Cryopreservation of a Whole Ovary As a Strategy for Restoring Ovarian Function

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Submitted March 4, 2004; accepted August 30, 2004

Purpose: To evaluate whether a total ovary can successfully be cryopreserved and thawed under maintenance of high proportions of structurally normal primordial follicles.

Methods: Porcine ovaries were explanted immediately after slaughter. Ten ovaries were cooled to -196°C using cryoprotective solution and a computer-controlled freezing technique. Five contralateral ovaries were fixed with formalin for histological examination, the five remaining ovaries were directly plunged in liquid nitrogen. After three weeks of storage, frozen ovaries were thawed and examined by light and electron microscopy. Viability was assessed by evaluation of intact primordial follicles with respect to cellular and intracellular structures.

Results: Histological viability of primordial follicles in computer-frozen ovaries was 84.4% (76.1–90.6%), as compared to non-frozen control samples (92–100%; mean 97.6%) and to ovaries plunged in liquid nitrogen without cryoprotection 21.1% (4.5–35.0%).

Conclusions: Cryopreservation of whole ovaries may present a feasible way to maintain high numbers of viable primordial follicles in ovarian tissue retransplantation.

KEY WORDS: Cryopreservation; fertility; fertility preservation; ovarian tissue banking; whole ovary.

INTRODUCTION

Over recent years, improvements in the treatment of cancer have lead to significant increases in long-term survival rates, especially in patients of younger ages. However, in the treatment of women aggressive chemotherapy and radiotherapy can severely deplete the follicular store, often compromising ovarian function (1,2). Women who suffer from ovarian failure as a result of cancer treatment develop infertility and premature menopause and are at higher risk of osteoporosis and cardiovascular morbidity. As survival

rates for young patients continue to improve, protection against iatrogenic ovarian failure assumes higher priority.

Women undergoing cytotoxic chemotherapy or radiotherapy have few options for fertility protection, all of which essentially center around the retrieval and preservation of reproductive products. One possibility is the cryopreservation of embryos conceived by in vitro fertilization after oocyte retrieval after the disease is diagnosed. However, this technique is inappropriate for children and for women without a male partner, unless sperm donation is an acceptable option. Another strategy is the frozen storage of mature oocytes, which is suitable for women who do not have a male partner, but rarely proves successful (3). Moreover, the cooling process and application of cryoprotectants may disrupt the organization of the meiotic spindle, leading to chromosome anomalies (4). Cryopreservation of oocytes may also stimulate the release of cortical granules (5) and initiate parthogenetic activation (6).

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An alternative to either embryo storage or oocyte banking is available in the harvesting and cryopreservation of ovarian tissue or isolated follicles. Autografting enables the storage of large numbers of primordial follicles and preserves the structural integrity of somatic and reproductive cells within the ovary. Primordial follicles, being small, undifferentiated and less metabolically active, might be more tolerant to freeze-thawing and may even tolerate a short period of ischaemia during grafting.

Small pieces (1 mm³) or thin slices (2 mm) of ovarian tissue of rat, mouse, marmoset, sheep, and humans have already been frozen successfully. In a study by Harp *et al.*, grafts of frozen-thawed mouse ovaries have restored cyclic function in ovariectomized recipients (7). Candy *et al.* reported the development of large antral follicles in xenografts of cryopreserved marmoset ovarian slices (8). In two other reports, live young were born after orthotopic transplantation of frozen-thawed ovarian tissue from mice (9,10). Likewise, cortical grafts returned to the orthotopic site either fresh or after frozen storage have successfully restored oestrus cycles and fertility in sheep and have resulted in two pregnancies, one originating from a fresh and the other from a frozen-thawed graft (11). Salle *et al.* reported pregnancies and live births using semi-ovary autografts in sheep (12), but a follow-up study also revealed a drastic reduction in follicular population of the grafts (13). In another study in sheep by Baird *et al.*, endocrine cycles continued throughout the study period of 22 months, although elevated follicle-stimulating hormone concentrations indicated imminent ovarian failure (14).

These successes in animal models suggest autografting of ovarian tissue a promising method to postpone cancer-treatment-induced premature menopause and to allow restoration of the natural state including conception *in vivo* with the patient's own germ cells in humans. First clinical cases of "ovarian tissue banking" have been reported in the scientific literature (15,16). The procedure involves the collection of ovarian cortical slices by laparoscopy at any stage of the menstrual cycle before the start of sterilizing chemotherapy and grafting back once treatment is completed and the patient is disease-free (17). However, no human pregnancies have been achieved as yet (18).

There is evidence that in tissue autotransplantation more follicles are lost during reperfusion ischaemia than by freezing and thawing (19). In a study on the effect of cryoprotectants on the survival of follicles, ovarian human biopsies were frozen-thawed in different freezing media and xenografted under the kid-

ney capsule of mice with severe combined immunodeficiency for 18 days. Upon retrieval, follicular survival within the graft was found to range from 44 to 84% in the best cryoprotective media. Yet, approximately 26% thereof were lost as a result of grafting *per se*, which presumably reflects the acute effects of ischaemia (20). During posttransplantation ischaemia, nearly all of the developing follicles disappear as they undergo apoptosis, and primordial stages in the cortical region are also lost, resulting in curtailed reproductivity. Mice receiving fresh or frozen-thawed cryopreserved mouse ovaries had a reproductive lifespan of 6.2 and 8.4 litters, respectively, compared with 13 litters in control animals (20). In a similar study, only 10 and 11% of ovariectomized mice orthotopically autografted with either fresh or frozen-thawed tissue maintained fertility for four litters, compared to 100% of sham-operated controls (21). These results indicate that follicular loss resulting from grafting significantly shortens the functional lifespan of the graft.

Ovarian cortical slices contain only small amounts of follicles, and even if a substantial proportion of follicles survive the grafting procedure, the total follicle population will still be low, so that ovarian function may only be regained for a short time. More follicles would probably survive with vascular anastomosis, reducing the period of ischaemia to a few minutes. Therefore, the aim of this study was to evaluate whether a total porcine ovary can successfully be cryopreserved on the basis of the survival of high proportions of structurally normal primordial follicles as determined by light and electron microscopy performed immediately after thawing.

MATERIALS AND METHODS

A total of 20 ovaries were obtained from 10 freshly slaughtered pigs at a local abattoir. They were assigned to one of three groups.

In the first group of ovaries ($n = 10$), the vascular pedicle was prepared and the *A. ovarica* cannulated with a venflon (Ohmeda), which was anchored with a 2-0 suture. After flushing the vessels with ice-cold RPMI-1640 solution (Sigma Aldrich), the ovaries were trimmed free of adhering tubes, mesosalpinx, and fat. They were placed in RPMI-1640 solution and transferred on ice to the hospital within 1 h of removal. The ovaries were placed in RPMI-1640 solution containing 1.5 M dimethyl sulfoxide (DMSO; Merck, Vienna, Austria) and 10% human albumin (Centeon Pharma GmbH, Vienna, Austria) that had been precooled to 4°C. They were equilibrated for

30 min on ice in the cryopreservation medium (22), with the ovaries perfused continuously with the freezing medium. The ovaries were transferred to a programmable freezer (IceCube 1810, SY-LAB) at a starting temperature of 4°C, cooled at 2°C/min to 0°C and held at 0°C for 12 min. The temperature was then lowered by 1.5°C/min to -9°C and after 8 min at -9°C by 0.5°C/min to -40°C. Cooling was continued to -150°C at the faster rate of 10°C/min. The vials were removed and plunged into liquid nitrogen and stored at this temperature (-196°C) for 3 weeks.

The second group ($n = 5$) consisted of contralateral ovarian specimens, which were frozen by plunging in liquid nitrogen without application of a cryoprotectant.

The other contralateral ovaries ($n = 5$) were fixed immediately in 4% paraformaldehyde for subsequent histological examination and served as controls to determine the initial numbers and morphology of the primordial follicles.

Thawing of cryoprotected and nitrogen-plunged ovaries was performed by rewarming in air for 2 min before ovaries were immersed in a water bath at 25°C until the ice had thawed. The cryoprotectant was quickly removed from the tissue by washing in saline and fresh medium, and low pressure perfusion with RPMI medium for evacuation of DMSO was performed.

Light Microscopy

For histological examination, ovaries were fixed in paraformaldehyde, embedded in paraffin and cut into 5 μm serial histological sections, which were then stained with haematoxylin and eosin. Control ovaries were prepared immediately after explantation, cryopreserved and nitrogen-plunged ovaries were after rethawing. Slides were coded.

To determine the proportion of structurally normal primordial follicles after freeze-thawing, the numbers of follicles recovered from fresh and frozen-thawed tissue were evaluated. Because the density of primordial follicles in ovarian tissue varies, counts were obtained from three different sections of each sample under standardized magnification ($\times 20$) and in a standardized area (field of vision). Results were expressed as mean values.

Follicles containing unfragmented oocytes in the dictyate stage of the prophase were considered intact. Contracting and clumping of the chromatin material and wrinkling of the nuclear or follicular membrane were regarded as signs of atresia.

The morphological appearance of fresh (control group, $n = 5$) and frozen-thawed (computer-frozen group, $n = 10$; plunged group, $n = 5$) follicles was recorded.

Electron Microscopy

Pieces of ovarian tissue with a maximum dimension of 1 mm³ were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde solution in 0.1 M cacodylate buffer at pH 7.4. After several washes, they were postfixed in 1% osmium tetroxide (OsO₄) in the same buffer for 2 h and placed in uranyl acetate for 45 min. They were dehydrated in a graded ascending series of alcohol concentrations at room temperature and finally embedded in Epon and polymerized at 60°C. The blocks were thin-sectioned for locating the tissue, before ultrathin sections (70–90 nm) were cut and examined under the electron microscope. The purpose of electron microscopy was to confirm the results of light microscopy with regard to follicular viability.

RESULTS

Light Microscopy

In total, 84.4% percent (min. 76.1%, max. 90.6%) of the primordial follicles in the computer-frozen ovaries ($n = 10$) appeared histologically viable, consisting of a quiescent oocyte and a single layer of surrounding flattened pregranulosa cells. Seventy-three percent did not differ from the fresh follicles obtained from unfrozen contralateral ovaries, 11.4% showed only slight alterations of their morphological appearance, and 15.6% were considered not viable due to their characteristics. In contrast, ovaries plunged in liquid nitrogen without cryoprotection ($n = 5$) showed a mean histological primordial follicle viability of only 21.1% (min. 4.5%, max. 35%). In control ovaries viability was 97.6% (min. 92%, max. 100%). Data are given in Table I.

Electron Microscopy

To confirm these results on an ultrastructural level we examined a representative number of follicles, in particular from the group with slight or moderate morphological alterations.

The oocytes of most primordial follicles in cryoprotected ovaries contained a large spherical nucleus with finely granular chromatin and a nucleolus. The

Table I. Percentage of Viable Follicles

Animals (<i>n</i> = 10)	Frozen-thawed Ovaries ^a (<i>n</i> = 10)	Fresh ovaries ^b (<i>n</i> = 5)	Plunged ovaries ^c (<i>n</i> = 5)
1	76.1	99.5	
2	90.6		12.0
3	86.6	96.7	
4	78.8		29.5
5	76.9	98.3	
6	90.3		21.2
7	86.9	97.0	
8	86.2		26.9
9	82.5	96.7	
10	88.9		15.7
Mean count	84.4	97.6	21.1

Note. Counts were obtained from three different sections of each specimen, with the percentages of vital follicles expressed as mean values. The groups were compared using two-tailed homoscedastic *t* test, all yielding highly significant results.

^a *p* < 0.001 (*t*-test comparison between first and second groups).

^b *p* < 0.001 (*t*-test comparison between second and third groups).

^c *p* < 0.001 (*t*-test comparison between first and third groups).

majority of cytoplasmatic organelles, particularly the mitochondria, were assembled close to the intact nuclear membrane (Fig. 1).

Primordial follicles that showed alterations after freeze-thawing had abundant mitochondria with reduced transverse cristae and endoplasmatic reticulum. Follicular cells showed a large nucleus containing irregularly distributed chromatin with a mass of granular chromatin located near the nuclear envelope. The mitochondria were small with a few cristae, endoplasmatic reticulum was present in small amounts, and the Golgi complex showed normal features. Numerous junctions (desmosomes) connected the follicular cells to each other. The follicular cells rested on a thin basal membrane.

Although we observed differences in the appearance of the primordial follicles between fresh and cryopreserved total porcine ovaries, these changes provided no evidence for compromised viability. In contrast, ovaries plunged in liquid nitrogen without cryoprotectant contained a substantial proportion of damaged primordial follicles (Fig. 2).

DISCUSSION

The cryopreservation of ovarian tissue is a promising new method for conserving the ovarian function of young patients about to undergo aggressive chemotherapy or radiotherapy (23). In the human ovary, more than 90% of the follicles are in the primordial stage. Primordial follicles have a paucity of potentially sensitive organelles, are less metabolically

Fig. 1. Viable primordial follicle. Mitochondria with transverse cristae assembled close to the intact nuclear membrane.

active, and their comparably low intracellular complexity makes them less susceptible to the adverse effects of low-temperature storage.

Oktay *et al.* reported the isolation of primordial follicles from human ovarian biopsies partially disaggregated with collagenase before or after cryopreservation, with follicles isolated by microdissection (24). Fifty-eight percent of follicles could be recovered from the ovarian tissue. Assessment of follicle viability using live–dead stains showed that 72% of whole follicles and 91% of oocytes survived the isolation procedure. Electron microscopy confirmed that the majority of the cells were free of ultrastructural signs of damage.

In another study in mice, ovarian grafts were suspended in plasma clots and transplanted to the ovarian bursas of oophorectomized mice, where they reorganized into morphologically and functionally indistinguishable tissue (25). Isolated follicles may also be grown to maturity *in vitro*. Even though follicle

Fig. 2. Damaged primordial follicle. Mitochondria with reduced transverse cristae.

culture technology is still in its infancy, the first complete success was reported in a murine model by Eppig and O'Brien (26), who developed a two-step strategy to achieve complete oocyte development in vitro. The ovaries of new-born mice were first grown in organ culture for 8 days, during which time the early follicular growth was initiated, and then the oocyte-granulosa cell complexes were isolated from these ovaries and cultured in vitro for an additional 14 days. Mature oocytes were subsequently collected, fertilized, and the transfer to pseudopregnant females resulted in the birth of a viable pup. In vitro activation of primordial follicles has also been achieved in cattle and primates, but only few follicles pass to the secondary step in development. (27).

Another strategy is to reimplant ovarian cortical slices or biopsies, even though this may result in significant follicular loss, potentially affecting long-term graft survival. Nugent *et al.* (28) investigated follicular survival after human heterotopic grafting. In women

undergoing laparoscopic assessment for reversal of sterilization, cortical biopsies of 5-mm diameter were sutured to the anterior surface of the uterus. Three months later, the grafts were recovered and all of them contained follicles with a survival rate of approximately 27%.

In contrast, total ovaries contain larger numbers of primordial follicles and permit autotransplantation with vascular anastomosis, so that higher rates of follicular survival can be expected. It will also be important to investigate whether oxidative stress occurs as a result of ischaemia and reperfusion following the grafting procedure and whether antioxidants moderate any resultant cellular injury.

Freeze-thawed ovarian tissue can be returned to the body as an orthotopic or heterotopic autograft. Even though heterotopic reimplantation to a site with a rich vascular bed can restore endocrine cycles, clinical assistance, including stimulation of the ovary, aspiration of follicles, and in vitro fertilization will be required to achieve pregnancy. Previous heterotopic sites have, for example, included the subcutaneous tissue of the axilla (29).

Another option is to return the ovarian tissue back to the original site, with orthotopic insertion of the cryopreserved ovarian tissue being the only method by which natural fertility can return.

A primary concern surrounding ovarian tissue cryopreservation is the possibility that the ovarian tissue might harbour malignant cells, inducing a recurrence after reimplantation. Thus, Shaw *et al.* showed that cancer cells can be transmitted with both fresh and cryopreserved ovarian tissue grafts. When small pieces (1 mm³) of ovarian tissue obtained from donors with lymphoma were grafted into healthy mice, 13 of the 14 recipients contracted lymphoma (30). Even though such a result is to be expected in patients carrying a blood-borne disease or metastatic spread to the ovaries, it is highly unlikely that a relapse would occur with Hodgkin's disease or Wilms' tumour. Moreover, cytogenetic or molecular markers can be used to screen the tissue for the presence of malignant cells.

If successful, reimplantation of frozen and thawed ovarian tissue can restore endocrine cycles, thus obviating the need for hormone replacement therapy in patients with ovarian failure, and permit a woman to regain natural fertility. Ovarian tissue storage clearly is the only option for preserving ovarian function in children and may also be applied to young women receiving cytotoxic chemotherapy for benign diseases. Patients with an autoimmune disorder treated

with alkylating agents could likewise profit from autologous ovarian grafting. Finally, it could have a role in healthy women who choose to delay conception into the years of diminishing ovarian reserve.

The aim of this study was to determine whether a total ovary can successfully be frozen and thawed and high rates of viable, structurally intact primordial follicles can be maintained. Light microscopy is a well-established method for evaluating the viability of frozen-thawed ovarian tissue. In our study, more than 80% of primordial follicles recovered from the frozen-thawed ovaries appeared viable, showing good histological integrity. Electron microscopy is capable of fine discrimination of damage to membranes and organelles. The observed differences of the primordial follicles between fresh and frozen-thawed ovaries indicate no impairment of viability. They possessed intact nuclear and cell membranes, abundant mitochondria with few cristae, and endoplasmic reticulum in the cytoplasm. The surrounding pregranulosa cells rested on a thin basal lamina and appeared to be ultrastructurally normal. These morphologic methods cannot provide final proof of viability, which clearly requires assessment of the in vivo function of cryopreserved whole ovaries. Meanwhile, we have performed autografting of a whole frozen-thawed ovary in sheep and delivered a healthy lamb (unpublished). However, the results presented here appear to be promising both in terms of the numbers of surviving follicles and their ultrastructural morphology.

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