

Restoration of ovarian steroid secretion and histologic assessment after freezing, thawing, and autograft of a hemi-ovary in sheep

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Objective: To evaluate the effects of freezing, thawing, and autograft of a hemi-ovary on steroid secretion, endometrial maturation, and ovarian histology in ewes.

Design: Experimental animal study.

Setting: Laboratoire de Zootechnie, Ecole Nationale Vétérinaire, Marcy l'Etoile, France.

Animal(s): Six lambs aged 6 months to 1 year old.

Intervention(s): Hemi-ovaries were prepared and frozen from the right ovary of six lambs and autografted 4 weeks later to the contralateral ovarian hilus. The autografts and the uterus were recovered 1 year later. Blood tests were performed each week to measure P concentration.

Main Outcome Measure(s): Number of primordial follicles; levels of plasma P.

Result(s): Histologic examination of ovarian slices after freezing showed no destruction of primordial, primary, secondary, or cavitory follicles. The frozen ovarian autograft showed good recovery of the macroscopic and microscopic ovarian structure. After autografting, histologic examination revealed primordial to cavitory follicles. Secretion of P started to rise 4 weeks after the autograft. Histologic analysis of the endometrium showed numerous glands, vessels, and mucous secretion.

Conclusion(s): Frozen ovarian autografts achieved P secretion and endometrial maturity. (Fertil Steril® 1999; 72:366–70. ©1999 by American Society for Reproductive Medicine.)

Key Words: Autograft, cryopreservation, ovary, follicle

In recent years, the preservation of fertility in patients undergoing radiotherapy and chemotherapy has become the objective of numerous research teams. Modern oncology therapies tend toward increasingly aggressive chemotherapy or radiotherapy. A large number of young patients are cured of their cancer but then develop definitive ovarian insufficiency.

Ovarian protection can be performed in several ways. IVF can be performed before treatment and the embryos frozen. However, this cannot always be done in the case of adolescents or single women. The administration of estrogen and progesterone or LH-releasing hormone analogues has not always proved effective. Ovarian transposition is possible only in the case of pelvic irradiation; it is of no use in

radiochemotherapy or in the case of aggressive chemotherapy.

Cryoconservation of ovarian fragments was first reported by Gosden et al. (1), and this procedure is now being considered by many investigators as a method for protecting fertility. After remission, an autograft of the frozen ovarian tissue may restore ovarian endocrine function and fertility.

We reported previously a preliminary study of freezing-thawing and autograft of nonfrozen ovarian fragments (2). The frozen and thawed ovarian fragments showed numerous primordial, primary, secondary, and cavitory follicles. We report the second step of our study: the freezing, thawing, and autografting of frozen hemi-ovaries in ewes.

Received December 8, 1998; revised and accepted March 18, 1999.
Supported by Organon, Serfontaine, France.

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0015-0282/99/\$20.00
PII S0015-0282(99)00238-1

MATERIALS AND METHODS

Surgical Procedure

Six "grivette" ewes aged 6 months to 1 year underwent laparotomy during October 1997 in the Zootechnical Laboratory of the Lyon Veterinary School, which is approved by the French Agriculture Ministry for animal experimentation. The right ovary was completely removed and was immediately transferred to B.M. 1 medium (Elios Bio Media, Paris, France). The ovary was divided in the sagittal plane into two equal parts. The ovarian medulla was dissected to obtain a cortical thickness no greater than 2 mm. One hemi-ovary was then frozen immediately, following the procedure described above. The other half was divided into two equal parts. One part was kept as a reference for histologic analysis, and the larger fragment was immediately frozen to evaluate the effects of the freezing-thawing process on the ovarian parenchyma.

After 1 month, ovarian autograft of the frozen-thawed hemi-ovary was performed after ablation of the left ovary. The surgical procedure was identical to the one we have described previously (2). The ewes were returned to pasture for 1 year. On the day of laparotomy, the second frozen ovarian fragment was thawed and sent for histologic analysis.

Starting 1 week after the autograft, blood tests were performed weekly to measure P (Amerlex M Progesterone; Ortho-Clinical Diagnostics, Amersham, Little Chalfont, UK). A P concentration >0.3 nmol/L indicated endogenous secretion by the ovary. The blood tests were suspended during July and August 1998. During this time, which is the reproduction period, no measurements were made.

At the beginning of September 1998, oophorectomy of the grafted ovary was performed during a third laparotomy. During the same operation, a hysterectomy was performed to evaluate the endometrial mucosa by histologic analysis. Both the ovarian and endometrial biopsy specimens were sent for histologic analysis.

Freezing and Thawing Procedures

The ovarian fragments were transferred within the hour to 10% dimethylsulfoxide for 5 minutes (Sigma, St. Louis, MO) and then frozen with a descent curve of $-2^{\circ}\text{C}/\text{min}$ to -140°C (Air Liquide, Paris, France). The seeding was semi-automatic at -7°C . The fragments were then kept for 15 days in liquid nitrogen at -196°C . Thawing was performed over 1 minute in a bain-marie at 37°C . The ovarian fragments were then washed for 30 minutes in survival medium (BM 1; Elios Bio Media) at 10°C . The fragments were then incubated for 30 minutes at 37°C .

Histologic Analysis

The different ovarian fragments (immediately sampled, frozen and thawed, grafted, and then sampled) and endometrial biopsy specimens were fixed for 2 days in 0.1:1,000 formalin. Each ovarian fragment was cut into serial $5\text{-}\mu\text{m}$

sections, stained with hematoxylin and eosin, and embedded in paraffin. All fragments were cut into sections, and all sections were examined with a microscope (magnification $\times 200$). Turnbull's description from 1997 was used to define primordial, primary, secondary, and cavitory follicles.

The endometrial stages were defined according to the classification proposed by Banks in 1981 (3). During proestrus, there is increasing vascularity and congestion in the connective tissue space. During estrus, epithelial and glandular proliferation continues and is more apparent. Secretory activity of the cells is marked, whereas agranulocytic infiltration of the epithelium continues. The connective tissue space is marked by maximal congestion, edema, and hemorrhage. Mestrus is characterized by high secretory activity, whereas the edema of the connective tissue space declines. Anestrus is characterized by a thin and lined epithelium where uterine glands are sparse. Ovulation in the ewes occurred at the end of estrus.

RESULTS

Five of the six ewes had favorable development. One ewe died at the end of August 1998. The P levels measured in this ewe showed only one rise in progesteronemia. A laparotomy was performed immediately, but it was impossible to recover the graft.

During the third laparotomy performed on the remaining five ewes, all the grafts were recovered. All contained growing follicles. The mean weight of the grafts was 3.72 g (range, 0.424–4.424 g). Each uterus was of normal size, confirming the endogenous secretion of ovarian steroids. All of the grafts were situated in a subperitoneal position but were easily identified beneath the peritoneal flap.

Histologic analysis of the fresh ovarian fragments showed primordial to cavitory follicles. Freezing and thawing of the control ovarian fragment did not damage the ovarian structure. The number and distribution of follicles were identical to those in the fresh control sample. The frozen and thawed autografts contained cavitory and numerous primordial, primary, and secondary follicles. However, the ovarian structure of the autograft was difficult to recognize and the number of primordial follicles was greatly diminished compared with the fresh control sections (Fig. 1). In one ewe, the ovary showed a dominant follicle. Analysis of the follicular fluid showed a mature oocyte. We did not count the number of primordial follicles per graft because of the spatial distribution, which was heterogenous.

All uterine histology results showed numerous endometrial glands, an abundant mucosa, and numerous vessels. Two endometria were classified as estrus and three as mestrus (Fig. 2).

Four weeks after the grafts, the first increase in P was detected. Each ewe had a cyclic increase in serum levels.

FIGURE 1

Cavitory follicle and its oocyte from a frozen ovary 1 year after autograft. A = cavity; B = oocyte. Original magnification, $\times 200$.



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Average P concentrations were 2.7 nmol/L (range, 0.4–11.5 nmol/L).

DISCUSSION

Many investigators are proposing the use of ovarian cryopreservation for patients treated with radiotherapy or chemotherapy. Some of the problems involved in freezing ovaries seem to have been resolved. The problem of ovarian cryoconservation is that of the future of the frozen fragments. In theory, there are four possibilities: heterotopic autograft, orthotopic autograft, in vitro maturation, and xenograft.

In vitro maturation would be the ideal solution. In vitro maturation from primordial follicle to mature oocyte has never been reported in humans.

Xenograft is a possible way of using the grafts. Xenografts of ovarian tissue implanted under the renal capsule of immunodeficient mice have demonstrated the viability of human or monkey ovarian tissue. Follicular growth up to the pre-antral stage was reported by Oktay and Gosden in 1998 (4).

Heterotopic graft has been proposed on several occasions. Sites for this type of graft are numerous and varied: rectus

muscle, abdominal subcutaneous tissue, the omentum, or saphenous vein. Heterotopic grafts were originally performed to prevent early premature ovarian failure and not to preserve fertility.

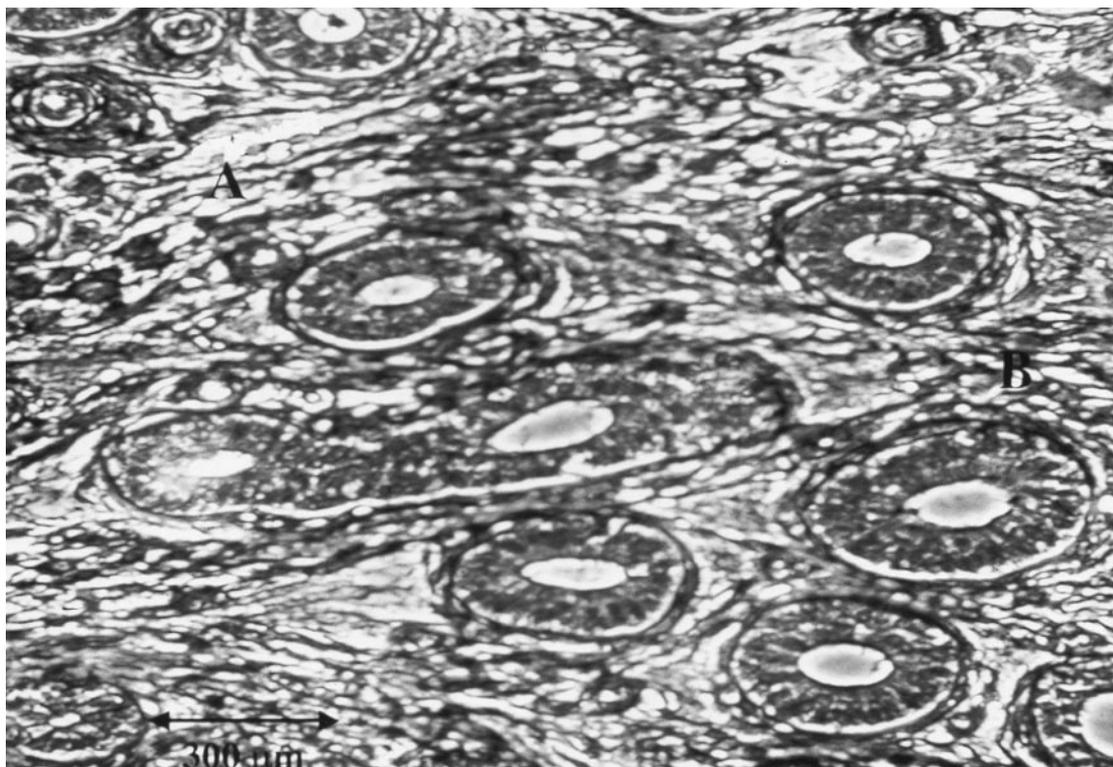
Orthotopic graft is the last possibility for cryopreserved ovaries. When an intact uterus and functional fallopian tubes are present, an orthotopic graft may restore normal fertility. Graft sites are less numerous than in the case of heterotopic graft. The peritoneum of the broad ligament has not given good results, whereas the contralateral ovary and uterus have accepted the graft without problem. Pregnancies have been obtained in the rat, mouse, and sheep.

Gosden et al. (1) were the first to report pregnancy in sheep after a freezing-thawing and autograft procedure. Five of six ovarian grafts contained primordial follicles, but in a much smaller number than in the control.

Our results confirm the work of Gosden et al. (1). Our five grafts contained primordial follicles, but also primary and cavitory follicles, confirming the revival of development and the start of follicular growth. We also noticed a diminution in the follicle density. The cause of this reduction could be the surgical procedure itself because of the time taken for revas-

FIGURE 2

Endometrium from the estrus ewe showing mitotic epithelium, mucous glands, and edema of the connective tissue. A = glands; B = edema. Original magnification, $\times 200$.



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cularization. Gosden et al. (1) showed that circulation is established in the grafts within 1 week after grafting, by which time little necrotic tissue remained. Nevertheless, the tissue would have been ischemic for a few days after grafting. To preserve a maximum of primordial follicles, ovarian grafts should be returned to the body with anastomosis of vessels to minimize the duration of ischemia. Unfortunately, this is impractical after a long period of oophorectomy, and frozen storage of whole ovaries is not possible at present.

The good histologic results from the freezing-thawing process are not necessarily indicative of the potential for revival of the ovarian fragments. The reduction in the population of primordial follicles after cryopreservation and autograft remains a serious problem. Faddy and Gosden's mathematical model (5) suggests that there is an acceleration in follicle loss when the population passes below 25,000 and that ovarian function is lost below 1,000. Only 10%–12% of mice having oophorectomy and then autograft with fresh or frozen ovarian fragments maintained their fertility for more than four cycles, compared with 100% of sham-operated controls.

In the sheep, Baird et al. (6) demonstrated that cycles

were maintained for 22 months at the same rate as in the control. After 1 year of graft, the cyclic secretion of P and the endometrial histology in our five ewes confirmed the continuation of endocrine ovarian function. The three mestrus endometria are in concordance with the luteal phase, when P concentrations are high. The two estrus endometria are in concordance with the follicular phase.

We did not obtain any pregnancies. Observations during the last laparotomy showed that the grafts were covered by peritoneum. The quality of ovulation may have been changed by adhesions. Adhesions may also explain why no gestation occurred. The levels of P that we measured do confirm a revival of cyclic ovarian secretion, but they were inferior to the levels required for gestation. As described previously (1), P concentrations began to rise 4 weeks after grafting.

Cryopreservation can now be proposed to patients (7). However, the future of the grafts remains somewhat hypothetical. Orthotopic graft seems to be the simplest solution at present, and it may allow spontaneous pregnancy. However, the craze that was started by the publication of Gosden et al. in 1994 (1) should not disguise the fact that the pregnancy obtained has been the only one in a large mammal to be

reported in the international literature. Patients must be made aware that the future of grafts is still unknown and that they have not proved their reliability. No pregnancy has been achieved in humans. The first demands for the use of cryo-preserved ovaries should occur in about 5 years' time. This leaves time for progress to be made by teams working in this field. Each experiment will bring its quota of information, which will further the understanding of the mechanisms involved in the revival of ovarian grafts. Experimental studies on large mammals are still necessary.

Acknowledgment: The authors thank Les Laboratoires Organon, Villefontaine, France, for their financial support.

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