

Long-term follow-up of cryopreserved hemi-ovary autografts in ewes: pregnancies, births, and histologic assessment

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Objective: To evaluate a 2-year follow-up of cryopreserved hemi-ovary autografts in ewes.

Design: Animal study.

Setting: University fertility center, Hospices Civils de Lyon; Ecole Nationale Vétérinaire de Lyon, INSERM U 418 Hôpital Debrousse, Lyon; and Hôpital Edouard Herriot, Lyon, France.

Patients(s): Grivette ewes.

Intervention(s): Recently we reported four pregnancies and six live births after transplantation of frozen-thawed hemi-ovary in six different ewes. The four remaining ewes were monitored for 2 years. After the last birth, the autografted ovary was removed in each ewe during a final laparotomy. The entire grafted ovary was sliced to estimate the remaining primordial follicle population 2 years after grafting.

Main Outcome Measure(s): Uterine ultrasound scanning was performed to diagnose pregnancy. Histological assessment of the grafted ovary was performed after delivery.

Result(s): The four remaining ewes began new gestations. For two of them, this was a second gestation obtained more than 2 years after the autograft. These two ewes delivered male lambs, which died immediately after delivery because of distocia. The lambs were both oversized for gestational age; autopsy found no malformation. A twin pregnancy of a healthy male and a healthy female occurred in May 2002, and a singleton male was born in February 2002. All grafted ovaries showed drastic reduction in follicle population.

Conclusion(s): Frozen-thawed ovary autograft allowed recovery of fertility a very long time after the procedure despite a drastic reduction in the total number of follicles. (Fertil Steril® 2003;80:172–7. ©2003 by American Society for Reproductive Medicine.)

Key Words: Long-term follow-up, cryopreservation, autograft, ovarian tissue, ewe, pregnancy

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After several years of publications assessing both freezing-thawing protocols and the possibility of using ovary fragments, it seems that ovary cryopreservation is now commonly performed worldwide. The aim of ovary cryopreservation is to restore fertility after cancer therapy. Most of the young women concerned present ovary failure after chemotherapy or X-ray therapy (1). There are now three theoretical ways to use frozen-thawed ovary grafts: in vitro maturation (2), xenograft (3), and autograft (4).

Today, given the ethical problems associated with xenografting (5) and the technical problems inherent in in vitro maturation (6), autotransplantation of frozen-thawed ovaries is

the procedure that has been developed the most (7). Gestation and live birth after ovarian autograft have been reported in three animal species: rats, mice, and sheep. To date, only two human patients have received their own ovaries back after freezing and thawing. Although no pregnancies were obtained in either case, ovary function did lead to one or two ovulation cycles (8, 9).

Most of the studies on ovary cryopreservation have focused on developing freezing protocols (10–14) and on the surgical techniques of autograft (15–18). Unlike other teams, we opted from the beginning for freezing whole hemi-ovaries and last year reported four gestations and six births after cryopreserved hemi-

TABLE 1

Development of the pregnancies after hemi-ovary frozen-thawed autografting (these data have been previously reported).

Ewe no.	Pregnancy	Delivery	Lamb
36	Simple	Vaginal	Male, 5 kg, died
514	Twin	Vaginal	Male and female, 3 and 3.1 kg
227	Twin	Cesarean section	Male and female, 1.5 and 2.8 kg, died
6	Simple	Vaginal	Male, 3 kg
538	0	0	0
635	0	0	0

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ovary autograft in six ewes (19). The present paper is an update on the four remaining ewes 2 years after ovary autograft.

MATERIAL AND METHODS

In 2002, we reported four gestations and six live births of lambs, three of which survived after frozen-thawed ovary autograft procedures (Table 1). The three lambs are presently in good health and are fertile. Ewe no. 36 was ovariectomized immediately after delivery to measure the follicle population in the grafted ovary. Ewe no. 227 died after cesarean section. The four remaining live ewes of this protocol were kept with males during the year after the births. No fertility treatment was administered, although serum P concentration was assayed weekly. The protocol was approved by the ethics committee of the Lyon National School of Veterinary Medicine.

Surgical Procedure

Six Grivette ewes between the ages of 6 months and 1 year underwent initial laparotomy at the Lyon Veterinary School, the Zootechnic Laboratory of which has French Ministry approval for animal experimentation. Right oophorectomy was performed during a median laparotomy. The right ovary was transferred immediately to BM1 medium (Elio Bio Media, Paris, France). The ovarian cortex was then divided into two equal hemi-pieces no thicker than 2 mm.

The two hemi-ovaries were immediately and separately frozen and stored in NO for 1 month. A frozen-thawed ovary autograft was performed 1 month later during a second laparotomy. The left ovary was removed, leaving only a small portion of the median hilus measuring 0.5 cm × 0.5 cm. The frozen-thawed tissue from the right ovary was then fixed to the left hilus with six sutures using Prolene 8/0 (Ethnor, Paris, France). The laparotomy was then closed, and the lambs were returned to pasture. During a third laparot-

omy, the frozen-thawed autograft was removed for histologic assessment of the remaining follicle population. The laparotomy was performed immediately after the delivery if the newborn lamb died and 2 months after delivery in case of live birth.

Freezing-Thawing Protocol

We used the freezing procedure that we reported elsewhere (16). In brief, the 12 hemi-ovaries were placed in cryogenic vials (Nunc Brand Products, Roskilde, Denmark) containing 1 mL of BM1 freezing medium (Elio Bio Media) supplemented with 10% fetal calf serum and the cryoprotectant. The BM1 contained human albumin, cholesterol, glucose, lactate, penicillin, streptomycin, vitamins, and inorganic salts.

The cryoprotectant was dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) at a concentration of 2 M. The fragments were incubated with DMSO at room temperature (20°C) for 10 minutes before freezing. After incubation, the cryogenic vials were placed in a programmable freezer (Minicool 40PC; Air Liquide Santé, Paris, France).

The freezing procedure was performed with seeding. The tubes were all cooled at the same rates: in the first cooling phase (2°C/minute), they were cooled to -35°C, and in the second cooling phase (25°C/minute), to -140°C. During the first descent, before reaching T2 (initiation of crystallization, -11°C), the latent heat generated was compensated by a release of negative calories within the freezing chamber (semiautomatic seeding).

The cooling curves of the cooling chamber and medium were recorded throughout the procedure by computer (Fig. 1). Temperature graphs were obtained for each freezing process. The cryogenic vials were transferred to liquid nitrogen and stored for 30–45 days.

Ovarian Tissue Thawing

After rapid thawing at 37°C in a water bath, the ovarian tissues were washed in BM1 medium for 5 minutes. The vials were shaken gently to promote efflux of the cryoprotectant from the tissue.

Histologic Analysis

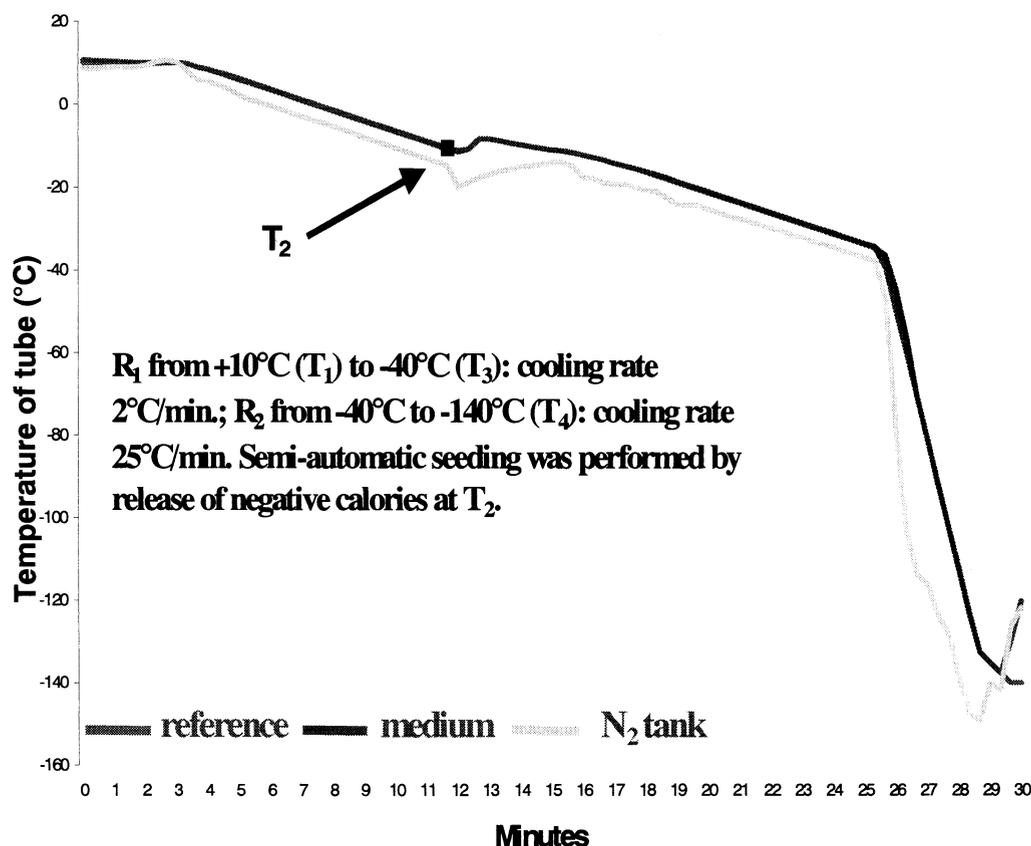
The frozen-thawed ovary autograft was fixed for 2 days in 0.1:1,000 formalin (10%). The fixed ovarian graft was embedded in paraffin, cut into serial 60- μ m sections, and stained with hematoxylin and eosin. The thickness of the slice was set at 4 μ m to count follicles only once. Follicles were taken into account only if the nucleus could be seen. All sections were examined by microscope (magnification ×100). Turnbull's (20) description was used to define primordial, primary, secondary, antral, and atretic follicles.

RESULTS

All four remaining ewes developed normally. All had regular P secretion, indicating normal function of the frozen-

FIGURE 1

Results of cooling with semiautomatic seeding.



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thawed autograft (data not shown). All four remaining ewes began gestation during the follow-up period (Table 2). Two ewes (nos. 6 and 514), which had delivered at the end of 2000 or at the beginning of 2001, began new gestations. Ewe no. 6 was the first to be diagnosed pregnant, in April 2001. On September 24, 2001, this ewe delivered a normal 5-kg

male, which died during delivery. Autopsy revealed no malformation. The second diagnosis of pregnancy was made on November 28, 2001 (ewe no. 514). This ewe, which had delivered two healthy twins in December 2000, delivered a 5-kg male, which also died during birth. The autopsy of this lamb was also entirely normal.

TABLE 2

Fertility of the four remaining ewes that received frozen-thawed hemi-ovary autograft and follow-up over 2 years.

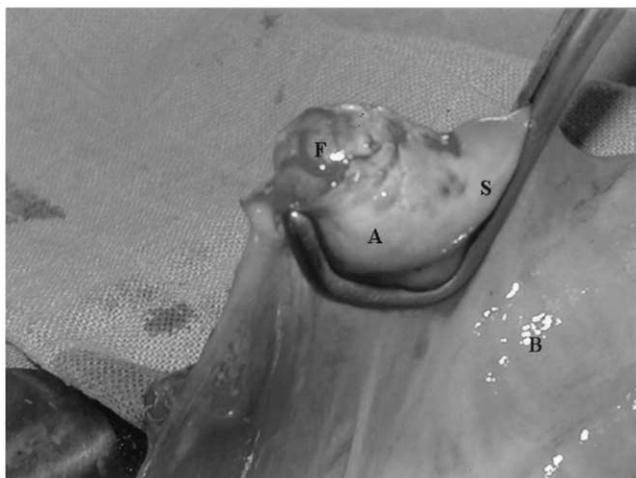
EWE no.	Autograft date	First delivery date	Second delivery date	Delivery route	Outcome
514	March 2, 2000	February 2, 2000	December 16, 2002	Vaginal	Male, 5 kg, died
6	January 21, 2000	January 22, 2001	September 24, 2001	Vaginal	Male, 5 kg, died
538	January 27, 2000	May 15, 2002	None	Vaginal	Male and female, 3 kg and 3.2 kg
635	February 3, 2000	February 2, 2002	None	Vaginal	Male, 3.1 kg

Note: Delivery route for all ewes was vaginal.

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FIGURE 2

Orthotopic frozen-thawed ovarian autograft 2 years after grafting (ewe no. 514). F = preovulatory follicle; S = sutures; A = autograft; B = uterus.



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The last two ewes (nos. 538 and 635), which had regular P secretion after autografting but had never begun a gestation, also began a pregnancy. Ewe no. 635 began gestation in November 2001, 21 months after autografting. It delivered a healthy, normal male lamb on February 2, 2002. Ewe no. 538 had a positive diagnosis for gestation in February 2002, 110 weeks after autografting, and on May 15, 2002, delivered a male and a female in good health, weighing 3 and 3.2 kg, respectively (Fig. 2).

During the laparotomy performed after the births, all of the frozen-thawed autografts were retrieved. The macroscopic aspect and the size of the grafts were extremely variable from one ewe to another, ranging from normal (Fig. 3) to atrophic ovaries.

The follicle population within the grafts revealed extreme variability (Table 3), ranging from extremely few (<50)

FIGURE 3

Twins born in 2002 from ewe no. 538 more than 2 years after the orthotopic autograft.



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follicles in three cases to a reduced population at best (ewe no. 514).

DISCUSSION

Ovarian autopreservation is a procedure that is still being evaluated. Although many teams have cryostored ovaries, only two cases of autotransplantation have been reported after freezing and thawing (8, 9). This is undoubtedly due to the poor results published in the literature. Pregnancies have been obtained in some animals, but there are only two teams who have reported gestations and live births in large mammals such as the ewe (19). This reluctance is also undoubtedly due to the lack of long-term studies of what happens to the grafted ovarian tissue, which could shed light on the time limits for obtaining pregnancy. Two studies have reported ovary transplant functioning after a time lapse of more than 50 weeks.

TABLE 3

Number and type of follicle and corpus luteum found in frozen-thawed autograft 2 years after orthotopic transplantation (per whole ovary).

EWE no.	Graft size (cm)	Primordial follicles	Secondary follicles	Antral follicles	Corpus luteum	Atretic follicles	Total
514	2.9 × 2 × 1.0	575	291	28	4	51	949
6	2.5 × 2.6 × 1.4	4	10	1	2	0	17
538	1.5 × 1 × 1.5	2	10	4	8	0	24
635	1.5 × 1.7 × 0.9	5	7	3	0	0	15

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Baird (21) reported nearly 2 years of survival for cryopreserved ovarian cortical strips in ewes. Gonadotropin rates remained near physiological levels for 60 weeks. Only 28% of the primordial follicles survived the procedure. After 22 months, all the transplanted ovaries contained large antral follicles and cysts, but very few primordial follicles were found on autopsy. However, Baird did not report significant differences in the length of estrus.

Shaw (22) studied fresh and frozen-thawed ovary grafts in fetal mice. Three of the seven mice that received a fresh fetal graft and four of the seven mice that received a frozen-thawed fetal graft began gestation. All of the grafted mice were monitored for 52 weeks. At 52 weeks, all the grafts contained small follicles but with a very reduced number. Shaw attributed the rather low pregnancy rates to anatomical particularities modified by surgery.

Our data show that fertility can sometimes be restored a very long time after ovary transplantation. Two ewes began their first gestation 1.5 and 2 years after autografting. These gestations were obtained despite the number of primordial follicles being drastically reduced after transplantation. Fewer than 50 follicles were counted 2 years after the autograft procedure. Only one graft contained a higher number of follicles, so that a third gestation might perhaps have been feasible. This decrease in follicle number can be explained in two different ways. The freezing and thawing procedure may destroy a small percentage of the primordial follicles. The survival rate is estimated to be between 80% and 92% (23). A more important factor is the time necessary for good revascularization of the graft. The delay and the quality of the neovascularization can induce a rapid fall in follicle population. Aubard et al. found less than 10% of the primordial follicles 1 year after grafting (10). It may be that the neovascularization in ewe no. 514 was of better quality than in the three remaining ewes. Nevertheless, the 514 remaining follicles were far less than the 165,000 normally expected (24).

Clearly, the crucial step in frozen-thawed hemi-ovary autografting lies in graft revascularization. Effort should be concentrated on this step. The plasticity of the ovary nevertheless enables functional resumption of fertility. Perhaps freezing entire ovaries with vascular anastomosis would be a solution. Jeremias et al. reported autotransplantation of an ovary associated with its pedicle to the lower epigastric vessels (25). The transplant was monitored for 7 days. Concentrations of E_2 and FSH did not change with successful autotransplantation. However, these were unfrozen ovaries and freezing entire ovaries has recently been reported in small animals (26).

We here report live births and two neonatal deaths. These deaths were due to the fetus being oversized for gestational age, our males being much larger than the females. None of the autopsies revealed any polymalformation syndrome.

To date, no pregnancies have been reported after orthotopic autograft of human ovarian tissue. One possible reason is that the protocols have only been started very recently. One of the two patients who received her own ovary had already undergone first-line chemotherapy before autopreservation. This could explain the relative failure of the procedure. If freezing and thawing protocols preserve a large majority of primordial follicles, autografting substantially reduces the number of viable oocytes. Research is needed to determine how long the grafts can survive. In the series we report here, it seems that more than 104 weeks is sometimes possible. These results have encouraged us to propose cryostorage of ovarian tissue using a similar procedure for women who may undergo early menopause. Such a procedure could allow us to propose a solution to conserve fertility.

However, the discussion about ovarian cryopreservation should focus on the indications, the timing of the procedure, and the size of the ovarian fragments. There are two types of indications (27): malignant and benign pathologies that need to be treated by small doses of chemotherapy over a very long period. The timing of preservation is also important: most of our patients who have had ovary tissue preservation had already undergone first-line chemotherapy (29), which may have reduced the number of primordial follicles. Each time, both hemi-ovaries were preserved. We believe that the frozen surface is not a limiting factor. The surface must be as thin as possible to enable good penetration by the cryoprotectants. The idea of freezing a large surface is to allow a large number of primordial follicles to be grafted to increase the chances of pregnancy (30).

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