The human postmenopausal ovary as a tool for evaluation of cryopreservation protocols towards whole ovary cryopreservation

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Abstract

Purpose Cryopreservation of a complete ovary may be a future method for fertility preservation in cancer patients. Difficulties exist in cryopreservation of the relatively large ovarian tissue mass. This study evaluates whether a human postmenopausal ovary can be used, as a complement to animal models, in studies of this research field.

Methods Postmenopausal human ovaries ($n=10$) were isolated and flushed through ovarian arteries with either the cryoprotectant dimethylsulphoxide or Ringer-Acetate, followed by slow freezing. After thawing, production of androgens during in vitro perfusion and morphology (light/electron microscopy) were assessed.

Capsule The study evaluates the use of human postmenopausal ovaries for ovarian cryopreservation research.

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Results The dimethylsulphoxide-cryopreserved ovaries showed larger secretion of androgens during perfusion than Ringer Acetate-cryopreserved ovaries. Light microscopy showed well preserved morphology in both groups. Electron microscopy revealed normal appearance of stroma and vessels in the dimethylsulphoxide group.

Conclusions The study demonstrates the potential to use the postmenopausal human ovary for further studies aiming at optimizing cryopreservation protocols, with special reference to ovarian vascularity and stroma.

Keywords Androgens · Cryopreservation · Morphology · Ovary · Perfusion

Introduction

The emerging clinical field of oncofertility aims at preserving/restoring fertility in women undergoing treatment for malignancies. Today, the only established option to preserve fertility in female cancer victims of reproductive age is IVF and embryo cryopreservation [1], which however only is possible in a partner relationship with a committed partner. In adult women of fertile age and with no partners the options for fertility preservation are cryopreservation of mature unfertilized oocytes [2] or ovarian cortex cryopreservation. This latter method is today the only alternative for fertility preservation among prepubertal girls and the retrieval part of this technique has been practised for at least a decade by several centres. The first pregnancy and live birth after ovarian cryopreservation followed by avascular orthotopic retransplantation was reported 6 years ago, and involved a patient treated for Hodgkin's lymphoma [3]. The efficiency of ovarian cortex

cryopreservation, in terms of longevity of the transplant and pregnancy, is rather low [4], mostly due to ischemic damage with loss of large amounts of primordial follicles during the time until the transplanted tissue has established new blood flow through neoangiogenesis [5].

As a possible alternative, cryobanking of an intact whole ovary with its vascular pedicle and later followed by autotransplantation with vascular anastomosis, has been brought forward [6]. This technique would theoretically avoid ischemic injuries since the transplant would be connected to the blood circulation after thawing and vascular anastomosis.

The microsurgical vascular anastomoses of the human ovarian arteries and veins is achievable as illustrated by successful heterotopic auto-transplantation [7, 8] and orthotopic transplantation between monozygotic twins [9]. A live birth was reported by the latter method [9]. The main challenge concerning human whole ovary cryopreservation is to overcome the complex cryobiological properties of the large ovarian tissue. Until today, no transplantations of a cryopreserved human ovary has been performed, although programs of cryobanking of whole human ovaries have been established [10].

It is clear that a large amount of additional research, both in animal models and in appropriate human ovarian tissue models, is needed before whole human ovary cryopreservation could be introduced as an alternative to ovarian cortex cryopreservation. This research on the human ovary has to deal only with ovarian tissue that is removed as part of the clinical routine of surgery. Postmenopausal ovaries may thus be a source to study aspects of viability of vascularity and stroma, since they are often removed during hysterectomy for benign uterine disease as a prophylaxis against future development of ovarian cancer or to avoid additional surgery for benign ovarian cysts [11].

There are to date only three reports on cryopreservation of human ovaries, with assessment of function in vitro after thawing [6, 12, 13]. The present study evaluates whether a postmenopausal ovary can be used in future experimental studies to optimize the procedure of whole ovary cryopreservation.

Material and methods

Patients

The study was approved by The National Committee for Medical and Health Research Ethics, Oslo, Norway. A total of 10 ovaries were harvested from 5 postmenopausal women undergoing hysterectomy for benign uterine disease (Table 1). The decision to perform bilateral oophorectomy had been taken before written and oral information about

the study was given to the patient. Written consent was obtained from all patients. Only clearly postmenopausal (>2 years after last menstrual period) women with no previous ovarian surgery and macroscopically normal ovaries were included.

Surgery and ovarian artery cannulation

The surgical hysterectomy procedures of the study population were abdominal total hysterectomy ($n=3$), laparoscopic subtotal hysterectomy ($n=1$) or vaginal hysterectomy ($n=1$). During abdominal hysterectomy, an early procedure was bilateral dissection of the infundibulopelvic ligaments to mobilize them from the pelvic sidewalls. The peritoneal layer over infundibulopelvic ligaments was removed and the ovarian artery was isolated from the larger ovarian veins (typically 2–3 in each ligament) and dissected free for a distance of at least 5 cm from the ovarian hilus. The tubo-ovarian and utero-ovarian vessels were ligated by 2–0 sutures and this was followed by clamping and severance of the proximal part of the infundibulopelvic ligament. The ovary, with its vascular pedicle, was then removed and handed to the lab staff.

In the case of laparoscopic subtotal hysterectomy ovarian retrieval was performed according to the method recently described [14], using vascular clips on the infundibulopelvic ligament, utero-ovarian ligament and Fallopian tube. When vaginal and laparoscopic subtotal hysterectomy with bilateral salpingo-oophorectomy was performed, the Fallopian tubes were removed from the ovary on the backtable after ligation of tubo-ovarian vessels.

The ovary, with its vascular pedicle, was placed on ice slush of Ringer-Acetate (RA; Braun Melsungen AG, Melsungen, Germany) to trim the arterial end of the vascular pedicle and to cannulate the ovarian artery by a 22 G Teflon cannula (Becton Dickinson Therapy AB, Helsingborg, Sweden). This procedure was aided by the use of surgical loupes (magnification $\times 3.5$). The cannula was fixed to the ovarian artery (Fig. 1) by two 6-0 sutures (Polysorb®, Covidien Norway AS, Asker, Norway). The time from ovarian retrieval until cannulation varied between 10 and 80 min (Table 2) and cannulation could not be obtained in all ovaries. After cannulation, the ovary was gently flushed by hand via the ovarian artery with 2 ml RA supplemented with heparin (50 IU/ml; Astra Zeneca AS, Oslo, Norway) and (xylocaine 0.4 mg/ml; Astra Zeneca AS, Oslo, Norway).

Cryopreservation and thawing

The ovary was then perfused ex vivo through the arterial cannula either with a cryoprotectant solution of 1.5 M dimethylsulphoxide (DMSO) and 2% human serum albu-

Table 1 Patient ages, surgical details and number of ovaries retrieved/used

Patient	Age	Diagnosis	Surgery	No. of ovaries retrieved	No. of ovaries used
1	57	myoma uteri	TAH, BSO	2	2
2	79	CIN 3	TAH, BSO	2	2
3	60	myoma uteri	LASH, BSO	2	2
4	65	CIN 3	TAH, BSO	2	1
5	63	uterine prolapse	VH, BSO	2	1

TAH total abdominal hysterectomy; BSO bilateral salpingoophorectomy; LASH laparoscopic subtotal hysterectomy; VH vaginal hysterectomy.

min in Leibovitz L-15 medium (Gibco, Invitrogen, Oslo, Norway) or RA. The solutions were kept at 4°C and flushing was performed using a pressure-infusion-device (Rudolf Riester GmbH & Co. KG, Jungingen, Germany) to flush at 80 mmHg for 30 min with a flow rate of 1–2 ml/min.

The ovary was then positioned inside 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 99% isopropanol and placed inside a –80°C freezer which resulted in a cooling rate of around 1°C/min, according to a previous study of the human ovary [12]. After 24 h in the –80°C freezer, the container with the ovary was transferred into liquid nitrogen and stored for >2 month until thawing.

The ovary was thawed by positioning the frozen vial for 10 min in a 37°C water bath, which was set to gentle shaking. A small wedge biopsy was at this stage cut out with a scalpel to be used for light and electron microscopy. The wedge site was sutured with 6-0 suture (Polysorb®, Covidien Norway AS, Asker, Norway). To remove DMSO/RA and metabolites, which had formed during the freezing and thawing procedure, flushing (flow rate 1–2 ml/min) was performed stepwise with solutions of Leibovitz L-15 medium with 2% human serum albumin and sucrose at 0.1 M, followed by supplementation with 0.05 M sucrose and at last no sucrose. The ovary was then subjected to in vitro perfusion (see below).

Table 2 Time to achieve ovarian artery cannulation (min)/type of cryoprotectant used for left and right ovary of patients 1–5

Patient	Right ovary	Left ovary
1	60/DMSO	80/RA
2	16/RA	10/DMSO
3	12/RA	10/DMSO
4	10/RA	–
5	–	12/RA

DMSO dimethylsulphoxide; RA Ringer Acetate; – cannulation not achieved

In vitro perfusion

An in vitro perfusion system [15] was used to evaluate the functionality of the ovary. This is a closed circuit system (Fig. 2) where 70 ml of recirculating perfusion medium is pumped into the ovarian artery with possibility to measure ovarian metabolites that accumulate in the medium. The perfusion medium consisted of M199 with Earl's salts, 26 mM NaHCO₃, 0.68 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) plus 2% bovine serum albumin (Roche Diagnostic GmbH, Penzberg, Germany) and was continuously equilibrated with 5% CO₂ in 95% O₂. The perfusion pressure was maintained at between 40 and 90 mmHg to acquire a flow rate through the ovary of around 2.5 ml/min. Samples of the perfusion medium were taken at the beginning of perfusion procedure and then every 30 min during the perfusion duration of 4 h for later analysis of testosterone and androstenedione concentrations.

Steroid assays

Androstenedione was analysed by an ELISA method (DRG International Inc., Mountainside, NJ USA) and testosterone was assayed by an immunofluorometric method (DELFLIA, Wallac OY, Turku, Finland). The androstenedione-assay had a detection limit of 0.019 ng/mL. The intraassay-CVs were 9% at 0.3 ng/mL, 5.6% at 2.6 ng/mL and 4.7% at 4.7 ng/mL. The interassay-CVs were 9.6% at 0.2 ng/mL, 12.1% at 2.3 ng/mL and 8.8% at 4.4 ng/mL. The testosterone-assay had a detection limit of 0.3 nmol/L with intraassay CVs of <6% at 1.21, 10.2 and 27.6 nmol/L. Interassay-CVs were 14.2% at 1.21 nmol/L, 8.7% at 10.2 nmol/L and 5.6% at 27.6 nmol/L.

Microscopy

For light microscopy analysis, biopsies taken before and after perfusion were fixed in 4% phosphate buffered formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Examinations of all samples of ovarian biopsies by light microscopy were performed independently by two persons blinded to the experimental data. The small biopsies (2×2×2 mm), to be used for electron microscopy, were fixed

Fig. 1 Photograph of a human ovary during cannulation of ovarian artery ex vivo

in 3% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer. They were then postfixed in 1% osmium tetroxide, dehydrated and embedded in epoxy resin. Three non-consecutive ultrathin sections contrasted with uranyl acetate and lead citrate were studied in an electron microscope.

Results

Outcome of cannulation

Two out of the 10 retrieved ovaries could not be cannulated due to inability to achieve an intraluminal position of the catheter tip (Table 2). The contralateral ovary of the patients with ovaries that resulted in the two unsuccessful cannulations exhibited a short cannulation time. Eight ovaries were successfully cannulated and the duration for the cannulation procedure varied between 10 and 80 min (Table 2), but with the long cannulation times (60 and 80 min) occurring in the first patient in this series.

In vitro perfusion

A total of seven out of the eight cryopreserved ovaries were subjected to in vitro perfusion, since one ovary was inadvertently lost during storage in liquid nitrogen. The restricted number of ovaries used in this feasibility study did not allow for statistical comparisons. The DMSO-preserved ovaries of patient 1 and 2 had the contralateral RA-preserved ovaries as controls and the DMSO-preserved ovary of patient 3 was compared to RA-preserved ovaries of patient 4 and 5 (Fig. 3). The testosterone secretion into the perfusion medium was greater in the 3 DMSO-preserved ovaries as compared to the RA-preserved controls (Fig. 3). In the RA-preserved ovary of patient 1, very little testosterone was

secreted albeit the androstenedione secretion was comparable to the DMSO-preserved ovary. The DMSO-preserved ovaries of patient 2 and 3 showed a slightly larger secretion rate of androstenedione than the RA-controls (Fig. 3).

Morphology

All investigated ovaries showed morphology representative of postmenopausal ovaries. The tissue was dominated by stromal tissue containing spindle shaped cells which were surrounded by dense collagen bundles (Fig. 4a, b). In the cortical area, occasional inclusion cysts were found (Fig. 4c) and in the medulla corpora albicantia were seen in most specimens. Primordial follicles, corpora lutea and atretic follicles were not seen. Electron microscopy confirmed the observation. All specimens were well preserved when examined by electron microscopy and no necrotic areas were seen. There was no major difference between ovaries of the two different storage media (Fig. 5a) but the ovaries frozen in DMSO tended to show a slightly better, “crisper” morphology (Fig. 5b). When blood vessels were found in the electron microscopical specimens, they had a normal morphology (Fig. 5c).

Discussion

Cryopreservation of ovarian cortex tissue is today a clinical procedure in fertility preservation and whole ovary cryo-

Fig. 2 Schematic drawing of the in vitro perfusion apparatus

Fig. 3 Androstenedione and testosterone production during in vitro perfusion. A1-A3/T1-T3 refer to androstenedione/testosterone secretion from ovaries cryopreserved with either DMSO (dimethylsulphoxide; *filled squares*) or RA (Ringer Acetate; *filled circles*) of patients.

A1 and T1 represent patient number one; A2 and T2 patient number two, A3 and T3 patient number three for DMSO and patient number four and five for RA

preservation is procedure still at its experimental stage. Although several animal models for whole ovary cryopreservation and auto-transplantation exist they are not fully appropriate to the clinical situation of a human ovary, mostly because of that the size of ovaries from the rodent and domestic animal models are considerably smaller in size than the human ovary. Thus, the tissue mass of a an ovary of the rat is around 1/1000 (our unpublished data) of the human ovary and the sheep ovary is about 1/6 of a human ovary [16]. Nevertheless, the sheep is the animal model which has been used most extensively in studies of whole ovary cryopreservation, most likely due to the

large experience of this animal model in reproductive physiology research, the similarity of ovarian tissue architecture as compared to the human, and since the size of the ovarian arteries of the sheep allows for surgical anastomosis [17]. However, it is also important to test principles of whole ovary cryopreservation in the human in order to optimize the procedure before clinical introduction. The scarcity of premenopausal ovaries for experimental purpose is natural and is also reflected by that the accumulated published research on human whole ovary cryopreservation only includes ovaries from 5 patients [6, 12, 13].

Fig. 4 Light microscopy of postmenopausal human ovaries cryopreserved with Ringer Acetate (**a**) or dimethylsulphoxide (**b** and **c**). The ovarian tissue appeared well preserved and the lumina of blood vessels were devoid of blood cells. The ovarian tissue was dominated

by a stroma composed of spindle shaped cells surrounded by abundant collagen bundles. Occasional surface epithelium inclusion cysts were found (**c**). The arrow indicates the ovarian surface

The present study represents the first study that has explored the possibility to use the human postmenopausal ovary as an experimental tissue aiming at assessing the viability after ovarian cryopreservation. A whole ovary cryopreservation procedure of a premenopausal ovary is of course aimed at preserving the pool of small follicles, but it is also important that the vasculature and the stroma of the ovary is preserved to enable the ovary to regain functionality after transplantation. Thus, in the experimental situation of the present study with postmenopausal ovaries depleted of follicles, only functionalities of the stromal and vascular components were evaluated.

In the present study the procedure of ovarian artery cannulation was associated with unexpected difficulties, as illustrated by a cannulation time of over 1 h in the first patient and that the ovarian arteries of two ovaries could not be cannulated properly, although the cannulation procedure had been practiced in 4 patients before the study. We did not specifically measure the width of the arterial lumen in our patients but noted great variations. The diameter of the ovarian artery lumen in premenopausal women is stated to be 0.5–0.8 mm [9] and it is

quite likely that the ovarian artery of a postmenopausal ovary is of smaller diameter, considering the decrease in ovarian blood flow after menopause. The backtable procedure, to trim the ends of the blood vessels and cannulation, would of course be aided by use of a dissection microscope, in line with our experience to cannulate the small common iliac arteries of the rat during flushing a uterine graft [18]. In our experimental approach we did not have to dissect the ovarian veins, which usually are 2–3 in numbers. It should be emphasized that careful cannulation, to avoid damage to the blood vessel walls, is important in organ cryopreservation since the blood vessels seem to be especially vulnerable to the freezing procedure [19].

In previous studies on the cryopreserved whole sheep ovary, it was shown that, in spite of proven long-term vascular patency, a majority of ovaries did not resume cyclicity [20]. That finding further points to the necessity to improve the cryopreservation procedure, since a large follicle loss was seen despite immediate and long-term blood flow.

To assess the viability of an organ that is cryopreserved as an intact tissue, it is important to test the functionality of

Fig. 5 Electron microscopy of ovaries cryopreserved with Ringer Acetate (**a**) or dimethylsulphoxide (**b** and **c**). No obvious preservation damage was seen but the tissue cryopreserved in dimethylsulphoxide (**b**) showed a slightly better morphology, suggesting better preservation.

Spindle-shaped stromal cells containing well-preserved nuclei and organelles were surrounded by extracellular collagen. Occasional capillaries with normal morphology were observed (**c**). *cap* capillary lumen; *e* endothelial cell

the whole organ after thawing. The natural test of functionality of a cryopreserved ovary is fertility after transplantation, as has been achieved in a minority of attempts with cryopreserved rat [21] and sheep [22] ovaries. The limited number of studies concerning cryopreserved whole human ovaries have used morphological assessment by light and electron microscopy, live dead analysis and apoptosis tests to assess the viability of the ovary after thawing. In the present study we used an ovarian in vitro perfusion system, which was developed in our laboratory 40 years ago [23]. This in vitro methodology represents an experimental situation which is close to an in vivo situation, with preserved ovarian tissue architecture and intravascular flow. Although the ovarian in vitro system was developed for the rabbit [23] and rat ovary [15], it has been adapted to the human ovary [24], with proven unaltered histological appearance and absence of oedema after short term perfusions of freshly isolated human ovaries.

In the present study we evaluated the secretion of the two major ovarian-derived androgens, androstenedione and testosterone during in vitro perfusion. It was found that concentrations of androstenedione and testosterone in perfusion media increased during the perfusion time. The postmenopausal ovary is composed mainly of stromal cells [25], with a portion of these cells being steroidogenically active [26] to produce androgens, although with large inter-individual variations [27]. In the present initial feasibility study, the sample size was too small to be able to statistically compare the androgen secretion from DMSO- and RA-frozen ovaries. However, the androgen production from DMSO-preserved ovaries was higher from all individual ovaries indicating the benefits of DMSO-preservation also for the human ovary, as we have previously shown for the cryopreserved whole sheep ovary [28]. In that study, we showed satisfactory equilibration using the uncontrolled slow freezing protocol method as also applied in the present study. It should be pointed out that the results of androgen production from these postmenopausal ovaries may not correspond to the follicular steroid production from cryopreserved human premenopausal ovaries, since it is known that the larger androgen-producing follicles have a high spontaneous rate of atresia after cryopreservation.

This paper reports detailed morphological evaluation of the human ovary after cryopreservation. Light microscopy confirmed the postmenopausal state of the ovaries and additionally that blood vessels were devoid of blood cells, and thereby properly flushed. Earlier studies in this field have shown that light microscopy is not a method that can detect tissue damage after cryopreservation and thawing [12], while electron microscopy would visualize intracellular degenerative changes. The electron microscopy evaluation of

the present study showed preserved ultramorphology, with signs of a better morphology of the DMSO-preserved ovaries. Importantly, the endothelial cells of the microvasculature and the stromal cells showed no signs of decreased cellular viability.

We applied an identical DMSO-cryoprotocol as described in our previous report on the sheep ovary [28] and in the first report on whole ovary cryopreservation in the human [12]. In the latter study, performed on premenopausal ovaries, follicular viability was about 75% and there was a normal histological appearance when examined by light microscopy. In a follow-up study, transmission electron microscopy demonstrated absence of ultrastructural alterations or apoptosis in cryopreserved intact human ovaries [13]. Another report on cryopreservation of human ovaries from two patients (44- and 46-year old), reported follicular viability of around 75% and no induction of apoptosis [6]. It may well be that the stromal cells are more sensitive to cryopreservation than the small follicles, as suggested by results of a study of cryopreserved bovine [29] and human ovarian cortex [30], using slow freezing protocol.

In conclusion, the present study points towards that the human postmenopausal ovary can be used for future studies aiming at evaluating and optimizing cryopreservation and thawing protocols for the human ovary towards introduction of a novel method for fertility preservation in young female cancer patients. This human postmenopausal ovary method would aim at studies of cryopreservation influences on the ovarian vascularity and the ovarian stroma.

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