

Normal pregnancies and live births after autograft of frozen-thawed hemi-ovaries into ewes

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Objective: To evaluate long-term outcome of autotransplantation of cryopreserved hemi-ovaries into ewes.

Design: Animal study.

Setting: University fertility center, Hospices Civils de Lyon; and Ecole Nationale Vétérinaire de Lyon.

Patients(s): Grivette ewes.

Intervention(s): Six hemi-ovaries from 6 ewes aged 6 to 12 months were frozen with a slow cooling protocol using 2 M of dimethyl sulfoxide as cryoprotectant. After dissection of the medulla, the hemi-ovarian cortex was stored at -196°C in liquid nitrogen. Freezing procedure was performed with a programmable freezer. Semiautomatic seeding was performed before crystallization. Four to 6 weeks after the first laparotomy, the left ovary was removed and the frozen-thawed hemi-ovary was sutured.

Main Outcome Measure(s): Mean plasma concentrations of FSH, LH, and progesterone after autotransplantation of frozen-thawed hemi-ovary. Ultrasonography was done to confirm pregnancy. Blood samples were collected weekly to measure FSH, LH, and progesterone. After the first birth, the autografted ovary was removed for histologic examination.

Result(s): Plasma progesterone concentration increased in a regular manner in all ewes except one 4 weeks after the graft. Concentrations of FSH and LH did not reach the menopausal level. Four pregnancies occurred, from which 6 lambs were born. The first delivery of a normal lamb occurred after 135 days of gestation; the lamb died immediately after birth. The second delivery of two normal lambs occurred after 130 days of gestation. A caesarean section was performed on the third pregnant ewe the 110th days of gestation because the ewe had a vaginal prolapsus. The two normal lambs and the ewe died after surgery. The fourth birth of a normal lamb occurred after 132 days of gestation. Histologic examination of the grafted frozen-thawed ovary showed a regressing corpus luteum and few primordial and antral follicles.

Conclusion(s): These four pregnancies in a ewe model may indicate that women who undergo preservation of their ovaries before chemotherapy or radiotherapy can have successful pregnancy. (Fertil Steril® 2002;77: 403–8. ©2002 by American Society for Reproductive Medicine.)

Key Words: Follicle, cryopreservation, autograft, ovarian tissue, ewe, pregnancy

Cryopreservation of ovaries may be a way to preserve the fertility of women who must undergo medical treatment that results in sterility (1). In vitro fertilization can be performed before chemotherapy and radiotherapy, and embryos can be frozen for later embryo transfer. If the patient has no partner, oocytes can be frozen for later fertilization. However, although a few pregnancies have been reported, the results of oocyte freezing are not yet satisfactory (2). In vitro fertilization and embryo freezing or frozen oocytes will always be limited by the time required to perform these

procedures and by the number of embryos or oocytes frozen.

Ovary cryopreservation is sometimes proposed by medical teams caring for very young women and prepubescent girls (3, 4), but the future of frozen ovaries remains hypothetical. Three alternatives are possible: ovarian autograft (5, 6), ovarian xenograft (7), and in vitro maturation of primordial ovarian follicles (8–10).

Deanesly (11) reported the first attempt at graft after freezing and thawing in the rat. Six

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years later, Parrott (12) reported on the fertility of mice after use of frozen grafted tissue. After the pregnancy reported by Gosden in 1994 (13), many teams reported fundamental research on ovarian histology or physiology after frozen-thawed autograft in ewes. Oktay and Karlikaya (14) performed autotransplantation of a cryopreserved ovary fragment behind the left pelvic peritoneum in a 29-year-old woman. Six months later, ultrasonography and serum levels of ovarian steroids showed that the graft was functioning in the medium term. However, the number of pregnancies reported in animal experiments after ovary autotransplantation remains low. No pregnancies have been reported in women. Pregnancies in mice (15) and rats (5), and a single pregnancy in a ewe (13), have been reported after freezing-thawing and autotransplantation.

We have been working for the past 5 years on ovary cryopreservation. We have reported our experience on the histology (16) of the ovary after freezing-thawing and the resumption of steroid secretion (17) in the ewe after a frozen-thawed autograft. We found a primordial follicle survival rate of 92% with a slow cooling protocol using dimethyl sulfoxide as cryoprotectant (18). Here, we report four pregnancies and three births after frozen hemi-ovarian autografting in ewes.

MATERIALS AND METHODS

Study Animals

Between September and November 1999, six Grivette ewes 1 to 1.5 years of age 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 and fallopian tube ligation were performed. After removal, the right ovary was sectioned sagittally. The medulla was removed by using scissors; the remaining hemiovarian cortex was 1 mm thick, 2 cm long, and 1 cm wide. It was immediately frozen in a programmable freezer and stored at -196°C in liquid nitrogen.

Thirty to 45 days later, during another laparotomy, the left ovary was completely removed under the microscope, leaving only the hilum. After thawing, the right hemiovarian cortex was sutured on the hilum by using 8 to 10 stitches of 9.00 Prolene suture, under a microscope (Ethicon, Somerville, NJ). During this laparotomy, great care was taken with the left fallopian tube extremity. After the laparotomy opening was closed, the ewes were returned to pasture and normal husbandry condition and were placed with fertile rams. Any treatment was administered, because restoration of spontaneous fertility was expected. The protocol was approved by the ethics committee of the Ecole Nationale Vétérinaire de Lyon.

Freezing-Thawing Protocol

We used the freezing procedure that we reported elsewhere (16). In brief, the 12 hemi-ovaries were placed into

cryogenic vials (Nunc Brand Products, Denmark) containing 1 mL of BM1 freezing medium (Elio Bio Media, Paris, France), supplemented with 10% fetal cord serum and the cryoprotectant. The BM1 contained human albumin, cholesterol, glucose, lactate, penicillin, streptomycin, vitamins, and nonorganic 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 cooled at the same rates: in the first cooling phase ($2^{\circ}\text{C}/\text{min}$), they were cooled to -35°C , and in the second cooling phase ($25^{\circ}\text{C}/\text{min}$), to -140°C . During the first descent, before reaching T_2 (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 to 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 cryoprotectant from the tissue.

Histologic Analysis

The frozen-thawed ovarian autograft was fixed for 2 days in 0.1:1,000 formalin (10%). The fixed ovarian graft was embedded in paraffin, cut into serial $5\text{-}\mu\text{m}$ sections, and stained with hematoxylin and eosin. All fragments were cut into sections, and all sections were examined by using a microscope (magnification, $\times 100$). The description of Turnbull and colleagues was used to define primordial, primary, secondary, and antral follicles (19).

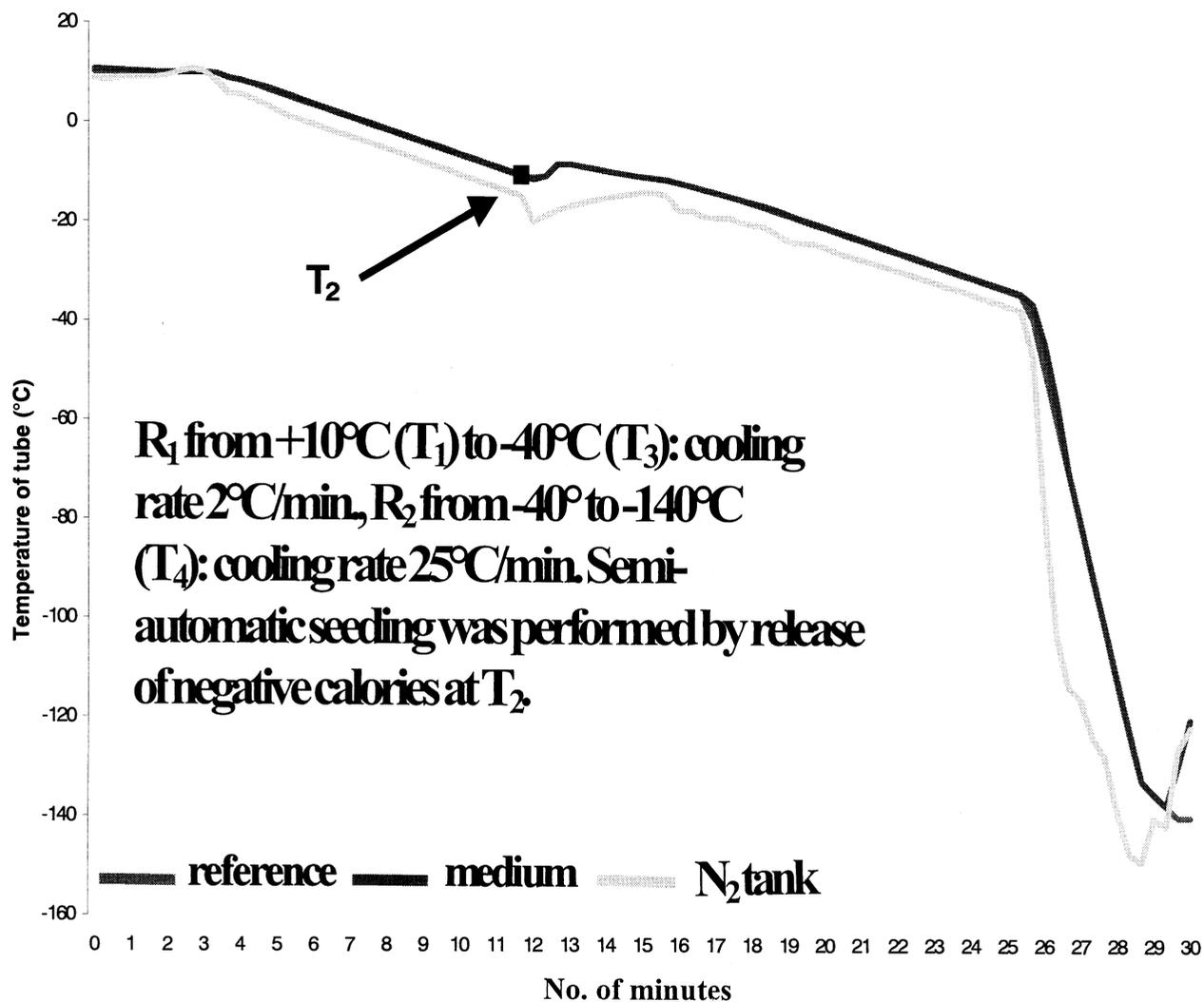
Hormone Dosages

Starting 3 weeks to 1 month after autografting, blood samples were obtained weekly to determine serum concentrations of progesterone, FSH, and LH. Progesterone concentrations were determined at the Ecole Nationale Vétérinaire de Lyon. Concentrations of FSH and LH were determined at the Institut National de Recherche Agronomique (INRA, Tours, France).

Gonadotropins were measured by using enzyme immunoassays. The intraassay and interassay coefficients of variation were 5% and 10%, respectively. Progesterone was also measured with by using an enzyme immunoassay; the intraassay and interassay coefficients of variation were both 12%. All blood samples were obtained during July and August 2000.

FIGURE 1

Results of cooling with semiautomatic seeding. The temperature of tubes containing ovarian tissue is plotted as a function of time. = reference; = medium; = N₂ tank.



Salle. Births after frozen-thawed ovarian tissue into ewes. *Fertil Steril* 2002.

RESULTS

Four weeks after autografting, serum progesterone reached a physiologic concentration. Only one ewe had progesterone levels <3 nmol/L. In the other ewes, the pattern of the increase in progesterone level indicated that the grafts had resumed function. Follicle-stimulating hormone and LH never reached concentrations equivalent to ovary insufficiency.

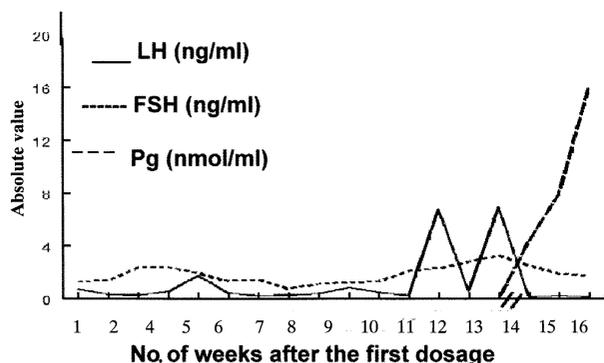
Figure 2 shows the mean secretion pattern on one ewe. Concentrations of FSH and LH decreased once progesterone concentrations increased, indicating a pregnancy. Four ewes started a gestation during the summer of 2000 (Table 1).

Ultrasonography in mid-September 2000 confirmed two single pregnancies and two twin pregnancies. Ultrasonography dated the beginning of the pregnancies to the summer of 2000.

The first ewe spontaneously gave birth to a male lamb. The lamb weighed 6 kg and lived for only a few hours. Autopsy revealed no malformations. The cause of this premature death is thought to be dystocia at the moment of delivery owing to macrosomia. The second birth of two lambs was uneventful; they are in good health and are progressing normally. Cesarean section was performed to deliver the second twin pregnancy because the ewe had a

FIGURE 2

Mean plasma concentration of progesterone (Pg), FSH, and LH in one pregnant ewe that received a frozen-thawed hemiovary on 17 February 2000.



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vaginal prolapsus. The two lambs died because of premature birth, and the ewe died the day after the cesarean section. The fourth pregnant ewe gave birth to a normal and healthy lamb.

Two days after the first birth, the ewe underwent a third laparotomy. The frozen-thawed ovarian autograft was of subnormal size. No peritoneal adhesions were seen. The anatomic relations between the grafted ovary and the fallopian tube were normal. The graft had a regressing corpus luteum. Histologic examination showed a regressing corpus luteum, a few primordial follicles, and antral follicles above the Prolene sutures. The connective tissue located below the Prolene ligatures contained no follicle elements (Fig. 3). We did not perform ovariectomy of the grafted ovary in the other 4 so that we could evaluate their long-term fertility. Ewes were placed with rams 2 months after the delivery.

TABLE 1

Evolution and outcome of pregnancies in lives after hemiovarian frozen-thawed autografting.

Ewe	Type of pregnancy	Days of gestation	Route of delivery	Outcome
36	Single	135	Vaginal	Male lamb, 5 kg; died
514	Twin	130	Vaginal	Male and female lambs, 3 and 3.1 kg
227	Twin	110	Caesarean section	Male and female lambs, 1.5 and 2.8 kg; died
6	Single	132	Vaginal	Male lamb, 3 kg
538	None	0	0	0
635	None	0	0	0

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DISCUSSION

Many studies of ovarian cortex cryopreservation have been reported (19). Most these studies have investigated the histologic consequences of ovarian freezing-thawing (20, 21).

There are still few reports of pregnancies after autotransplantation of ovarian fragments (22). Pregnancies have been reported in rats, mice, and ewes. However, it is difficult to draw conclusions that are relevant to humans from the pregnancies obtained in rats and mice. The fertility of these animals is much higher than in humans, and they have an ovarian bursa that greatly favors the graft surgical procedure.

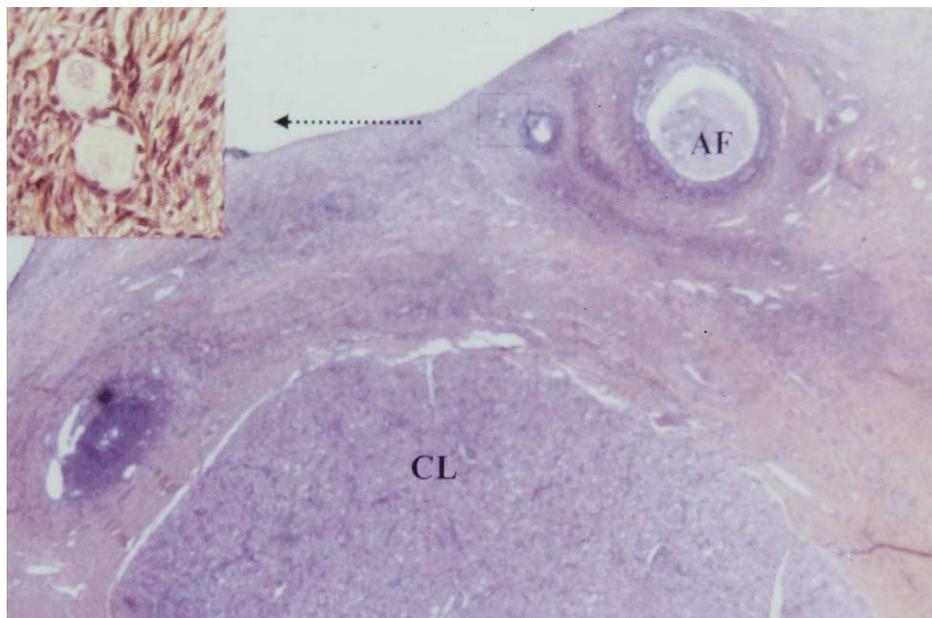
The sheep model is of great interest because sheep are large mammals whose ovarian architecture is very close to that of human women. Furthermore, the ovarian cortex is thick, the size of the ovary approaches that of the human ovary, and the number of subcortical primordial follicles is high. In ewes, it has been shown that autotransplantation of frozen ovarian fragments can last in the long term. After ovarian autograft, Baird et al. (23) reported that during the breeding season (6 months), all ewes 5 to 10 estrus cycles. Twenty-two months after the autograft, all transplanted ovaries contained antral follicles but very few oocytes, and the decrease in the number of primordial follicles was drastic. However, no pregnancies were reported after autograft of frozen-thawed ovarian fragments.

We observed a decreased number of primordial follicles 10 months after ovarian autograft. Histologic analysis of the entire graft was impossible because of the high volume of the grafted ovary. We checked the density of the primordial follicles in only one ewe after the first birth; the other ewes will not undergo ovariectomy so that we can evaluate the function of the graft and fertility in the long term. If ovarian tissue banking is to become more than an experimental procedure, both the reproducibility of our protocol and the permanence of the ovarian graft must be evaluated.

In our study, pregnancies were obtained 6 to 9 months after autografting. The originality of our freezing method and ovarian cortex preparation allowed us to freeze large-sized fragments. The most important factor in freezing is the thickness of the cortex; it must be as thin as possible, so that the cryoprotectants may penetrate the cortex, where the primordial follicles are stored. Our graft procedure is original because we removed the contralateral ovary in its entirety, leaving only the hilus but no residual ovarian cortex. The placement of the regressing corpus luteum and the appearance of the graft indicate that the follicle resulting in pregnancy came from the graft of the frozen-thawed hemiovarian cortex. Histologic analysis confirmed the presence of a regressing corpus luteum and primordial and antral follicles located at a distance and above the surgical sutures. This placement is important because it confirms the absence of residual follicles after the left ovariectomy. The FSH and LH

FIGURE 3

Histologic appearance of the frozen-thawed ovarian autograft. A regressing corpus luteum (CL) is present, representing a recent pregnancy. A few primordial (top right) and antral (AF) follicles are located beside the corpus luteum.



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concentrations that we observed never reached castration concentrations after the left ovariectomy; the volume and the surface of the frozen hemi-ovary may explain the lack of a high increase in FSH and LH rates.

Many medical teams have proposed or performed ovarian preservation. The future of autografting is an important problem: It is difficult to explain to a patient that it is still experimental and that no patient has yet had natural fertility restored with this procedure. Oktay and Karlikaya (14) reported on an autograft in a 29-year-old woman. Even though the graft resumed functioning, its subperitoneal position will not allow a spontaneous pregnancy.

The four pregnancies in ewes that we reported here, 7 years after the report of Gosden et al. (13), demonstrate how difficult the procedure is. Nonetheless, they offer great hope for women. Standardization of the freezing protocol and the surgical procedures will make ovarian preservation a viable option. However, many avenues of animal experimentation remain: the lifespan of the grafts, the ideal volume of the ovarian fragments, use of an autograft after treatment of a condition that may lead to metastases in the ovary, and improvement of the freezing protocol.

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References

1. Byrne J, Fears TR, Gail MH. Early menopause in long-term survivors of cancer during adolescence. *Am J Obstet Gynecol* 1992;166:788-93.
2. Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum Reprod* 2001;16:411-6.
3. Hovatta O, Silye R, Krausz T, Hardy K, Winston RM. Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants. *Hum Reprod* 1996;11:1268-72.
4. Gosden RG. Low temperature storage and grafting of human ovarian tissue. *Mol Cell Endocrinol* 2000;163:125-9.
5. Aubard Y, Lavignac C, Grandjean MH, Piver P, Teissier MP. Autogreffes orthotopiques de fragments ovariens chez le rat avec grossesse. *Contracept Fertil Sex* 1996;24:852-5.
6. Aubard Y, Piver P, Cognie Y, Fermeaux V, Poulin N, Driancourt MA. Orthotopic and heterotopic autografts of frozen thawed ovarian cortex in sheep. *Hum Reprod* 1999;14:2149-54.
7. Oktay K, Newton H, Gosden RG. Transplantation of cryopreserved human ovarian tissue results in follicle growth initiation in SCID mice. *Fertil Steril* 2000;73:599-603.
8. Cortvrindt R, Smitz J, Van Steirteghem AC. In vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepubertal mice in a simplified culture system. *Hum Reprod* 1996;11:2656-66.
9. Eppig J, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* 1996;54:197-207.
10. Picton HM, Gosden RG. In vitro growth of human primordial follicles from frozen-banked ovarian tissue. *Mol Cell Endocrinol* 2000;166:27-35.
11. Deanesly R. Immature rat ovaries grafted after freezing and thawing *J Endocrinol* 1954;11:197-200.

12. Parrott DMV. The fertility of mice with orthotopic ovarian grafts derived from frozen tissue. *J Reprod Fertil* 1960;1:230–41.
13. Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196°C . *Hum Reprod* 1994;9:597–603.
14. Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *N Engl J Med* 2000;342:1919.
15. Carroll J, Gosden RG. Transplantation of frozen-thawed mouse primordial follicles. *Hum Reprod* 1993;8:1163–7.
16. Salle B, Lornage J, Franck M, Isoard L, Rudigoz RC, Guerin JF. Freezing, thawing, and autograft of ovarian fragments in sheep: preliminary experiments and histologic assessment. *Fertil Steril* 1998;70:124–8.
17. Salle B, Lornage J, Demirci B, Vaudoyer F, Poirel MT, Franck M, Rudigoz RC, Guerin JF. Restoration of progesterone secretion and histological assessment after freezing, thawing and autograft of hemiovary in sheep. *Fertil Steril* 1999;72:366–70.
18. Demirci B, Lornage J, Salle B, Franck M, Frappart L, Guerin F. Follicular viability and morphology of sheep ovaries after cryoprotectant exposure and cryopreservation with different freezing protocols. *Fertil Steril*. 2001;75:754–62.
19. Turnbull KE, Braden AWH, Mattner PE. The pattern of follicular growth and atresia in the ovine ovary. *Aust J Biol Sci* 1977;229–41.
20. Newton H, Aubard Y, Rutherford A, Sharma V, Gosden R. Low temperature storage and grafting of human ovarian tissue. *Hum Reprod* 1996;11:1487–91.
21. Candy CJ, Wood MJ, Whittingham DG. Effects of cryoprotectants on the survival of follicles in frozen mouse ovaries. *J Reprod Fertil* 1997;110:11–9.
22. Candy CJ, Wood MJ, Whittingham DG. Follicular development in cryopreserved marmoset ovarian tissue after transplantation. *Hum Reprod* 1995;10:2334–8.
23. Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG. Long-term ovarian function in sheep after ovariectomy and transplantation of auto grafts stored at -196°C . *Endocrinology* 1999;140:462–71.