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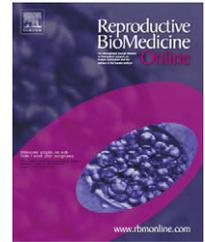
ARTICLE

Ovarian function 6 years after cryopreservation and transplantation of whole sheep ovaries

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Abstract Whole ovary cryopreservation and transplantation has been proposed as a method for preserving long-term ovarian function. This work reports ovarian function 6 years post transplantation of frozen–thawed whole sheep ovaries. Three 9-month-old Assaf sheep underwent unilateral oophorectomy to provide organs for the experiments. After perfusing with cold University of Wisconsin solution supplemented with 10% dimethyl sulphoxide, ovaries were cryopreserved using unidirectional solidification freezing technology. After thawing, ovaries were re-perfused and re-transplanted orthotopically by microvascular re-anastomosis, to the contralateral ovarian pedicle after removing the remaining ovary. Six years following transplantation and after inducing superovulation, the sheep were killed and the ovaries analysed. Two ovaries had normal size and shape showing some recent corpora lutea, while the third showed atrophic changes. A total of 36 antral follicles were counted by transillumination and four germinal vesicle oocytes were aspirated and matured in vitro to metaphase II. Serum progesterone concentrations were indicative of ovulatory activity in one of the three sheep. Histological evaluations revealed normal tissue architecture, intact blood vessels and follicles at various stages. Currently, this is the longest recorded ovarian function after cryopreservation and re-transplantation. Cryopreservation of whole ovaries, using directional freezing combined with microvascular anastomosis, is a promising method for preserving long-term reproductive capacity and endocrine function. RBMOnline

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KEYWORDS: anastomosis, cryopreservation, directional freezing, transplantation, whole ovary

Introduction

As increasing numbers of female patients of reproductive age survive cancer, the quality of life after treatment is becoming an important consideration. Among the toxic side effects of chemotherapy and radiotherapy, one of the greatest concerns for young women is the risk of sterility or premature menopause (Meirow, 2000; Wallace et al., 2005).

Today there are many options for the preservation of fertility, although all but embryo freezing are still considered investigational (Lee et al., 2006). Two fertility preservation options involve the freezing of ovarian tissue: (i) cryopreservation of ovarian cortical slices; and (ii) cryopreservation of the whole ovary.

One of the problems associated with the first technique is the ischaemic damage of the grafted tissue due to the time required to re-establish a vascular supply, causing significant follicular loss and thus truncating ovarian function after re-transplantation. In fact, although sheep autotransplants of frozen—thawed ovarian cortex (Baird et al., 1999) and hemi-ovaries (Salle et al., 2003) have resulted in deliveries and hormone production, the duration of function has never been longer than 2—3 years, and often much shorter because of ischaemia. This outcome was also observed with mouse ovaries (Liu et al., 2002). The experience in humans with ovarian cortex transplants has also confirmed the short lifespan and, thus, the suboptimal nature of this approach (Silber et al., 2008a). The second option, freezing of the whole ovary, at least in preliminary results from animal models (rat, rabbit, dog, sheep) (Arav et al., 2005; Chen et al., 2006; Imhof et al., 2006; Ishijima et al., 2006; Yin et al., 2003), seems to confirm prolonged ovarian survival and reproductive ability when the intact organ is cryopreserved. However, freezing whole organs requires a different methodological approach to conventional freezing devices that operate on the principle of multidirectional (equiaxial) heat transfer by convection. With convection, the temperature changes in the sample are dependent upon the thermal conductivity and the geometric shape of the container and thus cannot be uniformly controlled between the periphery and the core of the biological sample (Armitage, 1987; Karlsson and Toner, 1996). The freezing apparatus utilized in the experiments presented here, the multithermal gradient freezer, is based on the physical concept of directional freezing, which enables precise adjustment of the temperature gradients while moving the samples in a certain velocity within the thermal gradient in order to achieve an accurate cooling rate through the entire tissue.

This report is a continuation of a previous one for which Arav et al. (2005) documented ovarian function for up to 3 years after whole-organ cryopreservation in sheep. Furthermore, 1 month after transplantation, oocytes were aspirated indicating immediate hormonal activity and the ability of the follicles to survive the cryopreservation and transplantation processes. Normal conception was not achieved due to adhesions. At 6 years, this report in the same animal model is the longest-yet documented period of ovarian function after whole-organ cryopreservation, thawing and orthotopic microvascular transplantation.

Materials and methods

Three 9-month-old Assaf sheep (labelled 1—3) were used for the experiments. The research was approved by the Israeli National Animal Ethics Committee of the Agricultural Research Organization. Details of the ovarian resection, perfusion, cryopreservation and thawing have been described previously (Arav et al., 2005). Briefly, under general anaesthesia, longitudinal low median laparotomy was performed. Dissection and isolation of the right ovarian vascular pedicle enabled disconnection of the ovary and pedicle at a point near the origin of the ovarian artery. The ovarian artery was perfused under a dissecting microscope with 10 ml of cold (4°C) University of Wisconsin solution supplemented with 10% DMSO for 3 min and then placed into a glass freezing tube 16 mm in diameter (Manara Industries, Kibuts Manara, Israel) filled with the same cryoprotectant medium. The samples were frozen using the Multi Thermal Gradient (MTG) freezing apparatus (Core Dynamics, Nes Ziona, Israel). The samples were pushed along the thermal gradient at a very slow rate (0.01 mm/s), resulting in a cooling rate of −0.3°C/min (**Figure 1**), after which the tubes were plunged into liquid nitrogen.

Temperature measurements of the freezing process were performed on slaughterhouse sheep ovaries, in which two thermocouples of 50 μm each were placed in the ovary; one in the centre and the other in the cortex. The temperatures were recorded using a data equisition system (Almemo 2390—5; Ahlborn, Holzkirchen, Germany). The data was downloaded to a PC using AMR-control software (Ahlborn).

Thawing was performed from 2 weeks to 2 months after cryopreservation, by immersing the glass tubes into a 68°C water bath for 20 s and then into a 37°C water bath for 2 min. The cryoprotectant solution was rinsed out of the ovaries via cannulation of the ovarian artery using 10 ml HEPES-Talp medium supplemented with a single step 0.5 mol/l sucrose and 10 IU/ml heparin (Sigma, St. Louis, MO, USA) at 38°C.

The ovaries were all re-transplanted orthotopically, by performing microscopic end-to-end vascular reanastomosis (10—0 interrupted sutures, Ethilon; Johnson and Johnson, New Brunswick, NJ, USA) to the contralateral ovarian vascular pedicle after removal of the remaining ovary as previously described (Revel et al., 2004). Blood flow was verified by observing pulsation in the ovarian artery and
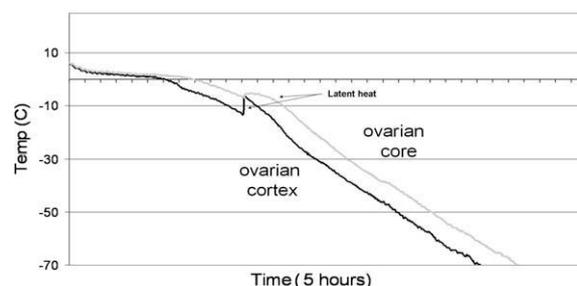
Figure 1 Multi-thermal gradient unidirectional freezing profile of a sheep ovary. Arrows = latent heat release.

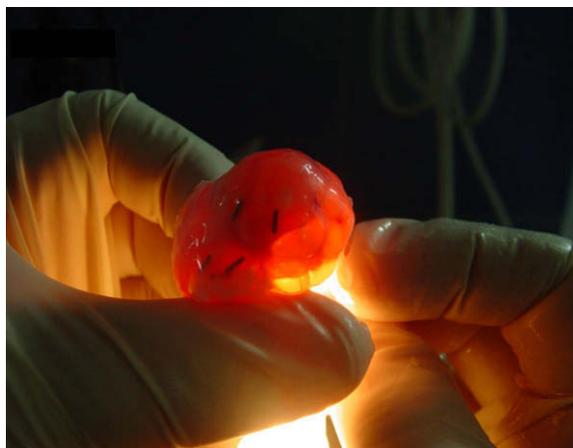the venous return was confirmed by normal distension of the ovarian vein.

To assess ovarian endocrine activity, progesterone concentrations were measured during the 5 weeks before the sheep were killed. Blood samplings from the neck vein were collected twice a week into lithium heparin-coated test tubes (Greiner Labortechnic, Kremsmuenster, Austria), centrifuged at 3000 rpm for 15 min and the plasma was stored at −20°C until analysis. Progesterone was measured using a Coat-A-Count kit (DPC, Los Angeles, CA, USA) as previously reported (Revel et al., 2004). The sensitivity of the kit is 0.1 ng/ml and a progesterone concentration of more than 1 ng/ml was considered as evidence of a functional corpus luteum (Arav et al., 2005).

Before the sheep were killed, they were superovulated using six injections of FSH (32 IU, Ovagen; ICPbio, Auckland, New Zealand) twice a day for 3 days. Ovaries were excised, visually inspected using transillumination, and visible follicles were aspirated using a syringe with a 20 G needle. The harvested oocytes were at the germinal vesicle stage and allowed to undergo in-vitro maturation using the method described by Zeron et al. (2001). The ovaries were placed in Bouin's fluid for fixation, followed by embedding in paraffin, sectioning at 5 μm sections and staining with haematoxylin and eosin.

Results

The three ovaries re-transplanted 6 years earlier all had intact and functional vasculature connections; ovaries from sheep no. 1 and no. 2 were of normal size and shape showing antral follicles by transillumination (Figure 2) and in no. 1 there was also a recent corpus luteum. Ovary no. 3 was decreased in volume, consistent with atrophic changes.

A total of 36 antral follicles were observed by transillumination of ovaries no. 1 and no. 2. After random aspiration, four oocytes at the germinal vesicles stage were harvested and matured to metaphase (MII) *in vitro* within 24 h. Histological evaluation of the same ovaries revealed a normal tissue architecture, including follicles at all developmental stages from primordial to antral and intact blood vessels (Figure 3).

Histologically, the ovary with the best survival (no. 1) was multifollicular, displaying antral follicles ranging up to several millimetres (normal ovulatory size in the sheep is 8 mm) and, in addition, there was additional proof of corpora lutea in ovaries no. 1 and no. 2. The animals were stimulated with exogenous gonadotrophins and the size of the antral follicles indicated that the two ovaries (no. 1 and no. 2) were responsive to exogenous FSH. The histology revealed normal-appearing theca cells, mural granulosa cells, cumulus cells and zona pellucida. Other sections contained atretic follicles that were almost completely collapsed with a retained oocyte. Most importantly, there were only occasional primordial follicles widely dispersed in the cortex. This indicates that the functional reserve of the ovary was almost exhausted. This observation is in line with normal age changes of the sheep ovaries, which by age 6 years have become almost depleted of follicles and leading to anoestrus.

Histologically, the ovary from sheep no. 3 was completely depleted of follicles; however, the ovarian parenchyma did not show necrotic changes and the blood vessels had intact intima.

For sheep no. 1, the endocrine function of the transplanted ovary, monitored by sampling serial progesterone concentrations, showed measurable concentrations in a cyclic manner, but the hormonal profile of the other sheep was below the detection level (0.1 ng/ml).

Discussion

For many years, attempts to cryopreserve large organs have been ineffective because of problems associated with heat transfer (Balasubramanian and Coger, 2005) and the non-homogeneous rate of cooling between core and periphery of the organ. To overcome these challenges, two methodologies have been proposed: (i) vitrification methodology (Baudot et al., 2007; Fahy et al., 1984); and (ii) directional multithermal gradient freezing (Arav et al., 2005; O'Brien and Robeck, 2006; Saragusty et al., 2007). Vitrification as a methodology for preserving large biological samples such as vascularized organs has many drawbacks: chemical toxicity and osmotic shock following exposure to very high (>50%) concentrations of cryoprotectant solutions, fractures caused to the vitrified organ by the vitrification procedure (Courbiere et al., 2005) and devitrification if the storage temperature is above the glass transition temperature (Fahy et al., 1984).

The novel multithermal gradient freezing methodology as shown by the experiments reported here and in other works (Elami et al., 2008) has solved this problem by maintaining a uniform cooling rate through the entire organ. Figure 1 shows, by using two thermocouples one in the cortex and the other in the centre part of the medulla, that the cooling rate is very uniform throughout the periphery and the core of the ovary. Maintaining this uniform cooling rate was made possible by building the freezing devices based on two principles: (i) large mass of conductive material which enables rapid evacuation of latent heat being released due to crystallization in the freezing front; and (ii) since the sample is moving through the temperature gradient at a

Figure 2 Excised ovary (sheep no. 1) showing antral follicles by transillumination.
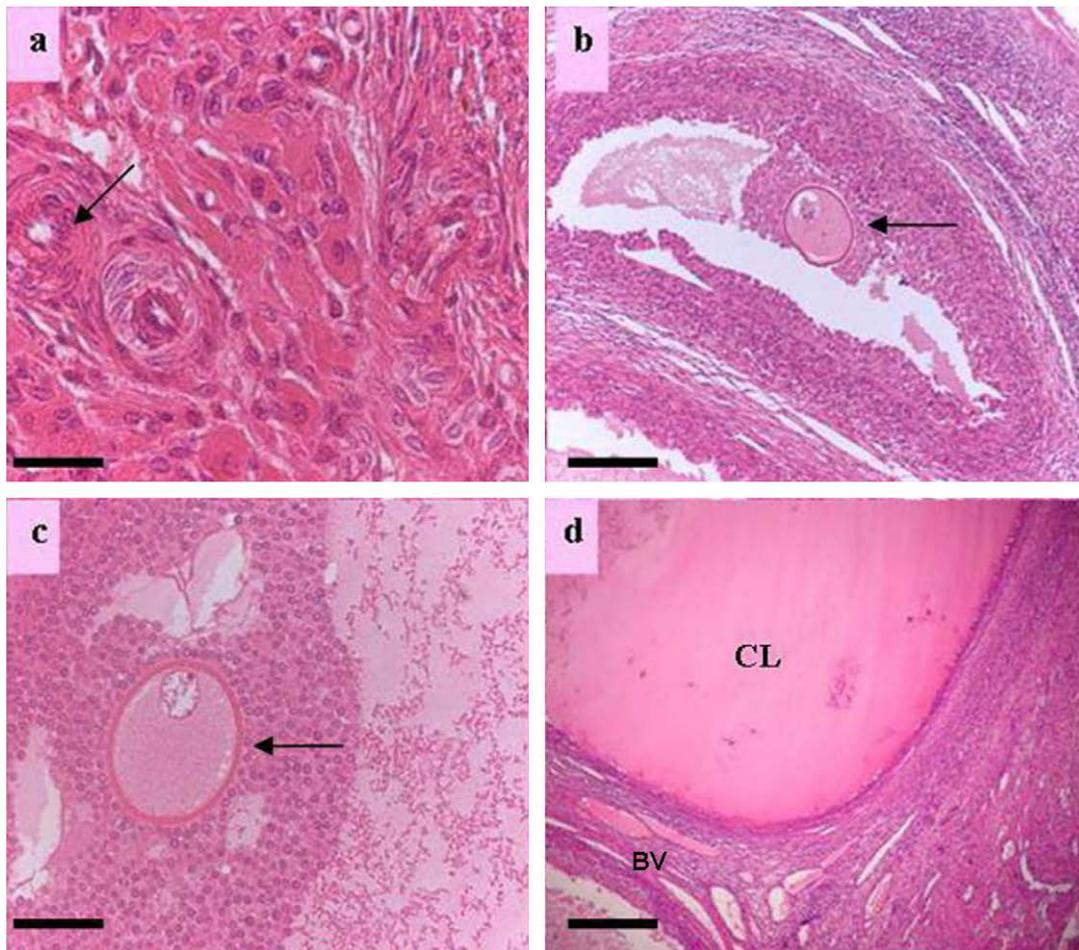
Figure 3 Haematoxylin- and eosin-stained ovarian sections (ovary from sheep no. 1) revealing normal tissue architecture including follicles at various developmental stages. Arrows pointing at: (**a**) primary follicle (×20, bar = 50 μm); (**b** and **c**) antral follicles (×10, bar = 100 μm); and (**d**) corpus luteum (×5, bar = 100 μm).

controlled speed, a very precise and uniform freezing front (the interface) is created, which allows efficient removal of the latent heat.

The alternative to whole ovary is the cryopreservation of cortical strips. However this method has important technical limitations since upon thawing and grafting of the cortical tissue, there is a large follicle loss during the period of ischaemia, before the tissue becomes revascularized (Baird et al., 1999; Silber et al., 2008b), compromising ovarian function.

The successful demonstration of a long survival of 6 years after whole-ovary freezing and re-transplant in a large animal bodes well for ongoing studies on whole human ovary cryopreservation and transplantation (Silber et al., 2008b). The information gathered from these animal experiments is very relevant to the human application since the sheep ovaries have strong similarities to the ovaries of young women displaying a high cortical primordial follicle density. There is hardly any data available on aged sheep ovaries since animals are normally culled long before 6 years of age (de Souza et al., 1998). The present study is the first time that long-term follow up of whole-ovary freezing and transplantation has demonstrated a large number of follicles.

Cryopreservation of an intact human ovary with its vascular pedicle as recently reported (Martinez-Madrid et al., 2007) is not associated with any signs of apoptosis or ultrastructural alterations in any cell types confirming that whole-organ vascular transplantation may thus be a viable option in the future.

In summary, directional freezing is a promising technology that allows freezing of large tissue and whole organs and it has been recently applied with success to other organs such as a whole heart (Elami et al., 2008) and a whole liver (Gavish et al., 2008) as well as tissue such as cartilage (Norman et al., 2006).

Concerning ovarian cryopreservation, with the advances in whole-ovary transplantation surgery, the availability of an effective cryopreservation methodology is paramount. The data gathered from this work are extremely encouraging to suggest whole-ovary freezing as a method of choice for preserving long-term reproductive capacity and endocrine function in young women.

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