches to examine the frozen–thawed tissue such as evaluations of histological parameters including fluorescent-based test (Martinez-Madrid et al., 2004) to estimate the proportion of live, dead and apoptotic cells (Martinez-Madrid et al., 2007) have been used to accumulate information about the effects of cryopreservation on sub-components of cells and tissue. Moreover, new viability tests such as determination of expression of the cell-cycle protein Ki67 and uptake of bromodeoxyuridine to assess DNA synthesis have been introduced as tests of cryopreserved ovine ovarian tissue (Onions et al., 2008).

The aim of the present study was to extend these studies by also incorporating new viability and functional tests such as in vitro perfusion and cell culture.

Materials and Methods

Animals

Sexually mature (2–4 years of age, weighing 40–70 kg) ewes (n = 14) of mixed breed were purchased from approved suppliers. The study was approved by the Animal Ethics Committee in Gothenburg. The ewes were synchronized to follicular phase by inserting vaginal sponges containing 60 mg of medroxyprogesterone (Sigma-Aldrich, St Louis, MI, USA) 10–12 days before surgery followed by an im injection of 500 IU eCG (Intervet, International, Boxmer, Holland) 24 h prior to surgery. After oophorectomy, for purposes of the present study, the animals were used for experiments of vascular anastomosis techniques and uterine ischemia in uterus transplantation research (Wranning et al., 2008). The experiments were performed as acute experiments with the animals euthanized during anaesthesia when ovaries had been removed and uterine autotransplantation had been completed.

Oophorectomy

On the day of the experiment, the animal was brought to the operating theatre and was given an iv injection of diazepam (0.2 mg/kg, Dupex-Alpharma, Copenhagen, Denmark) and shortly after pentothal (12 mg/kg, Abbot Scandinavia, Solna, Sweden) to induce anaesthesia. The ewe was then intubated with a tracheal tube, which was connected to a ventilator (Servo 900C; Siemens AG, Munich, Germany) to maintain anaesthesia by 2–4% isoflurane (Scherin-Plough Animal Health, Kenilworth, NJ, USA). A midline laparotomy was performed, and the ovarian vascular pedicles were identified. The curled ovarian arteries were dissected free from the underlying ovarian veins from 20 to 25 mm proximal of the ovarian hilus. The dissection was performed by the use of microsurgical forceps and within the aid of surgical loops (× 2.3). The ovarian pedicle was cut at this level with the ovarian artery clearly identifiable. Unilateral oophorectomy was performed in 13 animals and bilateral oophorectomy in 1 animal with a total of 15 ovaries available for the present study.

The ovaries were divided into three groups. Two experimental groups were subjected to the freezing–thawing procedure with or without cryoprotectant. The third group acted as control and was not subjected to freeze–thawing.

Washing

The ovary was submerged in cold (4°C) Ringer Acetate solution (Baxter, Deerfield, IL, USA), and the ovarian artery was cannulated with a 22 or 24 G Teril over-the-needle cannula (Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden) with the aid of a dissection microscope. The cannula was fixed with 4-0 silk suture. The ovary was then gently flushed by hand with a 1 ml syringe with a total volume of about 2 ml cold (4°C) Ringer Acetate supplemented with 50 IU/ml heparin (Leo Pharma, AB, Malmö, Sweden) and 0.2 mg/ml xylocain (Astra Zeneca, Gothenburg, Sweden) until clear fluid emerged from the vein.
Freezing

The first experimental group of ovaries (FROZEN-PROH, n = 5) was then flushed with a cryoprotectant solution. Infusion bags were filled with a solution composed of 1.5 M propandiol (PROH; Fluka, Buchs, Schweiz) 0.1 M sucrose (Sigma-Aldrich) and 2% human serum albumin (Sigma-Aldrich) in Leibovitz L-15 medium (Gibco, Scotland, UK). We chose propandiol as a cryoprotectant since it is used extensively for cryoprotection during slow freezing of embryos (Mandelbaum et al., 1998) and also for the cryopreservation of ovarian tissue and whole ovaries (Fabbrti et al., 2003; Gook et al., 2004; Courbiere et al., 2005). For the group without cryoprotectant (FROZEN-RA, n = 5), ovaries were flushed with a solution containing 0.1 M sucrose and 2% HSA in Ringer Acetate. A pressure infusion device (Rudolf Riester GmbH & Co. KG, Jungingen, Germany) was used to flush with cold solution for 30 min at a pressure of around 80 mm Hg resulting in a flow rate of 1–2 ml/min. The ovary, with the cannula in place, was placed in a 60 ml autoclavable straight-side wide-mouth polypropylene jar (Nalgene Nunc, Rochester, NY, USA) with 5–7 ml of the same respective solution. The cryovial was placed in a pre-cooled (4 °C) Cryo Freezing Container (Nalgene Nunc International, Rochester, NY, USA) containing isopropanol and placed inside a −80 °C freezer according to a previously published method for the human ovary (Martinez-Madrid et al., 2004). The theoretical cooling rate in this device is around 1°C/min. After 24 h in the −80 °C freezer, the specimen was transferred and stored in liquid nitrogen until viability/functionality tests (see below).

Thawing

The storage time in liquid nitrogen varied between 1 week and 9 months. The ovaries were thawed by placement of the frozen vial in a 37 °C water bath with gentle shaking. The duration of the thawing procedure was set to 10 min, and the cryovials with the ovaries were then removed from the water bath. Ovaries were judged to be thawed at this stage based on transparency and normal texture. A small (5 × 6 × 8 mm) wedge biopsy, on the opposite side to the hilus, was cut out with a scalpel and the live/dead assay was used to determine the percentage of viable cells (see below). The excision site was sutured with a 6-0 polypropylene suture. To remove the cryoprotectant and metabolites formed during the freezing and thawing procedure from the ovarian vasculature and tissue, the ovary was washed by flushing (flow rate 1–2 ml/min) at room temperature with a solution of 0.1 M sucrose and 2% HSA in Leibovitz L-15 medium for 10 min followed by a solution of 0.05 M sucrose and 2% HSA in Leibovitz L-15 medium for 10 min. Finally, the ovaries were flushed with 2% HSA in Leibovitz L-15 medium for 10 min before the ovary was mounted in the perfusion apparatus (see below).

In vitro perfusion and adenylate cyclase stimulation by forskolin

The FROZEN-PROH ovaries and FROZEN-RA ovaries were perfused after wash out of cryoprotectant as described above. The FRESH-RA control group (n = 4, cannulation not obtained n = 1) ovaries were perfused fresh, i.e. without having been frozen prior to perfusion. The in vitro perfusion apparatus was originally developed for rabbit ovaries (Bjersing et al., 1981) and modified for rat ovaries (Sogn et al., 1984; Brannstrom et al., 1987a, b). The system has been extensively used for the studies of ovarian physiology mostly in relation to ovulation (Brannstrom et al., 1987a, b). The perfusion medium was recirculated using a roller pump, and the pressure was continuously monitored (Fig. 1). The main parts of the perfusion apparatus were made of glass and Teflon tubing, and the system was water-jacketed to keep the temperature of the perfusion medium at 38°C.

The ovary was placed inside the sample beaker inside the perfusion chamber. The arterial catheter, which was inserted prior to freezing, was attached to the perfusion system with circulating perfusion medium. The medium consisted of M199 with Earl’s salts, 26 mmol/l NaHCO₃ and 0.68 mmol/l l-glutamine (Invitrogen, Carlsbad, CA, USA) and 2% bovine serum albumin (Roche Diagnostic GmbH, Penzberg, Germany) and was continuously equilibrated with 5% CO₂ in 95% O₂. The perfusion pressure was adjusted and maintained at 60–100 mm Hg, and the flow rate was monitored throughout the perfusion period. Average flow rate was 1–4 ml/min.

In the experiments, the ovary was perfused for 1 h before the addition of 30 μmol/l of forskolin (7ß-acetoxy-8,13-epoxy-1α,6ß,9α-trihydroxylabd-14en-11-one; Sigma-Aldrich), which is an adenylate cyclase stimulator.

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**Figure 1** Schematic drawing (a) and photograph (b) of perfusion set-up. 1, perfusion chamber; 2, bubble trap; 3, manometer; 4, roller pump; 5, outflow connector; 6, oxygenator reservoir; 7, gas humidifier; 8, sampling syringe; 9, shunt valve.
Forskolin acts by non-receptor-mediated (non-hormonal), direct stimulation of adenylate cyclase resulting in an increase in intracellular concentration of cyclic adenosine 3′,5′-monophosphate (cAMP). It has previously been used at the same concentration as in the present study, to induce ovulation in vitro perfusion of rabbit (Holmes et al., 1986) and rat (Brannstrom et al., 1987a, b) ovaries leading to a marked increase of cAMP levels in the perfusion medium. Samples of the perfusion medium were taken every 30 min and frozen for subsequent analysis of cAMP, progesterone and estradiol. The experiment was terminated 60 min after forskolin addition, and a second tissue wedge sample (~4 × 4 × 4 mm) was taken from the ovary for viability assessment using the 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). One out of the five FRESH-RA ovaries could not be adequately perfused due to mechanical disruption at the site of ovarian artery cannulation.

Histology
Biopsies taken before and after perfusion were fixed in formaldehyde, embedded in paraffin, sectioned and stained with the Van Gieson standard morphology staining method. Light microscopy examination of ovarian biopsies was performed independently by two persons blinded to the experimental data.

Cell culture
For cell culture, the tissue was prepared according to a previously described method in our laboratory using collagenase as the major enzyme to digest ovarian tissue (Runesson et al., 2000). The ovarian sample, consisting of both cortical and medullar tissue taken at a site with no protruding large follicle or corpus luteum, was cut with scissors into 1–2 mm pieces and then digested in a solution of 5 ml phosphate-buffered saline (PBS) supplemented with 2.5 mg/ml collagenase type IA (Sigma-Aldrich) and 100 µg/ml DNAse I (Roche Diagnostic GmbH) in a sterile container. The container was placed in a 37°C water bath with agitation for 60 min. The digestion was terminated by the addition of 0.5 ml fetal calf serum (FCS, Gibco, Invitrogen, Paisley, UK) and 5 ml cold PBS. The cell suspension was passed through a 100 µm mesh cell strainer ( Falcon, BD Biosciences, Franklin Lakes, NJ, USA) and centrifuged at 200g for 5 min. The pellet was washed twice in RPMI cell culture medium (Gibco), with 10% FCS. Viability of the cells was assessed using Trypan Blue exclusion, and 5 × 10^4–10 × 10^5 live cells per well were plated in a 24-well cell culture plate (Falcon). The cells were left for 24 h to attach to the plastic surface. After rinsing the cells, hCG (10, 100 or 1000 IU/ml) was added to the medium. The cells were left for 20–24 h after which the cell culture supernatant was collected and frozen for subsequent analysis of progesterone and estradiol. Two out of five cell culture preparations of FROZen-RA ovaries were excluded since insufficient numbers of cells were obtained after collagenase digestion or because too few cells adhered to the cell culture wells.

Viability assay (live/dead assay)
To evaluate the proportion of cells surviving the freeze–thaw procedure, the live/dead assay (Live/Dead® viability/cytotoxicity kit; Molecular Probes, Eugene, OR, USA) was used. The ovarian sample, consisting of both cortical and medullar tissue, was cut into small pieces and washed free from propandiol or Ringer Acetate. It was then enzymatically digested or because too few cells adhered to the cell culture wells. DNAse I (Roche Diagnostic GmbH) in a sterile container. The container was placed in a 37°C water bath with agitation for 60 min. The digestion was terminated by the addition of 0.5 ml fetal calf serum (FCS, Gibco, Invitrogen, Paisley, UK) and 5 ml cold PBS. The cell suspension was passed through a 100 µm mesh cell strainer (Falcon) and centrifuged at 200g. The pellet, resuspended in 100 µl PBS, was incubated for 30 min at 37°C with 100 µl of live/dead reagent at a concentration of 4 µmol/l calcein AM 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 was counted under UV light in a fluorescence microscope, and the proportion of live (green) and dead (red) cells was calculated independently by two observers. The inter-observer variance was <10%, and the mean of these was taken as a data point.

Steroid assays
Progesterone and estradiol concentrations in the perfusion medium were analysed by an immunofluorometric method (DELFAIA, Wallace OY, Turku, Finland) modified for cell culture medium (Bengtsson et al., 1995). The average inter- and intra-assay coefficient of variations for progesterone was 6.9 and 13.0%, respectively, and for estradiol 6.6 and 4.1%, respectively.

cAMP assay
The cyclic AMP content of the perfusion medium was analysed using a modified DELFAIA microtitre plate method for cells and tissue, and a high sensitivity acetylation protocol. The assay was performed according to the instructions by the supplier but with some minor modifications. 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 finally prepared acetylated volume. The acetylation was achieved by adding 5 µl acetylation reagent to 180 µl of perfusion medium sample or 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. Aliquots of 50 µl of the total 212 µl of sample and standards were added in duplicate to the microtitre plate. The inter-assay variations were 8.5% for 87.8 fmol (n = 10), 9.6% for 9.4 fmol (n = 9) and 12.2% for 3.3 fmol (n = 10) cAMP. The intra-assay variations at these concentrations were 3.8, 8.6 and 14.4%, respectively (n = 10). The detection limit of the assay was 46 pmol/l.

Statistics
The data was statistically evaluated using Kruskal–Wallis ANOVA (for independent groups) or Friedman’s two-way ANOVA (for dependent groups). Pair wise comparisons were made by Mann–Whitney U-test or Wilcoxon’s matched pairs test.

Results
Histology
Light microscopy examination of ovaries showed the presence of primordial, primary, secondary and pre-antral follicles with normal morphology in all groups (Fig. 2). Healthy looking antral follicles were only seen in the FRESH-RA group (Fig. 2a and b). Moderate oedema was commonly seen in the biopsies taken from the frozen–thawed ovaries after in vitro perfusion (Fig. 2d and f), which was not detected in biopsies taken before in vitro perfusion (Fig. 2c and e). In biopsies taken from frozen–thawed ovaries, the stromal cells in many areas were widely separated after perfusion, suggesting disrupted intracellular connections (Fig. 2d and f). Examination at higher magnification showed that the small follicles of FRESH-RA and FROZen-PROH groups appeared healthy both before and after perfusion (Fig. 3a–d). However, these small follicles of the FROZen-RA group became shrunk after perfusion (Fig. 3f) although they appeared normal before perfusion (Fig. 3e).
In vitro perfusion

Ovaries were pre-perfused for 60 min before the addition of adenylyl cyclase stimulator forskolin and were then perfused for a further 60 min giving a total perfusion period of 120 min. The ovaries of the FRESH-RA group responded to forskolin with a marked increase of cAMP production, detected as a 25- to 45-fold increase in the cAMP levels in the perfusion media at 90 and 120 min, when compared with basal levels prior to forskolin addition (Fig. 4). Cyclic AMP levels in the perfusion media of the FROZEN groups of ovaries showed a continuous increase (Fig. 4, inset) but without any further increase after forskolin addition. There were no statistically significant differences in cAMP levels between FROZEN-PROH and FROZEN-RA, although FROZEN-PROH showed a tendency to higher cAMP levels at time points 30–120 min. The concentrations of both progesterone (Fig. 5) and estradiol (Fig. 6) were measured in the perfusion media. All ovaries were primed to be in follicular phase but judging from the levels of progesterone in the medium it was apparent that three ovaries (one in each group) contained a newly formed progesterone-producing corpus luteum. Thus, the three ovaries whose perfusion gave rise to progesterone levels >1.2 nmol/l were excluded from the analysis of progesterone data.

The progesterone and estradiol levels were markedly higher in the FRESH-RA than in the two FROZEN groups (Figs 5 and 6). There were no differences in progesterone and estradiol levels at any time points when comparing FROZEN-PROH and FROZEN-RA.

Cell culture

Ovarian cells were cultured for 24 h with different concentrations of hCG (10, 100 and 1000 IU/l) after a 24 h pre-culture period.
The levels of estradiol in the medium were low, and no differences were seen in the levels between the groups at any hCG concentration or between the control (no hCG) and any concentration of hCG within groups (data not shown). Progesterone levels in the cell culture medium were significantly higher in the cultures of FRESH-RA ovarian cells when compared with cultures of ovarian cells of FROZEN groups at 0–1000 IU/l hCG. Progesterone levels were significantly higher in cell culture media of FROZEN-PROH at 10 and 100 IU/l hCG when compared with FROZEN-RA (Fig. 7).

**Discussion**

One way to achieve a better survival of primordial follicles after ovarian tissue cryopreservation and thawing is whole ovary cryopreservation and autotransplantation with vascular anastomosis after thawing. An improved survival rate of these follicles would then be due to decreased ischaemic injury since blood flow in the ovarian tissue would occur shortly after autotransplantation and not rely on the slow process of neoangiogenesis. Thus, several days of warm ischaemia would be avoided by the immediate connection to the circulation of the recipient.

**Live/dead assay**

The percentages of live cells were between 60 and 75% in all groups. The assay did not demonstrate any significant differences between the groups, either before or after perfusion. Although a slightly lower percentage of live cells were detected in FROZEN-RA cell suspensions, when compared with FROZEN-PROH and FRESH-RA cells, this difference was not statistically significant (Fig. 8).
Whole ovary cryopreservation followed by successful autotransplantation by vascular anastomosis has been performed in experimental animals such as the rat (Yin et al., 2003), the rabbit (Chen et al., 2006) and the sheep (Revel et al., 2004). Moreover, fertility in these animal models, with delivery of offspring, was demonstrated in the rat (Wang et al., 2002) and the ewe (Imhof et al., 2006). The results of these two studies are promising although the pregnancy rates were low with one out of nine ewes pregnant and one out of seven rats pregnant. It should be pointed out that two other studies in the sheep, using a vitrification technique, were not able to achieve pregnancy (Bedaiwy and Falcone, 2007; Courbiere et al., 2008). The difficulties in establishing a patent vascular anastomosis for ovaries with long-term functionality are illustrated in a study in the sheep where different techniques were compared (Bedaiwy and
Thus, whole ovary cryopreservation and autotransplantation by vascular anastomosis is still a highly experimental procedure with very low success rates in animal models. Several technical aspects of this procedure should be optimized in research on experimental animals before the procedure can be of clinical significance.

The objective of the present study was to evaluate different methodologies that may be used to develop better cryopreservation protocols for such a large organ as the ovary. We have used the sheep ovary model, since this is the most widely used animal model of whole ovary cryopreservation and is of comparable size to the human ovary (Bedaiwy et al., 2003; Revel et al., 2004; Arav et al., 2005; Courbiere et al., 2005, 2006, 2008; Imhof et al., 2006; Baudot et al., 2007; Bedaiwy and Falcone, 2007; Onions et al., 2008). The in vitro methods that have been used for the evaluation of viability of cryopreserved whole ovaries are conventional light microscopy (Bedaiwy et al., 2003, 2006; Martinez-Madrid et al., 2004; Arav et al., 2005; Courbiere et al., 2005, 2006; Imhof et al., 2006; Baudot et al., 2007), transmission electron microscopy (Martinez-Madrid et al., 2007), immunohistochemistry for vascular and follicular proteins (Arav et al., 2005; Bedaiwy et al., 2006), viability assessment by trypan blue exclusion or live/dead assay (Bedaiwy et al., 2003, 2006; Martinez-Madrid et al., 2004; Arav et al., 2005; Courbiere et al., 2005, 2006; Imhof et al., 2006; Baudot et al., 2007), assays of apoptotic markers (Bedaiwy et al., 2003, 2006; Martinez-Madrid et al., 2007) and the cell-cycle marker Ki67 as well as bromodeoxyuridine uptake into DNA (Onions et al., 2008). In the present study, van Gieson staining was used to evaluate whether there were any apparent morphological differences between the groups. We could not detect any major differences between the FRESH and the FROZEN-PROH groups before perfusion when evaluating morphology of the smaller follicles and the ovarian stroma. However, no antral follicles were seen in the FROZEN-PROH group. The stroma of frozen–thawed ovaries (FROZEN-PROH and FROZEN-RA) exhibited oedema after perfusion. Notably, the primordial/primary follicles of the FROZEN-RA group showed shrinkage after perfusion and this was not seen in ovaries of FROZEN-PROH. Collectively, these data may indicate that the vasculature of the frozen ovaries exhibits increased permeability and that presence of the cryoprotectant PROH is beneficial for the viability of small follicles compared with when no cryoprotectant is used. The lack of major histological differences between FRESH and FROZEN-PROH ovarian tissue before perfusion is in line with the results of light microscopy evaluation of whole sheep (Arav et al., 2005) and human ovaries (Martinez-Madrid et al., 2004) cryopreserved using a slow freezing method. In a detailed ultra-morphology study, utilizing transmission electron microscopy more than 95% of primordial/primary follicles and endothelial cells appeared healthy looking (Martinez-Madrid et al., 2007) although possible differences compared with fresh ovaries were not evaluated.

A difference may exist between slow freezing and vitrification protocols, since vitrified whole sheep ovaries in contrast to slow frozen ovaries contained substantially reduced numbers of primordial follicles and blood vessels with some degree of damage (Courbiere et al., 2005, 2006; Baudot et al., 2007).

The absence of structural differences in ovarian tissue before and after cryopreservation should be interpreted in the light of the in vitro perfusions results of our study. The in vitro ovarian perfusion method has been extensively used to evaluate the function of the ovary. The first attempts to perfuse ovaries in vitro to study metabolism and morphology were performed about 80 years ago (Carrel and Lindbergh, 1935). Later this method was employed mostly for studies of steroidogenesis in the ovary (Werthessen et al., 1953; Romanoff and Pincus, 1962). Ovulations induced in vitro were first reported in the isolated perfused human ovary (Stahler et al., 1974). The perfusion system was adapted by us for the rabbit ovary (Janson et al., 1982) for the purpose of studying morphological changes at the apex of the follicle and also the local ovarian biochemical events involved in ovulation. The surgical technique of isolating the rabbit ovary was later modified for isolating the ovary from the immature PMSG-primed rat (Koos et al., 1984; Brannstrom et al., 1987a, b) and mouse (Brannstrom and Flaherty, 1995). An advantage of this in vitro perfusion method compared with in vivo methods is that the ovary is available both for observation and treatment with various agents and for sampling of ovarian products in the medium for a relatively extended time period. In the present study, the adenylate cyclase stimulator forskolin was used to evaluate the responsiveness of the tissue. The diterpene forskolin is a potent non-receptor/specific activator of adenylate cyclase in a variety of mammalian tissues (Seamon et al., 1983) and exerts its action in a rapid and reversible fashion. Our group has previously utilized forskolin to induce ovarian cAMP response and ovulations in perfusions of rabbit (Holmes et al., 1986) and rat ovaries (Brannstrom et al., 1987a, b).

The results of the present study showed that the cAMP response to forskolin differed considerably between the FRESH and FROZEN groups of ovaries. The response to forskolin in the FRESH group showed a similar time course as previously observed in perfusions of fresh pre-ovulatory rat (Brannstrom et al., 1987a, b) and rabbit ovaries (Holmes et al., 1986). We had predicted that the cAMP response in the FROZEN-PROH group would be similar or only slightly decreased compared with the FRESH group. The observed big difference indicates that important cell membrane-related functions are damaged by the cryopreservation procedure, which is also

**Figure 8** Percentage of live cells as determined by live/dead assay of ovarian cells taken before perfusion (before) and after perfusion (after).
illustrated by the moderate oedema and separation of stromal cells. Another explanation could be that it takes more than the 2 h of perfusion to restore normal function. Nevertheless, the slight cAMP increase in FROZEN-PROH when compared with the FROZEN-RA indicates that the presence of a cryoprotectant in the cryopreservation solution is beneficial. The continuous increase in cAMP concentration in FROZEN groups could of course reflect the release of cAMP due to cell lysis but the concentrations in the perfusion medium were not higher in FROZEN groups than the FRESH group during the 60 min before the addition of forskolin, a fact that indicates that the production of cAMP was not due to cell lysis. The accumulation of estradiol and progesterone in the perfusion media showed a pattern similar to cAMP with regard to differences between FRESH and FROZEN groups. The present study showed healthy antral follicles only in fresh ovaries. This finding corresponds to the finding of high estradiol and progesterone in cultures at 10 and 100 IU/l hCG from cells obtained from ovaries that had been frozen with PROH compared with culture of a single ovarian cell type (Arav et al., 2005). Whole sheep ovaries that were cryopreserved with vitrification after perfusion of the tissue with cryoprotective solutions VS1 and VS4 had considerably decreased follicular viability after cryopreservation, as evaluated by trypan blue exclusion test (Courbiere et al., 2005). The lack of a difference in results of live/dead assay between fresh and frozen tissue in the ewe after slow freezing as seen in the present study and in a previous study (Arav et al., 2005) is not in line with the finding in human ovaries (Martinez-Madrid et al., 2004), and this indicates that species differences may exist. A recent report suggests that the number of cells positive for the cell-cycle marker Ki67 may be useful as an indicator of viability after cryopreservation of ovarian tissue (Oions et al., 2008).

In conclusion, this study indicates that, after cryopreservation of whole ovaries with PROH as cryoprotectant, ovarian function is impaired after thawing. There are a number of methods to assess the effects of cryopreservation, which differ in their capability to detect signs of impaired ovarian function/viability.

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