

# Orthotopic microvascular reanastomosis of whole cryopreserved ovine ovaries resulting in pregnancy and live birth

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**Objective:** To evaluate the feasibility of contralateral autotransplantation of cryopreserved whole ovaries with microanastomosis of the ovarian vascular pedicle.

**Design:** Animal study.

**Setting:** Department of Biomedical Sciences, General Hospital of Vienna, Austria.

**Animal(s):** Nine ewes, six month of age.

**Intervention(s):** Laparotomic unilateral oophorectomy was performed. Ovaries were frozen using a controlled-rate freezing system. After frozen storage, contralateral laparotomic oophorectomy was performed, and the thawed ovaries were returned to the contralateral orthotopic site with microsurgical vascular anastomosis.

**Main Outcome Measure(s):** Histologic examination and serum follicle-stimulating hormone and progesterone levels.

**Result(s):** Four sheep showed postoperative luteal function. One sheep conceived after spontaneous intercourse and delivered a healthy lamb 545 days after transplantation. Histologic examination of the ovaries 18–19 months after transplantation showed that the structural integrity of the ovarian stroma had largely been retained in six out of nine animals. Follicular survival rate in the grafted ovaries was 1.7%–7.6%.

**Conclusion(s):** Microvascular anastomosis of whole ovaries and orthotopic transplantation after cryopreservation is technically feasible and a promising procedure in ovarian tissue banking. (Fertil Steril® 2006;85(Suppl 1): 1208–15. ©2006 by American Society for Reproductive Medicine.)

**Key Words:** Cryopreservation, fertility preservation, microvascular anastomosis, ovarian tissue banking, ovarian transplantation, orthotopic autotransplantation, whole ovary

Recent years have seen dramatic improvements in the treatment of cancer and significant increases in long-term survival rates. But aggressive radiotherapy and chemotherapy can exhaust follicular stores and compromise ovarian function (1, 2), thus constituting a considerable demand for the protection of fertility. Meanwhile, indications for fertility preservation have increased far beyond cancer treatment.

Currently, patients have few options. An overview is provided in a recent article (3). The frozen storage of embryos after oocyte recovery and IVF is inappropriate for children and for women without a partner, unless they accept sperm donation. The frozen storage of mature oocytes for future in vitro fertilization (IVF) is suitable for women without a partner, but results have been poor so far (4, 5). Moreover, it leads to an unacceptable delay in the treatment of cancer, and the number of obtained embryos or oocytes is limited. In addition, oocyte cryopreservation is challenged

by low survival and fertilization rates, which could be attributed to the biologic attributes of oocytes making them extremely sensitive to adverse influences (6–8).

Another approach is the in vitro maturation of primordial follicles after harvesting and freeze-thawing (9). In vitro growth and maturation of primordial ovarian follicles was reported in murine models (10, 11), and the in vitro activation of primordial follicles has been achieved in cattle and primates, but only few follicles passed to the secondary step in development (12). The in vitro maturation of human primordial follicles is currently not an option (13).

Efforts have therefore focused on the cryopreservation of ovarian tissue containing large numbers of primordial follicles (14–16). In the human ovary, more than 90% of the follicles are in the primordial stage. Human ovarian cortex is tolerant to freezing and thawing (17, 18), and primordial follicles can survive freeze-thawing in small ovarian cortical slices (19, 20).

The first report on the successful transplantation and subsequent development of follicles in a frozen-thawed autologous graft in a human was published in the year 2000 (21), when pieces of tissue were thawed and sutured beneath the pelvic peritoneum. Later, several groups have autografted

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human ovarian cortical strips into heterotopic as well as orthotopic locations and have restored short-term ovarian function (22–24), but no fertility was achieved. Recently, the first human pregnancies following orthotopic ovarian tissue banking (OTB) have been reported (25, 26).

A major problem in ovarian tissue transplantation is follicular loss due to ischemic reperfusion injury. Studies suggest that many more follicles are lost during revascularization than by the freezing and thawing procedure (18, 27), and the key factor responsible for follicular survival is the post-grafting ischemia, resulting in significant follicular loss and affecting long-term graft functionality (28–30). In a recently published study by Kim et al. (31), the correlation between ischemic tissue damage and the duration of ischemia was verified. Those researchers found that ovarian cortex could tolerate ischemia at least for 3 h, whereas stromal cells appeared to be more vulnerable to ischemia compared to primordial follicles.

A promising approach to reduce ischemic damage is cryopreservation of an intact ovary with its vascular pedicle (32). Application of the freezing medium via vessels should enable faster penetration of the tissue by the medium as well as fast perfusion after regrafting. Recently, we have shown that freezing of a whole porcine ovary explanted by microvascular anastomosis enabled the storage of a high rate of primordial follicles and preserved the structural integrity of somatic and reproductive cells (33).

In this study, we demonstrate the grafting of whole frozen-stored ovine ovaries by microvascular anastomosis. The sheep model was chosen because the sheep's ovary is similar to the human ovary. It has a dense fibrous stroma, a relatively high primordial follicle density in the cortex, and a size most closely analogous to that of the human ovary. This article reports the first successful cryopreservation and orthotopic retransplantation of a whole ovine ovary with reanastomosis of the vessels resulting in a live birth.

## MATERIALS AND METHODS

### Explantation

Between December 1998 and July 1999, nine female sheep six months of age underwent laparotomy. The study was approved by the Tierversuchskommission der Universität Wien. Access to the ovary was achieved through midline laparotomy. One whole ovary including its supplying vessels was completely removed. The ovarian vascular pedicle was dissected under the operating microscope, and the ovarian artery (0.8 mm outside diameter) and vein (1 mm outside diameter) were isolated. The vessels were ligated with a 6.0 Prolene suture (Ethicon, Somerville, NJ) approximately 5 cm from the ovary. Special care was taken when removing the organ to avoid damage to the adjacent fimbria and tube.

The artery was immediately cannulated with a 24-gauge venflon (BOC Ohmeda, Helsingborg, Sweden), which was carefully anchored with a 6.0 Prolene suture. The ovary was

gently flushed through the artery with precooled (4°C) histidine-tryptophan-ketoglutarase solution (Custodiol; Pharmapal, Athens, Greece) followed by 20 mL cryoprotective solution, which consisted of cell culture medium (RPMI 1640; Sigma-Aldrich, St. Louis, MO) supplemented with 1.5 mol/L dimethyl sulfoxide (Merck, Vienna, Austria) containing 10% autologous sheep blood serum.

Flushings were performed manually using a 20-mL syringe adapted to the cannulus intubing the ovarian artery and a central venous measurement system via three-way adapter. Perfusion pressure was regulated manually not to rise above 50 cm H<sub>2</sub>O (54.4 cm H<sub>2</sub>O ≡ 40 mm Hg). Duration and perfusion rate were not measured.

Afterwards, the whole ovary was placed at 4°C for 30 min to allow equilibration. After completing local hemostasis, the abdomen was closed with a three-row technique for fascia, muscle, and skin closure. Postoperatively, all animals received a prophylactic injection of 3 × 10 mol/L IE Penicillin G (Hoechst Marion Roussel, Vienna, Austria).

### Freezing and Thawing Procedure

The whole ovaries were transferred to a programmable freezer (IceCube 1810; Sy-Lab, Purkersdorf, Austria) at a starting temperature of 4°C, cooled to 0°C at a rate of 2°C/min and held at 0°C for 12 min. Then the temperature was lowered by 1.5°C/min to -9°C, and after 8 min at -9°C by 0.5°C/min to -40°C. Owing to the size of the organ, no seeding-step was applied. Cooling was continued to -150°C at the faster rate of 10°C/min. Finally, the samples were removed, plunged into liquid nitrogen, and stored at -196°C.

At the time of retransplantation, the ovaries were rewarmed in air for 2 min before being immersed in fresh RPMI medium in a water bath at 25°C for 6–7 min. The cryoprotective solution was quickly removed from the tissue by perfusion at 35–40 mm Hg with RPMI medium. Special care was taken to restrict perfusion pressure to prevent the endothelium of the capillaries from being harmed.

### Retransplantation

Three to five weeks after the initial unilateral laparotomy, the sheep underwent contralateral laparotomy. One ovary had been left in situ to avoid the formation of adhesions and to provide an unharmed vascular pedicle for reimplantation. Now, the vascular pedicle of the second ovary was dissected under the operating microscope, clamped with a microclamp, and incised. This ovary was completely removed and the frozen-thawed ovary was autografted by microvascular end-to-end anastomosis to its remaining vessels.

First, the ovarian artery and, subsequently, the ovarian vein were joined with 9.0 Ethilon sutures (Ethicon, Somerville, NJ) using an operating microscope under 25× magnification. Then the ovary was secured in place by 6.0 Prolene sutures to prevent

vascular kinking. Ischemia time before complete reanastomosis of the ovarian blood supply was approximately 30 min. In two cases (sheep no. 5 and 7), reanastomosis took 45–50 min, because a second suture had to be set owing to leakage.

### Histology

The autografted ovaries were removed during a final laparotomy 18–19 months after retransplantation. The macroscopic appearance of the ovaries was recorded, making particular note of periovarian adhesions. The entire grafted ovaries were fixed in 7.5% paraformaldehyde for subsequent histologic examination. The ovarian cortex was sectioned into four pieces of equal size.

Afterward, the sections were embedded in paraffin and sliced into 5- $\mu$ m serial histologic sections, which were stained with hematoxylin and eosin to evaluate the remaining primordial follicle population. Of each ovarian quarter, 20 slices representative of the histologic status of the respective quarter were chosen for primordial follicle count. Counts were obtained in a standardized region of interest under standardized magnification (20 $\times$ ).

### Hormone Assays

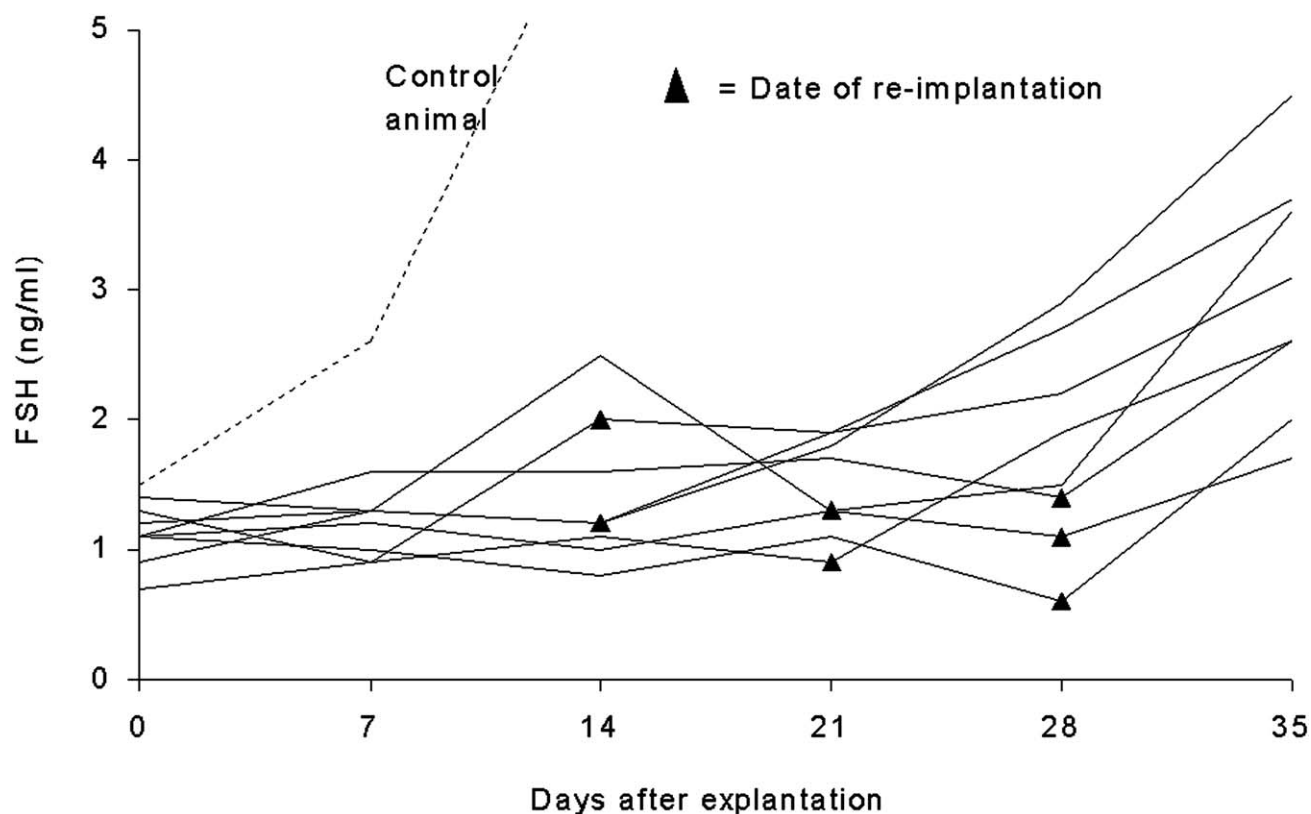
Blood samples from the jugular vein were collected preoperatively and at weekly intervals from the date of the first operation for hormone assay. Follicle-stimulating hormone and P concentrations were determined at a weekly basis for six weeks. After a single gap of two weeks, measurements were continued at a frequency of 4 weeks.

Follicle-stimulating hormone was measured by a radioimmunoassay previously validated for ovine plasma (34). The assay used an antiserum against ovine follicle-stimulating hormone (oFSH) (AFPC-5288113), oFSH tracer (AFP-4117A) and oFSH standard (AFP-4117A), provided by the National Hormone and Peptide Program (NHPP, Torrence, CA) and the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK, Bethesda, MD).

The samples were run in duplicate in one assay, and the analytical sensitivity of the assay was 0.3 ng/mL. The intra-assay coefficients of variation, calculated from the precision profile, were less than 10% for concentrations between 1 and 31 ng/mL. Serum P concentration was determined by commercial EIA kits (Vetoquinol, Lure, France).

**FIGURE 1**

Follicle-stimulating hormone course after explantation (day 0) and regrafting.



Imhof. Cryopreservation of whole ovine ovaries. *Fertil Steril* 2006.

Three to four weeks after retransplantation, the animals returned to pasture and normal husbandry conditions and were placed in a flock with other ewes and a fertile ram.

## RESULTS

### FSH and P

Before the initial explantation, FSH levels ranged between 0.7 and 1.4 (mean 1.14) ng/mL in all animals. The removal of one ovary lead to no drastic FSH increase in eight ewes. Sheep no. 9 showed an exceptionally strong increase. When the second ovary of this sheep was removed, it turned out to be mostly atrophic. The ovary was not replaced by the frozen-stored one, and this sheep served as control for FSH and progesterone (P) measures.

In general, FSH kept rising for about 3 months after grafting and decreased afterwards slowly to reach normal physiologic level 6 months or more after transplantation

(Figs. 1 and 2). Sheep no. 8 was killed two months after grafting, and the graft was removed to assess the freezing-induced damage histologically.

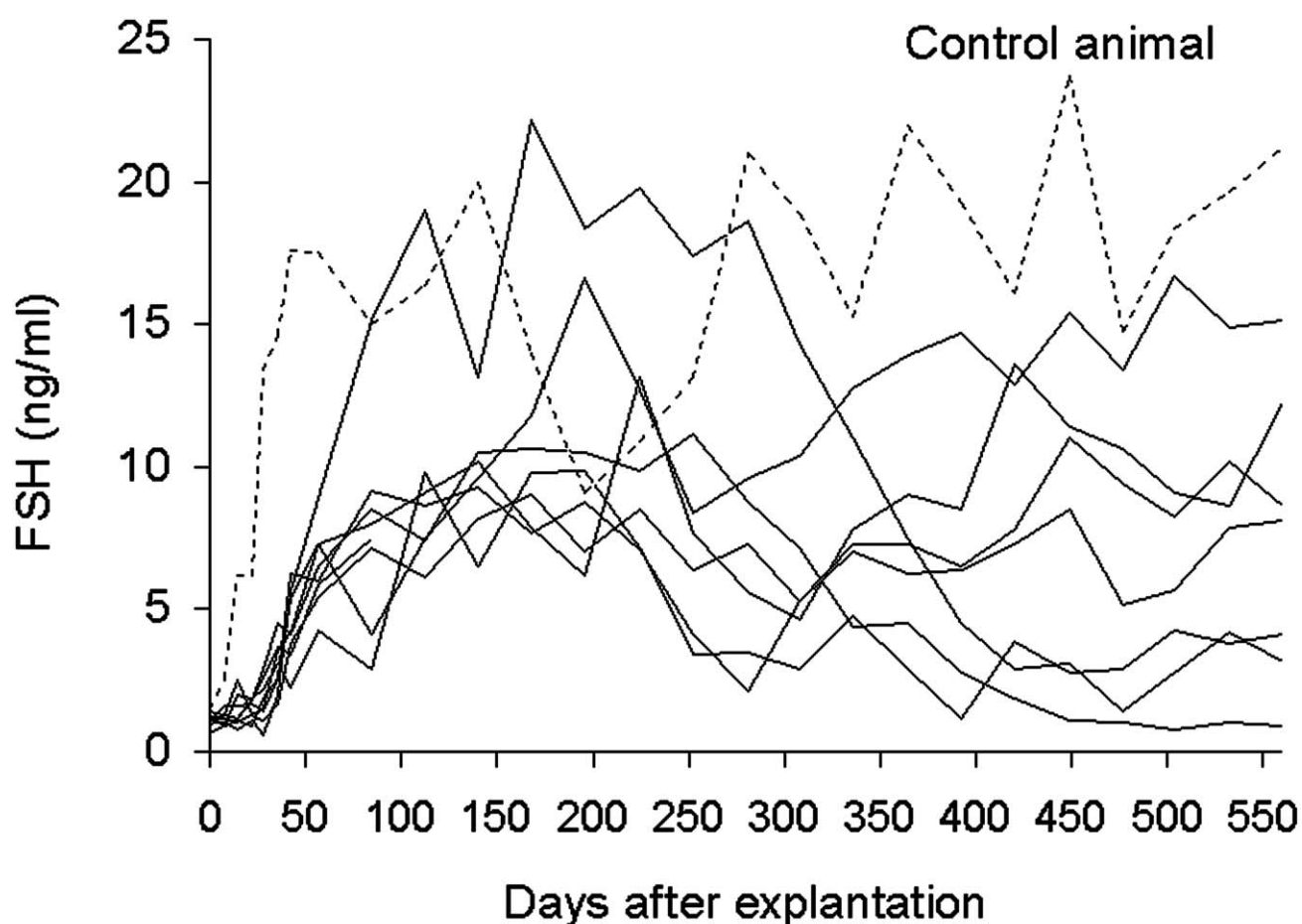
At a threshold value of 0.6 nmol/L, serum P was undetectable for about a year. In three animals (nos. 1, 4, and 6), P was detected 12–14 months after implantation at an initial level of 1.6–1.9 nmol/L. Subsequently, two of them (nos. 1 and 6) entered a P course indicating regular ovarian function, which was maintained until the end of the study. Sheep no. 4 reached a first peak of 16 nmol/L P on day 392, indicating a possible ovulation.

This sheep later was found to be pregnant. During pregnancy a sustained elevation of P up to >30 nmol/L was noticed.

The other four (nos. 2, 3, 5, and 7) of the seven sheep carrying frozen-stored ovaries and the spayed control animal (no. 9) showed no P throughout all measurements.

**FIGURE 2**

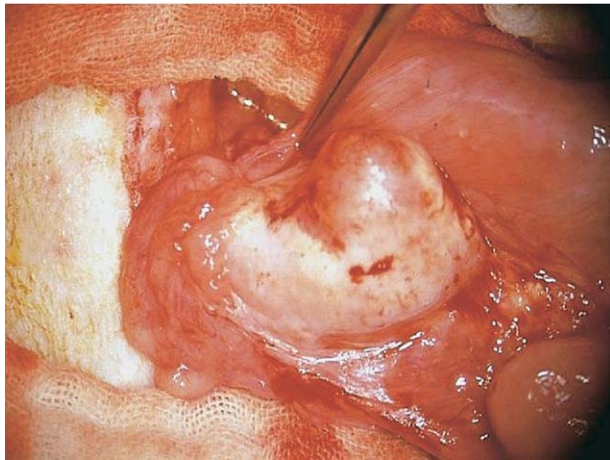
Long-term follicle-stimulating hormone course after ovarian transplantation.



*Imhof. Cryopreservation of whole ovine ovaries. Fertil Steril 2006.*

### FIGURE 3

The ovary of sheep no. 8 at final explantation, exposing a mature follicle three months after regrafting.



Imhof. Cryopreservation of whole ovine ovaries. *Fertil Steril* 2006.

### Histology

All sheep recovered well from the operation. The graft from sheep no. 8 was recovered for examination two months after retransplantation. On visual examination, periovarian adhesions were not noticed and the ovary was well vascularized. Scarry areas were observed that made up approximately a third of the surface, as estimated by visual examination. Histologic examination revealed that they correlated with deeper tissue damage.

In these areas, fibrosis had happened and primordial follicles were destroyed or completely absent. A mature follicle was presented on the frozen-thawed ovary (Fig. 3), confirming regained ovarian function. The structural integrity of the ovarian stroma had mostly been retained (Fig. 4).

Six sheep were killed 18–19 months after transplantation, when they had either re-entered regular hormonal cycles or showed no sign of ovarian activity. In four sheep (nos. 1, 2, 3, and 6), the ovaries were well vascularized and perfused but showed local damage. Basically, these areas made up about 30% in sheep nos. 1 and 6 and about 50% in sheep nos. 2 and 3. These two latter ovaries had failed to produce P, as indicated by the measurements. In two sheep (nos. 5 and 7), the frozen-thawed ovaries had largely undergone atrophy.

One sheep (no. 4) conceived after spontaneous intercourse more than a year after grafting. The pregnancy was diagnosed by manual examination and ultrasonography on day 527. A healthy lamb was delivered on day 545 (August 2, 2000). Ninety-seven days after delivery, the ovary was explanted for examination. Visually, the vascular system seemed intact.

The major ovarian vessels were free of thrombosis. A large corpus luteum was present on the grafted ovary, obviously remnant of the ovulation resulting in the pregnancy. In addition, a mature follicle was explored, indicating sustained function of the ovary. Local thrombosis of the capillary system was found in about 30% of the ovary.

Follicular count revealed a considerable loss of viable follicles. At 18–19 months after transplantation, between 90 and 406 viable primordial follicles were found in the slices taken from the grafted ovaries, with a mean of 229. In slices of the fresh and the frozen-thawed ungrafted ovary, 5,348 and 705 follicles, respectively were found (Table 1).

### DISCUSSION

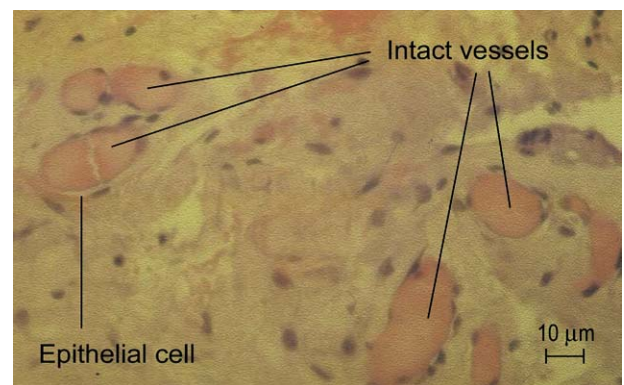
Freezing of ovarian tissue provides an interesting opportunity for the preservation of ovarian function and fertility. An overview of the different approaches and the perspectives of fertility preservation and particularly of OTB is provided in a series of recent articles (35–38).

Concerning graft location, heterotopic autotransplantation may restore hormone and ovulatory activity but conception requires assisted reproduction. The restoration of a natural state, providing regained endocrine activity, enabling natural conception, and obviating lifelong hormone replacement therapy, is orthotopic grafting. In addition, orthotopic grafting circumvents the problems of oocyte retrieval and IVF.

In the sheep model, cryopreserved cortical strips returned to the orthotopic sites have successfully restored estrus cycles for nearly two years and resulted in pregnancies (39, 40). More recently, the use of cryopreserved hemiovaries has resulted in live births in sheep (41). Orthotopic transplanta-

### FIGURE 4

Histologic examination of a transplanted frozen-thawed ovary showing small vessels with healthy endothelial cells.



Imhof. Cryopreservation of whole ovine ovaries. *Fertil Steril* 2006.

**TABLE 1****Results of the histologic examination of the ovaries.**

	Graft size (mm)	Follicle count per ovary				Total	Percentage (fresh ovary = 100)
		1st quarter	2nd quarter	3rd quarter	4th quarter		
Fresh ovary	2.6 × 1.2 × 1.3	1,325	1,526	1,213	1,284	5,348	
Frozen-thawed ovary	2.0 × 1.4 × 0.9	236	112	165	192	705	13.18
Sheep no. 1	2.7 × 2.0 × 1.2	42	106	132	98	378	7.07
Sheep no. 2	1.5 × 1.1 × 1.2	38	49	26	48	161	3.01
Sheep no. 3	1.9 × 1.7 × 0.9	44	39	66	25	174	3.25
Sheep no. 4	2.7 × 2.6 × 1.6	122	83	89	112	406	7.59
Sheep no. 5	1.5 × 1.3 × 0.9	18	47	11	14	90	1.68
Sheep no. 6	2.1 × 1.7 × 1.2	106	67	83	55	311	5.82
Sheep no. 7	1.9 × 1.2 × 0.8	12	32	24	27	95	1.78
Sheep no. 8	1.7 × 1.8 × 1.1	74	45	47	51	217	4.06

*Imhof. Cryopreservation of whole ovine ovaries. Fertil Steril 2006.*

tion of frozen-stored ovarian tissue has also enabled a first spontaneous human pregnancy after OTB (25).

A major concern of OTB is follicular loss. The use of thin cortical slices enables quick freezing and thawing, but grafting of ovarian cortex is challenged by reperfusion ischemia during revascularization. A recent attempt tries to avoid these problems by injecting frozen-thawed minced ovarian fragments into the cortex of an ovary that had previously been infertilized by irradiation (42).

Revascularization can be circumvented by cryopreservation of a whole ovary with its vascular pedicle. The vessels provide quick access to deeper tissue during freezing and thawing, and reanastomosis to the blood supply should reduce the period of post-grafting ischemia to a few minutes and thus improve the reproductive potential of the transplant.

However, only a few studies have addressed reanastomosis of a frozen-stored whole ovary in the sheep. It has been shown that vascular perfusion of the ovarian artery and slow freezing enabled preservation of an intact sheep ovary and restoration of fertility (43).

Frozen-stored whole sheep ovaries were grafted to the abdominal wall by microvascular anastomosis and examined after 8–10 days in situ (44). Three ovaries were viable without signs of necrosis, and vascular conclusion was found in eight animals. Follicular survival was compared with cortical strips, and the authors concluded that cryosurvival of whole ovary was as good as cortical strips, at least in the sheep.

In the present study, six out of eight sheep showed major ovarian vessels that were free of thrombosis. The ovaries were well perfused, although there were areas of histologic damage which made up 30%–50% of the respective ovary.

This suggests that the damage occurred owing to reasons such as local thrombosis and capillary fibrosis.

The ovaries from two sheep (nos. 5 and 7) had largely undergone atrophy. A possible explanation may be found in the transplantation time, which had been approximately 20 min longer in these two animals as compared to the others and in a reduced reperfusion owing to leakage of the first suture. Although we have no hard evidence that this delay was the decisive factor affecting the grafts, this seems to support a crucial role for fast reperfusion regarding tissue integrity.

In the ovary examined after two months (no. 8), we found a mature follicle, indicating regained function. Postoperative luteal function was indicated by the measured P concentrations in three other animals. Interestingly, these four cases coincide in that the respective ovaries showed histologic damage of about 30%.

In contrast, the two ovaries with a damage of about 50% failed to regain function, although macroscopic and light-microscopic examination revealed no significant differences beyond the portion of damage. This finding may indicate something like a threshold value for successful autotransplantation depending on the grade of histologic damage.

Follicular loss turned out to be higher than expected after visual examination. We hypothesize that this indicates adverse changes on the ultrastructural level. Primordial follicle counts in the examined slices ranged from 90 to 406, equivalent to a follicular survival rate of 1.7% to 7.6% (mean 4.3%). Although these results may not appear impressive, they show a good and rather uniform outcome compared to a long-term follow-up study in sheep with nonvascularized hemiovaries (45). In that study, two years after grafting 575

primordial follicles were present in one of four grafts, but only 2–5 in the others. This may indicate greater reliability of anastomosis.

Tissue damage and follicular loss may have happened during freeze-thawing and owing to insufficient perfusion during and after reanastomosis. The exact role of the respective factors could not be evaluated by our methods. However, a follicular survival rate of 13.1% in the frozen-thawed nontransplanted ovary suggests freeze-thawing rather than reanastomosis to be the major source of damage.

In the present study, the ewes were kept postoperatively under normal pasture conditions, together with other ewes and fertile rams. They were neither intentionally mated nor continuously observed. However, all animals had the opportunity to have intercourse, and we believe that most of them actually had. The unplanned pregnancy of sheep no. 4 gives us the opportunity to report the first live birth after orthotopic grafting of a whole ovary. A healthy lamb was born without assistance and grew up well, until it was slaughtered in late 2003, at the age of 3 years 4 months.

This article reports a pioneering study, which was primarily focused on the surgical issue of orthotopic microvascular reanastomosis. Freeze-thawing was performed in 1999 and the freezing protocol was not specifically optimized. No coagulation inhibitors were administered to prevent thrombosis, nor was any post-transplantation hormonal stimulation.

The restoration of endocrine function in four sheep and the unintended birth of a healthy lamb 19 months after grafting are encouraging, although the restoration of natural fertility may be more difficult in humans than in sheep. By now, it seems that there is no advantage of the whole ovary transplantation by vascular anastomosis compared with ovarian tissue transplantation, because of the still-existing poor follicular survival and higher surgical risks.

Further studies are required to improve freeze-thawing protocols for whole organs, reveal mechanisms of cell-to-cell interactions in cryopreserved tissues, and examine the possible role of agents in follicular survival if cryopreservation of whole ovaries is to become a clinical application.

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