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Normal gestations and live births after orthotopic autograft of vitrified–warmed hemi-ovaries into ewes

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BACKGROUND: The aim of this study was to evaluate the long-term outcome of autotransplantation of vitrified warmed hemi-ovaries into ewes. METHODS: Six hemi-ovaries from six ewes aged 6 to 12 months were vitrified. After dissection of the medulla, the hemi-ovarian cortex was stored at –196°C in liquid nitrogen. Four to six weeks after the first laparotomy, the left ovary was removed and the vitrified–warmed hemi-ovary was sutured. RESULTS: Plasma progesterone concentration increased in a regular manner in all ewes. Three pregnancies occurred, from which four lambs were born. The first delivery of a normal lamb occurred in February 2003. The second delivery of two normal lambs occurred in March 2003 (a 2.5 kg male and a 2.8 kg female). The last lamb had a normal delivery but had a malformation of the left leg and the oesophagus. This lamb died two months after delivery from pneumariae. Histological examination of the grafted vitrified ovaries showed few primordial and antral follicles. CONCLUSIONS: These three pregnancies in a ewe model may indicate that ovarian vitrification gives results as good as those from a slow cooling protocol in autograft. It is impossible to establish a link between the vitrification procedure and the malformation of the last lamb, and further studies are needed to evaluate the feasibility of ovarian vitrification.

Key words: autograft/cancer/fertility preservation/ovary/vitrification

Introduction

The preservation of ovarian function and fertility in patients before intensive chemotherapy can now be performed by ovarian cryopreservation (Oktay and Buyuk, 2004). All other procedures have disadvantages that make them too long to carry out. The long-term viability of the cryopreserved ovarian fragments is the main problem and many solutions are proposed (Sonmezer and Oktay, 2004).

Autograft figures as the most promising approach (Gosden *et al.*, 1994): *in vitro* maturation of frozen primordial follicles is still in its infancy (Gook *et al.*, 2004), while xenografts pose two problems—one of ethics and the other of infection (Oktay *et al.*, 2000). Ovarian autograft, on the other hand, may be orthotopic or heterotopic, and can involve ovarian fragments or hemi-ovaries or, indeed, whole ovaries with their vascular pedicle (Jeremias *et al.*, 2002). Gestations and births have been managed in many animal species, but not yet in humans. There have been case reports of ovarian autograft following cryopreservation, but with only a few ovulation cycles. Oktay *et al.* (2004) and Tryde *et al.* (2004) removed immature oocytes and were able to mature and fertilize them *in vitro*; one embryo was obtained and transferred, but did not lead to pregnancy. One performed an heterotopic autograft (Oktay *et al.*, 2004) and the other an orthotopic autograft (Tryde *et al.*, 2004). Donnez *et al.* (2004) recently reported the first live birth in women after orthotopic transplantation of cryopreserved ovarian tissue.

Most freezing procedures have used slow cooling protocols such as that proposed by Newton *et al.* (1996) and modified by Demirci *et al.* (2001). The post-freezing viability of primordial follicles is estimated at between 85% (Gook *et al.*, 1999) and 92% (Demirci *et al.*, 2001).

Vitrification is a so-called 'ultra-fast' cryopreservation technique, which aims to avoid any ice crystals forming during the drop in temperature by trapping all the aqueous solutions in a solid known as the 'vitreous phase'. Vitrification has many advantages in cryobiology. First, it avoids the formation of ice crystals, which damage cells. Secondly, it avoids the need for a programmed freezing device (Rall and Fahy, 1985). It does, however, require high cooling rates—though these can be moderated given a sufficient amount of cryoprotectant.

Cells and tissue are largely composed of water. Cryoprotectants therefore have to be deployed to deal with nucleation and ice crystal growth. They have the effect of increasing intra- and extra-cellular milieu viscosity so as to reach a hyperviscous state at the moment of contact with the liquid nitrogen. The choice of cryoprotectant solution is critical. It involves two requirements: low cellular toxicity and a strong tendency to vitrify (Kuleshova and Lopata, 2002).

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We have been working on ovarian cryopreservation for many years (Salle *et al.*, 1998, 1999). We developed a slow cooling protocol and reported its effects on follicle viability. (Demirci *et al.*, 2001) We then obtained gestation and births in ewes after orthotopic frozen-thawed hemi-ovary autograft (Salle *et al.*, 2002). In 2002, we reported the impact of an ovary vitrification protocol on tissue in ewes: 86.20 ± 1.32% of primordial follicles were alive after exposure to VS1 cryoprotectant (Rall and Fahy, 1985) and 82% after exposure to cryoprotectants and vitrification.

The aim of this study was to evaluate the results of vitrified–warmed hemi-ovary autograft into six ewes.

Materials and methods

Study animals

Between September and November 2001, six Grivette ewes aged 1–1.5 years were included in the protocol. Our study was approved by the ethics committee of the Ecole Nationale Vétérinaire de Lyon (ENV de Lyon, Marcy l'Etoile, France). During the first laparotomy, a right ovariectomy was performed. After removal, the right ovary was sectioned sagittally. The medulla was removed using scissors; the remaining hemi-ovarian cortex was 1 mm thick, 2 cm long and 1 cm wide. It was immediately vitrified and stored at –196°C in liquid nitrogen for a minimum of 15 days.

During another laparotomy 30–45 days later, the left ovary was removed completely under the microscope, leaving only the hilum. After thawing, the right hemi-ovarian cortex was sutured under microscope on the hilum using 8–10 stitches of 9.00 Prolene suture (Ethicon, Somerville, NJ, USA). During this laparotomy, great care was taken with the left fallopian tube extremity. After the graft procedure, the ewes were returned to pasture and normal husbandry conditions, and placed with fertile rams. No treatment was administered because restoration of spontaneous fertility was expected.

All the pregnant ewes had normal delivery. After the last delivery, a third laparotomy was performed and the transplanted ovaries were removed for histological analysis. The average size of the 1-year transplant was 12.6 × 20.67 × 1 mm.

Vitrification protocol

VSI is a reference vitrification solution first described by Rall and Fahy (1985) containing four cryoprotectants (Sigma Aldrich, St Louis, MO, USA)—dimethylsulfoxide (2.62 M), acetamide (2.60 M), propylene glycol (1.31 M) and polyethylene glycol (0.0075 M) in modified Dulbecco's saline (HB1) as a dilution medium. In our work, HB1 was replaced by BM1 (Eurobio, Les Ulis, France)—a synthetic medium enriched with human albumin, adapted to gamete preparation. BM1 is a synthetic saline medium containing human albumin 10 g/ml, cholesterol, pyruvate, amino acids and antibiotics (penicillin and streptomycin). The various cryoprotectant baths were prepared by diluting the basic 100% VS1 cryoprotectant solution in BM1.

The vitrification protocol consisted of three phases.

In phase 1 (dehydration),fragments were exposed to increasing cryoprotectant concentrations of VSI (12.5, 25, 50 and 100%). The first two steps lasted 5 min at room temperature, and the next two for 15 min at 4°C. The ovarian fragments were then transferred into cryogenic vials (Nunc Brand Products, Roskilde Denmark) containing 1 ml of 100% VS1 solution. Each 1.8 ml vial contained one ovarian fragment in 1 ml of solution.

Phase 2 was vitrification. This ultra-fast method of cryopreservation involves plunging the cryotubes containing the ovary fragments directly into liquid nitrogen at –196°C. The cryogenic vials were preserved in liquid nitrogen and stored until the day of the autograft. The cryogenic vials were placed onto a plate containing warmed water at 37°C for 10 minutes.

Rehydration in phase 3 consisted of extracting the cryoprotectants in baths of decreasing VS1 concentration (50, 25, 12.5%) and final washing in BM1.

In 2001, we evaluated the toxicity of this protocol on the primordial follicle population (unpublished data). Five hemi-ovaries were vitrified, stored for 15 days in liquid nitrogen and then thawed. After dissection, a vital stain was performed with haematoxylin and eosin. We counted a hundred follicles and calculated the percentage of follicles that were alive. Before vitrification (fresh ovary), an average of 95.33% were alive compared with 82% after vitrification and thawing.

Histological analysis

The frozen-thawed ovarian autograft was fixed for 2 days in picroformalin. (Sigma Aldrich, St Louis, MO, USA) The fixed ovarian graft was embedded in paraffin, cut into serial 4 μm sections, and stained with haematoxylin and eosin (H&E). All fragments were cut into sections, and all sections were examined by microscope (magnification ×320). The description provided by Turnbull *et al*. (1977) was used to define primordial, primary, secondary and antral follicles.

Hormone assays

Starting 3 weeks to 1 month after autografting, bloods samples were obtained weekly to determine the serum concentrations of progesterone. Progesterone concentrations were determined at the ENV de Lyon by enzyme immunoassay; the intra- and inter-assay coefficients of variation were both 12%.

Results

Four months to one year after transplantation, all ewes had recovered normal progesterone secretion patterns, indicating a restoration of ovarian function (data not shown). Three ewes began gestation during the summer of 2002 (Table I). Ultrasonography in mid-September 2002 confirmed two singleton

Table I. Results of the transplantation of vitrified thawed hemi-ovary

Ewe No.	First rise in progesterone (weeks after transplant)	Gestation	Outcome	Follicle density (number/cm^3)
61	44	Yes	Male 3.28 kg	23.5
68	43	Yes	Male 2.52 kg Female 2.83 kg	15.5
70	26	No	–	14.6
73	20	No	–	1.3
65	17	Yes	Male 4.20 kg with malformation; died 2 months after delivery	25.5
69	19	No	–	4.0

and one twin pregnancy. The first ewe gave birth spontaneously to a healthy male lamb. The lamb weighed 3.28 kg and had a normal development. The twin birth (one male and one female lamb) was uneventful; both lambs were in good health and progressed normally. The third ewe (no. 65) gave birth to a 4.2 kg male lamb that had a malformed leg and which died 2 months after delivery; autopsy showed two malformations—an equine varus foot and missing palate; death followed gastro-esophageal reflux, flooding the lungs.

After the last delivery, the ewes underwent a third laparotomy. The autografted ovaries were of subnormal size (the normal size of a ewe ovary is 2×1.5 cm). No peritoneal adhesions were seen. The anatomical relationships between the grafted ovary and fallopian tube were normal. The grafted ovaries of those ewes that had been in gestation bore a regressing corpus luteum. Histological examination of the transplanted ovaries showed a total of 6–58 primordial follicles per transplant. Primordial follicle density was 1.3–25.5 follicles/cm^3 (Table I).

Discussion

Vitrification could be an alternative to conventional slow cooling protocols. Conventional cooling protocols are performed routinely in the case of homogeneous biological systems or cells. Ovarian tissue, however, is heterogeneous, and each of its cell types has its own optimal cooling speed (Liebermann *et al.*, 2002). This makes it difficult to select an appropriate cooling speed for the whole tissue. The first vitrification protocol described was applied to mouse embryos and gave a survival rate of >80% (Rall and Fahy, 1985). However, such embryos have a low *in vivo* development rate (17%) (Rall *et al*., 1987).

Vitrification protocols are now being applied successfully at various stages of embryo development in mammals and in humans. Pregnancy and births have been obtained in humans after vitrification of 2-pronucleus zygotes (Jelinkova *et al*., 2002), pre-implantation stage blastocysts (Mukaida *et al*., 2003) and metaphase II oocytes (Yoon *et al.*, 2003). Research is therefore turning towards cryopreservation of ovarian tissue, enabling a large number of primordial follicles to be frozen, with oocytes that are less differentiated and less cryo-sensitive. In humans, good results in terms of follicle morphology after cortex fragment vitrification have been obtained (Isachenko *et al.*, 2003).

Data on the human ovarian cortex, however, remain limited. There have been several reports of successful vitrification of ovarian tissue in mice, rats, hamsters and cows. At present, there are only three reports of successful vitrification of whole ovaries in mice and rats, with encouraging first results (Sugimoto *et al*., 2000; Salehnia, 2002; Migishima *et al*, 2003). Using VS1 vitrification solution with rat ovaries, Sugimoto *et al*. (2000) found intact follicles, but also degenerative aspects such as pyknosis and vacuoles. After vitrification and orthotopic transplantation in mice, Migishima *et al.* (2003) reported continued spontaneous fertility with births, although less than in controls. Salehnia *et al.* (2002), studying follicle and oocyte ultrastructure following ovary vitrification in mice, found no significant alterations with respect to controls.

During this study, progesterone assays were positive at 3 months post-graft, with extreme values, over 5nmol/l at one year. As with slow cooling protocols, we obtained three gestations and three live births (Salle *et al*., 2002). The malformations in the lamb born to ewe no. 65 cannot be attributed to the vitrification protocol. Further studies are needed to determine whether the origins of such malformations are accidental or due to the vitrification. It is difficult to evaluate the malformation rate in sheep population. Nevertheless, such malformations are very rare. Oocyte and embryo vitrification studies have not suggested any increased risk of fetal malformation. Although there are no reports of an increase in fetal malformation with vitrification, high rates of aneuploidy have been observed following vitrification of animal oocytes (Kola *et al.*, 1988).

As in frozen tissue grafting with a slow cooling protocol, it would seem that the key to vitrified ovarian tissue autograft lies in the time needed for graft revascularization. The number of follicles found two-years post-graft in our ewes was indeed very small (6–58). Thus, recovery of ovarian function and gestation was possible, but only in the first year post-graft. This is a main difference with respect to slow cooling protocols because gestation and birth two years post-graft have been reported (Baird *et al.*, 1999; Salle *et al.*, 2003). It is difficult to explain why the follicle count in this study was so low one year after transplantation. The time to recover neovascularization may be an explanation, but an increase in primordial follicle fragility after vitrification may also explain this low count. A previous study using a similar vitrification cocktail to that used in the present study showed a low number of follicles with normal morphology following vitrification (Isachenko *et al*., 2003).

Ovarian cryopreservation is a procedure that is still in evaluation phase. Interesting results in humans may have been reported, but we are still far from having a standard procedure.

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