FERTILITY AND STERILITY®
VOL. 70, NO. 1, JULY 1998

Published by Elsevier Science Inc.
Printed on acid-free paper in U.S.A.

Freezing, thawing, and autograft of ovarian fragments in sheep: preliminary experiments and histologic assessment

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Objective: To evaluate the effects of freezing and thawing on the histologic changes in ovarian fragments from sheep and to determine the feasibility of ovarian autografts.

Design: Histologic evaluation of follicles that survived after freezing at −196°C for 2 weeks. Histologic evaluation of ovarian fragments 6 months after the autografts.

Setting: Laboratoire de Zootechnie, Ecole National Véterinaire, Marcy l'Etoile, France.

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

Intervention(s): Cortical fragments were prepared from the right ovary of 6 lambs and were grafted immediately to the contralateral ovarian hilus or were cooled slowly to −196°C in medium containing dimethyl sulfoxide for 2 weeks. The autografts were recovered 6 months later.

Result(s): Histologic examination of ovarian slices after freezing showed no destruction of primordial, primary, secondary, or cavitary follicles. The ovarian autograft showed good recovery of the macroscopic and microscopic ovarian structure. After autografting, histologic examination revealed primordial to cavitary follicles.

Conclusion(s): Freezing of ovarian fragments is possible without damaging the follicles. Ovarian autografts showed good recovery of ovarian structure. (Fertil Steril® 1998;70:124–8. ©1998 by American Society for Reproductive Medicine.)

Key Words: Autograft, cryopreservation, ovary, follicle

Many young patients undergo pelvic radiotherapy or chemotherapy for pelvic tumors or for malignant hemopathies. The trend among oncologists is to administer more intensive radiotherapeutic and chemotherapeutic treatments (1). The harmful side effects of these treatments on ovarian function are well described and are proportional to the dose and duration of treatment (2).

A growing number of these patients are presenting with ovarian insufficiency (3). It is impossible to predict with any accuracy when the onset of this condition might be (4). Oophorectomy with ovarian conservation during treatment would be an ideal solution for these patients. After remission, an autograft of ovarian tissue would preserve both ovarian endocrine function and the patient's fertility.

There are practical problems (5), however. For example, it is impossible to conserve whole organs for longer than several days, and the ovary is no exception to this rule. Nevertheless, freezing of ovarian fragments is possible to recover ovarian function after autografting (6).

We report a preliminary study of orthotopic autografting of ovarian fragments. We also studied the histologic consequences of freezing and thawing of the ovary.

MATERIALS AND METHODS

Surgical Procedure

Six "Grivette" ewes, between the ages of 6 months and 1 year, underwent an operation at the Lyon Veterinary School. The Zootechnic Laboratory is approved by the French ministry for animal experimentation. Right oophorectomy was performed during a median laparotomy. The right ovary was transferred immediately to BM 1 medium (Elio Bio Media, Paris,

Received November 4, 1997; revised and accepted February 27, 1998.

Financial support provided by Organon, Serifontaine, France.

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0015-0282/98/$19.00
PII S0015-0282(98)00095-8

France). The ovarian cortex was dissected down the length of one side using a scalpel to obtain pieces no thicker than 2 mm. The cortex was then divided into 3 equal pieces: one for histologic analysis, one for freezing, and one piece for the ovarian autograft.

The ovarian autograft was performed during the same laparotomy. The left ovary was sutured at the level of the 2 lateral pedicles using Prolene 3/0 (Ethnor, Neuilly, France). It was then dissected 2 cm along the ovarian pedicle, and the pedicle was clamped. The entire ovary was dissected, leaving only a small portion of the median hilus measuring 0.5 cm × 0.5 cm. The ovarian tissue obtained from the right ovary was then fixed to the left hilus with 6 sutures using Prolene 8/0 (Ethnor). The ovarian pedicle was released and then was sutured using 2 stitches of Prolene 4/0 (Ethnor). The laparotomy was then closed, and the lambs were returned to pasture for ≥6 months. During a second laparotomy, the autograft was removed and sent for histologic analysis.

Freezing and Thawing Procedures

The ovarian fragments were transferred within the hour to 10% dimethyl sulfoxide for 5 minutes (Sigma, St. Louis, MO) and then were frozen with a descent curve of 2°C/min to −140°C (LC 40; CFPO, Bussy Saint George, France). The seeding was semiautomatic at −7°C. The fragments were then kept for 15 days in liquid nitrogen at −196°C.

Thawing was performed for 1 minute in a receptacle that was placed in water with a temperature of 37°C. The ovarian fragments were then washed for 30 minutes in survival medium (BM 1) 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) were fixed for 2 days in 0.1:1,000 formol. Each ovarian fragment was cut into serial 10-μm sections, stained with hematoxylin and eosin, and embedded in paraffin. All fragments were cut into sections. All sections were examined with a microscope at a magnification of × 20. The description of Turnbull et al. (7) from 1997 was used to define primordial, primary, secondary, and cavitary follicles.

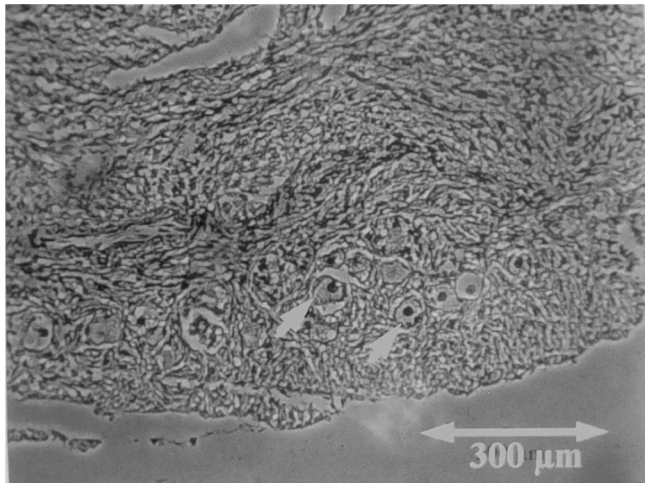
RESULTS

The animals recovered well from the operations. Freshly exised cortical fragments contained numerous primordial, primary, and secondary follicles. Several cavitary follicles were visible. Freezing and thawing caused no damage to the primordial follicles. The nuclei and cytoplasm were unaltered by freezing at −196°C. Primary and secondary follicles were not diminished in number after freezing and thawing (Fig. 1).

All of the grafts were retrieved during the second laparotomy. The autograft ovaries had an ovoid form, and their volume was similar to that of the normal ovary. Two ovaries were covered by peritoneal adhesions but were easily identifiable beneath the peritoneum.

FIGURE 1

Ovarian cortical slice after 2 weeks of freezing and thawing. The tissue contains primordial follicles (*arrows*). *Bar* = 300 μm.



Histologic examination of the grafts showed an increased density of vessels, indicating the restoration of vascularization. The tissue was more inflammatory and fibrosed than normal. The histologic results are summarized in Table 1. With the exception of one graft, all contained primordial, primary, secondary, and cavitary follicles (Figs. 2 and 3). The follicles were not counted because the original number present in the implants was unknown and was not controlled, but we believe that there were slightly fewer follicles. Nevertheless, this reduction of primordial follicles did not unfavorably affect recovery of the maturation cycle; all of the grafts contained primary and secondary follicles as well as cavitary follicles. The distribution of primordial follicles was identical in the frozen and grafted samples.

DISCUSSION

Ovarian transplantation has been performed for research purposes for many years. In 1906, Carrel and Guthrie (8) transplanted a cat ovary and pedicle to another animal. After a second attempt at ovarian transplantation in 1906, this time in a woman (9), it would be another 50 years before more experimental work was done in this field (10–13). The aims of these early ovarian transplant procedures were not clear. Some investigators used this procedure as a simple method to measure the ovarian steroid level, whereas others thought it would be beneficial to remove the ovary and pedicle from the irradiation field in oncology.

Ovarian grafting without vascular anastomosis was de-

TABLE 1

Follicular survival and development in grafted fresh and frozen stored ovarian slices.

Sheep no.	Biopsy type	Survival and development of indicated follicles			
		Primordial follicles	Primary follicles	Secondary follicles	Cavitary follicles
684	Control	+	+	+	+
684	Frozen	+	+	+	+
684	Grafted	+	+	+	+
1,130	Control	+	+	+	+
1,130	Frozen	+	+	+	+
1,130	Grafted	−	−	−	−
731	Control	+	+	+	+
731	Frozen	+	+	+	+
731	Grafted	+	+	+	+
1,137	Control	+	+	+	+
1,137	Frozen	+	+	+	+
1,137	Grafted	+	+	+	+
997	Control	+	+	+	+
997	Frozen	+	+	+	+
997	Grafted	+	+	+	+
998	Control	+	+	+	+
998	Frozen	+	+	+	+
998	Grafted	+	+	+	+

veloped to avoid the complications of microsurgery (14). The procedure of ovarian implantation is quite simple and well described. The ovary is cut into fragments of cortex of a thickness not exceeding 1–2 mm. The fragment is then fixed by fine sutures to a heterotopic or orthotopic site. Our study demonstrates recovery of the implant in an orthotopic site of grafting. Histologic analysis confirms the survival of primordial follicles and the beginning growth stages of some of these after autografting.

In the early stages of our study, we were not yet concerned with obtaining pregnancies, but merely assessed the feasibility of the surgical procedure. An orthotopic autograft may allow spontaneous pregnancies (15). Heterotopic autografts can restore ovarian secretion. Medical assistance would be necessary to obtain a pregnancy (16).

It has always been important to preserve the fertility of

FIGURE 2

Nonfrozen ovarian autograft recovered 6 months after autografting shows primordial follicles (*arrows*). *Bar* = 300 μm.

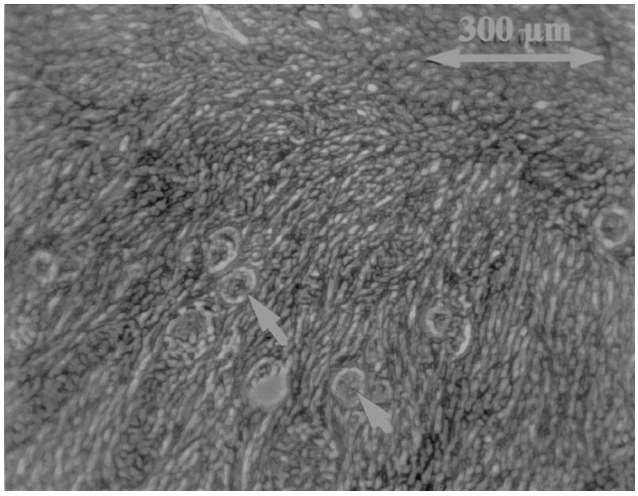
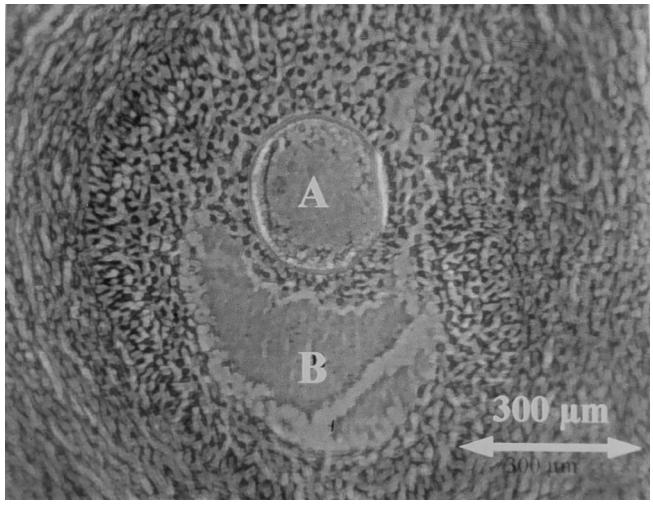


FIGURE 3

Nonfrozen ovarian autograft recovered 6 months after autografting shows cavitary follicle. *Bar* = 300 μm. *A* = oocyte; *B* = cavity.



female patients undergoing cancer treatment. Several potential strategies exist for helping such patients. Embryos may be stored frozen after IVF and oocyte recovery (17). Unfortunately, this procedure cannot be used for children or for unmarried women. The urgency of anticancer therapy and the patient's frailty also impede the success of IVF.

In the case of maternal death, these embryos are "orphans." In France, the 1994 law on ethics requires both biologic parents to be alive for the transfer of embryos. Oocyte donation can be considered in the case of early menopause, but donation raises many ethical and practical problems because of the scarcity of donors. Another issue is the suitable treatment for women who do not have a partner (18).

Despite recent publications on oocyte freezing, this technique is still hypothetical. The freezing process affects the meiotic spindles and the zona pellucida (19). Chemical protection of the ovary by estrogen-progesterone or by hypophyseal inhibition with LH-releasing hormone analogues has not yet been proved successful. By not stimulating the ovarian parenchyma, one hopes to preserve the follicular reserves. The restrictions inherent in all of these methods do not indicate that there is a place for the freezing of ovarian tissue (20).

Developing autografted follicles have been observed after freezing, thawing, and autografting of ovarian fragments in the mouse and rat. The destruction of developing follicles is not a real problem because it represents $<10\%$ of the ovarian follicular reserves. We have noted on histologic examination that the primordial follicles are totally conserved after freezing and thawing.

In 1994, Gosden et al. (6) froze sheep ovarian fragments. Autografts of these frozen fragments were successful, and 2 normal lambs were delivered 9 months after grafting (6). Candy et al. (20) demonstrated in the marmoset monkey that frozen pieces of ovarian cortex could survive thawing and that follicular growth was reestablished after xenografting under the renal capsule of mice with severe combined immunodeficiency. Newton et al. (21) had shown that follicle development was possible during xenografting in these mice. Follicular growth continued up to the antral stage (22).

An interesting alternative to autografting would be the maturation of primordial follicles in vitro. To our knowledge, publications have dealt only with the maturation in vitro of follicles collected at the cavitary stage in humans (23, 24). In 1996, Eppig and O'Brien (25) reported successful in vitro maturation of primordial follicles in mice. One mouse was born live per approximately 475 primordial follicles (25). In 1997, Wandjy et al. (26) reported in vitro maturation of primordial follicles in the baboon.

We thought it was prudent to study the survival of primordial follicles after freezing (27) to verify the evolutionary recovery of grafts before undertaking experiments on frozen sheep ovary fragments. Our results are encouraging, as are those of other investigators, but the days of an ovary bank similar to the sperm bank are not yet here (28).

Many questions remain (29), including the best choice of cryoprotectant and the reimplantation site for the grafts, which could be orthotopic for natural fertilization or heterotopic in the case of IVF. Furthermore, we cannot calculate the survival time of a graft or tell how many follicles are needed for a resulting pregnancy. Will it be possible one day to freeze the ovary and its pedicle in toto? It would then be possible to perform termino-terminal anastomoses with the near certainty of an evolutionary revival. After the freezing and thawing process, would the products of conception be identical to those obtained by natural fertilization?

The prospects for the transplantation of ovarian fragments are vast, but fundamental research on large mammals is required before we can propose autotransplantation to women who must undergo anticancer therapy.

Acknowledgments: The authors are grateful to Marie Thérèse Poirel from the Ecole Nationale Vetérinaire de Lyon for technical assistance. They also thank Les Laboratoires Organon, Puteaux, France, for their financial support.

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