Restoration of ovarian function after autotransplantation of intact frozen-thawed sheep ovaries with microvascular anastomosis

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Objective: To test the feasibility of transplanting an intact frozen-thawed ovary with microvascular anastomosis of the ovarian vascular pedicle to the deep inferior epigastric vessels.

Design: Chronic survival study.

Setting: Biological Resources Unit, The Cleveland Clinic Foundation.

Animal(s): Adult merino ewes.

Intervention(s): Bilateral laparoscopic oophorectomy was performed on 17 synchronized ewes. In one group of animals (Group I, n = 11), both ovaries were cryopreserved intact with their vascular pedicles. In another group of animals (Group II, n = 6), ovarian cortical strips were prepared from each ovary and cryopreserved. After thawing, follicular viability and apoptosis rates were assessed using one ovary. The other ovary was transplanted to the abdominal wall with microvascular anastomosis (Group I). In Group II, the ovarian cortical strips were placed in the anterior abdominal wall. Ovaries were harvested after 8–10 days in situ and subjected to histological evaluation.

Main Outcome Measure(s): Blood flow, apoptotic signals, follicular viability, serum estradiol (E2), follicle-stimulating hormone (FSH), and histology.

Result(s): No significant differences were found in the mean values of apoptosis (mostly in the atretic and some secondary follicles) and follicular viability in both groups. In Group I, immediate and long-term patency were documented in 100% and 27% (3/11) of the grafts, respectively; and postoperative FSH levels were similar to preoperative values in animals with patent vessels. In Group II, postoperative FSH levels were significantly higher than the preoperative ones (P = .03).

Conclusion(s): Transplantation of an intact frozen-thawed ovary is technically feasible. Using this approach, immediate restoration of vascular supply and ovarian hormonal functions is possible. (Fertil Steril® 2003;79:594–602. ©2003 by American Society for Reproductive Medicine.)

Key Words: Cryopreservation, ovarian transplantation, vascular anastomosis, apoptosis, follicular viability

Cryopreservation of ovarian tissues carries the hope of safeguarding the reproductive potential for women receiving sterilizing cancer therapy (1). Transplantation of frozen-thawed ovarian cortical strips and hemiovaries without vascular anastomosis gave rise to pregnancies/live births in many studies in several species such as sheep (2, 3), mouse (4), mouse intact ovaries (5), and rats (6). The human trials have shown promising results that have some limitations that require some more research.

The limited length of ovarian function in some human ovarian transplant cases using nonvascularized grafts may be partially due to ischemic injury until revascularization occurs (7–9). Therefore, the reduction of ischemia time and immediate revascularization are crucial for long-term survival and function of the grafts (10). We postulate that transplanting intact ovaries with vascular anastomosis may circumvent the problems associated with the use of the ovarian tissue slices. A previous study
demonstrated that a successful pregnancy was achieved after transplantation of intact frozen-thawed rat ovaries in the upper genital tract (11).

Autotransplantation of intact fresh sheep ovaries with vascular anastomosis to the deep inferior epigastric vessels is technically feasible (12). In this study, long-term patency of the anastomosis was achieved in three of the six cases, with an excellent outcome of the transplanted ovary showing no significant signs of tissue damage. The cryopreservation of an intact ovary with its vascular pedicle is quite a challenging procedure due to:

1. Factors affecting the cryosurvival of ovarian tissues are mostly unknown.
2. Tissue cryopreservation presents serious physical constraints related to heat and mass transfer.
3. The dynamics of cryoprotective agents are poorly understood (13).
4. Adequate diffusion of cryoprotectant into large tissue masses is difficult.

We postulated that it would be feasible to transplant an intact cryopreserved-thawed ovary with a vascular anastomosis. To test this hypothesis, the study was designed to:

1. Evaluate the feasibility of cryopreservation and transplantation of intact sheep ovary with vascular anastomosis.
2. Compare it with transplantation of frozen-thawed ovarian cortical strips without vascular anastomosis.

**MATERIALS AND METHODS**

**Animals**

This study was approved by the Institutional Animal Research Committee of the Cleveland Clinic Foundation. Seventeen adult, nonpregnant merino ewes, weighing 55–70 kg, were included in this chronic survival study. Surgical procedures were performed at the Cleveland Clinic Foundation Biological Resources Unit, in accordance with the facility’s Standard Operating Procedures (IACUC protocol). The animals were cared for according to the standards of the U.S. Public Health Policy of the Humane Care and Use of Laboratory Animals (PHS Manual, Ch. 143).

All animals were synchronized by using megestrol acetate, Ovaban (Schering Plough, Kenilworth, NJ) in a dose of 0.55 mg/kg/day for 10–12 days, followed by 5,000–10,000 IU Profasi (Serono, Randolph, MA) 34–36 hours before surgery. Animals were divided into two groups:

1. Animals for intact ovary cryopreservation and transplantation (Group I, n = 11).
2. Animals for ovarian cortical strips cryopreservation and transplantation (Group II, n = 6).

**Laparoscopic Oophorectomy**

After induction of general anesthesia and endotracheal intubation, the animal was secured on the operating table. A 10-mm 0° laparoscope was inserted through an umbilical incision. Pneumoperitoneum was created with CO2 at 10 L/min and 12 mm Hg of intraperitoneal pressure. The animal was placed in a slight Trendelenburg position, and inspection of the pelvis was performed. Ancillary 5-mm trocars were inserted through the right and left lateral skin incisions 5 cm below and 8 cm lateral to the umbilicus. A third 5-mm trocar was placed at the level of the camera port.

The ovarian vessels were identified, and their course was traced from the hilum cephalad. The ovary was dissected off the uterine horn oviduct, and the infundibulopelvic blood vessels were dissected of the surrounding tissue. The skeletonized blood vessels were double ligated as proximal as possible with nonabsorbable 1-0 multifilament silk suture using intracorporeal ligature technique. The ovary was removed through one of the 5-mm trocars and biopsied. The same process was repeated on the other side.

**Cryopreservation**

**Cryopreservation of the Intact Ovary**

The grafts were perfused with heparinized Ringer’s solution, followed by perfusion and immersion in a bath containing Leibovitz L-15 medium (Irvine Scientific, Santa Ana, CA), 10% fetal calf serum (Irvine Scientific), and 1.5 M dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO) immediately after oophorectomy. The ovarian vessels and excess hilar tissue were dissected, and ovarian ligaments were trimmed. The grafts were perfused via the ovarian artery with the cryoprotective mixture using Horizon Nxt Modular Infusion System (McGaw Inc., Irvine, CA) to maintain a flow rate at 1.3 mL/min until continuous replenishment of the reservoir.

After perfusion, the ovary was transferred to a 5 mL 12.7 mm × 92 mm cryovial (Corning Coaster Corporation, Cambridge, MA) containing the cryoprotective mixture for controlled freezing using Planer cryochamber (Planer Freezer Ltd., Middlesex, United Kingdom). Cooling began at 4°C and at 2°C.min⁻¹ until ice nucleation was induced at −7°C. The temperature was then reduced at 2°C.min⁻¹ until −35°C and, subsequently, at 25°C.min⁻¹ until −140°C before the cryovials were plunged into liquid nitrogen.

**Cryopreservation of the Ovarian Cortical Strips**

The ovarian cortical strips were prepared as described by Gosden et al. (2) and cryopreserved using the previously mentioned protocol but without perfusion.

**Thawing**

**Thawing of the Intact Ovary**

One week later, the vial was removed from the Dewar and held for 1 minute at room temperature before plunging and freezing.
swirling it in a bath of water at 37°C with gentle shaking. The contents of the vial were quickly emptied into a Petri dish containing Leibovitz L-15 medium supplemented with 10% fetal calf serum. The ovary was washed and immediately perfused with Leibovitz L-15 medium supplemented with 10% fetal calf serum, using the same flow rate as described earlier, for 20 minutes. The cryoprotectant was gradually eliminated by pumping Leibovitz L-15 medium, supplemented with 10% fetal calf serum, into the reservoir.

**Thawing of the Ovarian Cortical Strips**

Ovarian strips were thawed using the same procedure described previously with Group I. Then, they were washed and held in Leibovitz L-15 medium containing 10% fetal calf serum for 20 minutes, and then transplanted.

**In Vitro Assessment of the Cryopreserved-Thawed Ovarian Tissue**

For each animal, one ovary was transplanted while the other was used for in vitro studies including viability and apoptosis assays.

**Evaluation of the Ovarian Follicular Viability Using Trypan Blue Test**

In both groups, ovarian fragments were thinly sectioned in Leibovitz L-15 medium supplemented with 1 mg/mL (200 IU/mL) type 1 collagenase (Sigma), incubated at 37°C for 2 hours, and pipetted every 30 minutes. Collagenase activity was inhibited by 50% fetal calf serum. The suspension was filtered through a 70-μm nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ) and centrifuged at 400 g for 5 minutes. The precipitate was diluted with 50 μL of Leibovitz L-15 medium and conserved in a water bath at 37°C. Trypan blue (0.4%, Sigma) was added to the suspension containing the follicles (20 μL), deposited on a glass slide, and examined under an inverted microscope (400×). For each fragment, 100 small, intact follicles (<6 μm in diameter) were examined, while the partially or completely denuded oocytes were excluded.

**Evaluation of the Ovarian Apoptosis Using Terminal Deoxynucleotidyl Transferase (TdT)-Mediated Deoxyuridine Triphosphate (dUTP)-Digoxigenin Nick-End Labeling (TUNEL)**

The corresponding tissue sections were used for the TUNEL assay to detect apoptosis (QIA33-TDT-FragEL™ kits; Oncogen Research Products, Boston, MA). Briefly, after deparaffinization and dehydration, the sections were rinsed in phosphate-buffered saline (PBS) (pH 7.2), digested by proteinase K (20 μg/mL) at room temperature, and then rinsed in distilled water. The peroxidase activity was blocked by 2% hydrogen peroxide. After the application of an equilibration buffer, slides were incubated in TdT enzymes for 1.5 hours (at 37°C). The reaction was halted by a stop solution; then, the slides were rinsed in PBS. The incorporated nucleotides were identified by adding peroxidase streptavidine-conjugate.

Slides were then incubated with 3,3′-diaminobenzidine and counterstained with 5% methyl green. Positive controls were obtained from the manufacturer. The controls consisted of formaldehyde-fixed, paraffin-embedded sections from HL60 promyelocytic leukemia cells and HL60 cells, which were incubated with 0.5 μg/mL actinomycin D for 19 hours in order to induce apoptosis. Some ovarian tissue specimens were used as negative controls by substituting a microliter of distilled water for the deoxynucleotidyl transferase from the protocol, as suggested by others (14, 15). For the initial assessment of TUNEL-positive cells, following other researchers (16, 17), the results of TUNEL assay were evaluated according to the signal intensity as follows: −, weak; +, moderate; +++, intense; +++++, very intense. Then, all slides were examined at 400× magnification. Positively labeled cells were counted in a defined visual field. Ten different areas of each follicular wall were examined, and the mean number of positively stained cells was determined.

**Autotransplantation**

**Autotransplantation of the Intact Ovary (Group I)**

The animal was prepared as mentioned previously. An incision (3–4 cm) was made on the anterior abdominal wall, near the site of the 5-mm trocar, in order to expose the deep inferior epigastric vessels. A branch with a caliber matching that of the ovarian vessels was identified and dissected. Standard microsurgical instrumentation and Acland vascular microclamps were used for the anastomosis. Microvascular anastomosis was performed using 8–10 interrupted sutures (9-0 or 10-0 prolene) in an end-to-end fashion under Zeiss surgical microscope (Carl Zeiss, Oberikochen, Germany). Diluted heparin was applied topically as required. The ovary was fixed between the bundles of the rectus muscle. After surgery, all animals were anticoagulated using 5,000 IU sodium heparin (Eli Lilly and Company, Indianapolis, IN) s.c., b.i.d., for 3 days.

**Autotransplantation of the Ovarian Cortical Strips (Group II)**

A small incision was made and a segment of the deep inferior epigastric vessels was dissected in order to create a pocket in the rectus abdominis muscle. The cortical strips of one ovary were aligned in the created pocket and fixed using few sutures (9-0 or 10-0 prolene).

**Postoperative Assessments**

After 8–10 days, the transplant was inspected for macroscopic appearance, presence of visible follicles, and pulsations at the arterial anastomosis and patency of microvascular anastomoses. Viability of the ovarian transplant was also evaluated by checking the bleeding from the edges at a small incision. The transplant was then removed and dissected for further evaluation.
Assessment of baseline and postgrafting endocrine functions, and in vitro assessment of follicular viability and apoptosis assay of both groups.

| TABLE 1 |
|---------------------------------|-----------------|-----------------|----------------|
|                                  | Intact ovary (Group I, n = 11) | Patent vascular anastomosis (n = 3) | Occluded vascular anastomosis (n = 8) | Ovarian cortical strips (Group II, n = 6) |
|---------------------------------|-----------------|-----------------|----------------|
| Baseline mean ± SD              | Postgrafting mean ± SD | Baseline mean ± SD | Postgrafting mean ± SD | Baseline mean ± SD | Postgrafting mean ± SD | P value |
|---------------------------------|-----------------|-----------------|----------------|
| FSH (pg/mL)                     | 182 ± 70.3      | 120 ± 99.7      | 210 ± 109      | 105 ± 66.1      | 249 ± 80.8      | 0.03   |
| E₂ (ng/mL)                      | 166 ± 50.0      | 199 ± 115       | 282 ± 132.0    | 129 ± 33.4      | 154 ± 28.9      | 0.19   |

Note: SD = standard deviation; FSH = follicle-stimulating hormone; E₂ = estradiol.


Hormonal Assay

Venous blood from the animal was aseptically drawn in sterile 10-mL tubes, containing 0.2 mL of heparin at a concentration of 1,000 U/mL, before laparoscopic oophorectomy and before removal of the graft. The collected blood samples were centrifuged at 1,600 rpm for 7 minutes, and the serum was isolated and stored at −70°C until examination.

Evaluation of the Serum Estradiol (E₂)

Quantitative measurement of serum E₂ was performed using the Automated Chemiluminescence Immunoassay System (ACS: 180°; Bayer, Tarrytown, NY).

Evaluation of Follicle-Stimulating Hormone (FSH)

The measurement of the ovarian FSH levels, by radioimmunoassay, was performed at the Reproductive Endocrinology Laboratory at Colorado State University, Fort Collins, Colorado, as previously described. This measurement was performed using NIH antiovine FSH (A780) as an FSH-antibody and A853 (ARGG) as a second antibody. The data were analyzed using the RIANAL program (Colorado State University). Baseline FSH levels were obtained on the day of surgery (before oophorectomy) and on the day the transplant was removed.

Histological Evaluation

The ovarian tissues were Bouin-fixed, paraffin-embedded, H&E stained for histological evaluation of the primordial follicle count, presence of primary and secondary follicles, infarction, and apoptosis following previously established criteria (18). These criteria included: condensed nuclear fragments nuclei with marginated chromatin; multiple nuclear fragments; a single condensed nucleus; membrane-bound structures containing variable amounts of chromatin and/or cytoplasm; and eosinophilic cytoplasm (19).

Statistical Analysis

Analysis of variance (ANOVA) was used to compare the preoperative to postoperative values (P<.05) using SAS version 8.1 software (SAS Institute Inc., Cary, NC).

RESULTS

The Follicular Viability

The primordial follicular viability in cryopreserved intact ovaries (83.2 ± 3.79) was comparable to that of the ovarian cortical strips (82.2 ± 4.12) as evidenced by viability staining (P=.621) (Table 1 and Fig. 1).

TUNEL Assay

None of the negative controls showed TUNEL-positive immunoreactivity, while positive signals were consistently observed in the positive controls. The evaluation of apoptosis in ovarian tissues revealed the lack of apoptotic signals in the healthy (primordial, primary, and most of the secondary) follicles. The TUNEL-positive signals were mainly detected in the nuclei of granulosa cells of the atretic and, occasionally, in some secondary follicles. These positively stained nuclei were observed either in the central layers of the membrana granulosa, at the antral surface, or floating in the follicular antrum. Apoptosis was absent both in the interstitial cells and in the theca cells of the atretic follicles. The mean values of apoptotic cells in cryopreserved intact ovaries (2.44 ± 0.867) were comparable to that of the ovarian cortical (2.05 ± 0.99) as evidenced by combined histological
assessment and TUNEL assay (*P*=.12). Most of the apoptotic bodies were observed in secondary and atretic follicles (Table 1 and Figs. 2A and 2B).

**Immediate Vascular Patency**

Upon removing the microclamps at the end of the microvascular anastomosis, blood flow was documented for at least 20 minutes in all 11 transplants, documenting immediate postoperative success. In all transplants, microsurgical anastomosis of the ovarian artery and vein to branches of the deep inferior epigastric vessels was performed in end-to-end fashion. Matched segments of the deep inferior epigastric vessels were selected to avoid any caliper discrepancy.

**Long-Term Vascular Patency**

Eight to ten days following transplantation, the previously vascularized grafts were evaluated in situ and then removed for further evaluation. At the follow-up, 27% (3/11) of the transplants were viable and did not show any signs of necrosis. Arterial bleeding was documented at an incision made at the surface of the ovaries, documenting long-term patency of the anastomosis. In eight cases, the anastomosed vessels were completely occluded, leading to immense tissue loss. The ischemia time, which was defined as the sum of the time from removal of the ovary to cryopreservation plus the time from thawing to transplantation, was not significantly different between patent and occluded vessel groups (3.92 ± 0.355 vs. 4.03 ± 0.162 hours).

**Serum E₂ and FSH**

In Group I, no significant change occurred in serum E₂ levels before and after transplantation in either the patent vessel group (166.0 ± 50.0 pg/mL vs. 163.0 ± 32.6 pg/mL) or the nonpatent vessel group (199.0 ± 115.0 pg/mL vs. 282.0 ± 132.0 pg/mL), indicating that a small remnant of surviving ovarian cortex tissue was sufficient for the resumption of E₂ production (Table 1). In Group II, no significant difference was found in the level of E₂ before and after the transplantation (129.0 ± 33.4 pg/mL vs. 154.0 ± 28.9 pg/mL).

Serum FSH in the patent vessel group did not change significantly from pre- to posttransplantation (182.0 ± 70.3 ng/mL vs. 172.0 ± 42.0 ng/mL, *P*=.84), whereas a significant rise was observed in the nonpatent vessel group (103.0 ± 89.7 ng/mL vs. 268.0 ± 109.0 ng/mL, *P*=.0053), suggesting ovarian failure. In Group II, a significant difference was also found in the level of FSH after the transplant (147.0 ± 66.1 ng/mL vs. 249.0 ± 80.8 ng/mL, *P*=.038) (Table 1).

**Histology**

In Group I, the mean count of primordial follicle number, per high magnification field after transplantation, revealed a significantly higher number of follicles in the patent as compared with the nonpatent vessel group (3.67 ± 2.08 vs. 0.250 ± 0.463, *P*=.001, Fig. 3A). Scattered areas of necrosis were observed on histological assessment of the patent vessel group, whereas transplants in the nonpatent vessel group showed severe necrosis with thin peripheral rim of viable tissue.

For the patent vessel group, histological evaluation revealed:

1. No signs of necrosis in the 1-mm cortex rim.
2. Necrosis of most of the growing follicles, denoting that they did not survive the freeze-thaw process and the ischemia-reperfusion injury (Fig. 3B).
3. Viable patent vessels with an intact muscularis layer; however, some of the intraovarian vessels showed partial transmural necrosis. These vessels showed active regeneration of the vascular endothelium over the necrosed segment (Fig. 3C).

In both groups, the granulosa cells with the histological features of apoptosis were mostly observed in atretic and occasionally in some secondary follicles. These cells were observed either within the central region of the membrana granulosa layer or loosely attached to the membrana granulosa near its antral surface or in the antral follicular fluid (Figs. 3B and 3C).

**DISCUSSION**

Although the intact small-sized ovaries from mice and rats can survive freezing, successful cryopreservation of intact large-sized ovaries from other mammals such as human is still challenging due to: heat and mass transfer limitations; ischemia reperfusion injury; and, more important, intravascular ice-formation problems (20). The rate of cryoprotective agent/cellular water exchange is affected by the amount of tissue through which that agent must diffuse.
During the cooling stage of cryopreservation, the relative distance of cells in the interior of the ovary from the exterior affects the rate at which these cells undergo cooling. Therefore, cryopreserving small pieces (1–2 mm³) of ovarian cortex is considered technically necessary. Because current transplantation procedures do not utilize vascular anastomosis, grafts are completely dependent on posttransplantation vascularization. In this regard, the ability of the cells in the graft to obtain nutrients from their surroundings before permanent revascularization depends upon:

(A). The postthaw histological characteristics of the primordial (1), primary (2), secondary (3), and atretic (4) ovarian follicles from intact ovary; (B). TUNEL assay: Arrows in Figure B1 show primordial follicles; B2 shows secondary follicles; and B3 shows granulosa cells next to follicular antrum, all of which showed TUNEL-negative signals. The arrow in B4 shows positive apoptotic signals in atretic follicle. All the photographs were obtained from Group I (magnification 79×).
the vascularity of the transplant recipient site; the diffusion rate of these nutrients to the transplant; and the time needed for full revascularization to be completed. The process of graft preparation from removal of the ovary to the end of the cryopreservation steps was not associated with increased apoptosis in either group, emphasizing the impact of post-grafting revascularization in graft survival. These results are in agreement with a recent report by Liu and associates (21). Many reports in the literature have attributed a high rate of follicular loss to postgrafting ischemia (13, 22).

The practical solutions for this problem are to:

1. Develop an efficient cryopreservation protocol that allows cryopreservation of an entire ovary, with its vascular pedicle, with minimal tissue damage.
2. Develop a surgical technique that allows successful transplantation of the frozen/thawed ovary with microvascular anastomosis to an anatomically suitable site.

In this study, we tried to address these problems.

Previous studies proved that cryoinjury mainly affects the central part of the ovary. To diminish this problem, we perfused the ovary with the cryoprotectant via its vascular channel. Optimum cryoprotectant, perfusion volume, perfusion temperature, and perfusion pressure are mandatory for the success of this procedure. Using a special pump system to deliver the cryoprotectant in the ovarian vascular network might be of value to ensure even diffusion among ovarian tissue.

Our cryopreservation protocol proved to be satisfactory as evidenced by the in vitro studies performed. Primordial follicle viability in an intact cryopreserved ovary proved to be comparable to that of ovarian cortical strips, proving that cryopreservation by perfusion does not affect the immediate postthaw viability of the primordial follicles.

Moreover, in agreement with a previous study (23), cryopreservation and freeze-thaw trauma were associated with insignificant apoptotic changes.

In our study, the mean values of these apoptotic changes in the secondary and atretic follicles of intact frozen-thawed ovaries were insignificantly higher as compared with those in frozen-thawed ovarian cortical strips. The underlying mechanisms of such a lack of significant increase in the apoptotic activity in the intact ovary group, as compared with the ovarian cortical slices, are still open for further investigations. Although the effects of freeze-thaw trauma could not be refuted (24–26), these insignificantly higher apoptosis mean values, mostly observed in the atretic follicles, may reflect the preexisting atretic changes instead of the effect of cryopreservation or the occasional apoptosis in the endothelial cells. The absence of apoptotic signals in the primordial follicles lends further support to the fact that apoptosis is a downstream event that requires active metabolism and usually takes several hours or even days to become manifest (27–29).
In the second part of this experiment, we vascularized thawed ovaries using the deep inferior epigastric vessels by means of microvascular anastomosis under surgical microscope. Immediate patency of the vascular pedicle of all transplanted ovaries confirmed that the ovarian vascular channel system was not grossly damaged by the cryopreservation protocol used; however, long-term patency was obtained in 27% of the vascularized grafts. The possible explanations for the long-term failure in eight cases are:

1. The extreme tortuosity of the ovarian vessels, making them at high risk for thrombosis.
2. Cryopreservation might have had adverse effects on the intima of the blood vessels, predisposing them to thrombosis and anastomotic failure.

No significant rise of postoperative FSH occurred in sheep with patent anastomosis, denoting that, within 1 week of the transplant, the hypothalamo-pituitary ovarian axis was intact and little tissue damage occurred. Although secondary follicles were necrotic, tissue survival was excellent in the cortical part of the ovaries but with areas of necrosis near center.

A recent primate study showed spontaneous ovulation after subcutaneous transplantation of ovarian cortical strips (30). Furthermore, spontaneous ovulation occurred in a recently reported human case (8). Overall, pregnancy was not achieved in the primate study or in the human case, where pregnancy was desired. This may be partially explained by the initial posttransplant ischemic injury of the graft or inadequate follicular maturation.

Although no significant difference in E2 levels between animals with patent anastomoses and those with occluded ones, FSH levels were significantly lower in animals with patent anastomoses. This could be explained by lack of inhibin production in animals with occluded anastomosis (31). Spontaneous recovery of ovarian function after transplantation can be delayed by 3 to 4 months because ischemia results in the loss of virtually the entire growing follicle population, in addition to the 50% of the primordial follicles. The growth of a primordial follicle to a large preantral stage takes about 85 days. Some animal studies, however, have shown secretion of progesterone after 4 weeks (32) and, therefore, a return to normal FSH levels before the 3- to 4-month period.

Rapid regeneration of the intimal layer of the blood vessels over a partially necrotic vascular wall was a striking feature among the deeper vessels of the ovaries. This observation confirms that, even when tissue damage is induced by the cryopreservation insult and ischemia-reperfusion injury, it still has the potential to regenerate. This is particularly important for the supporting tissue of the ovary. It appears that complex mechanisms from ice formation during the freezing process, and from reperfusion injury, play a role in cellular or architectural damage to the capillary bed as well as the vascular endothelium. This is also evidenced by our observation in histological examinations of specimens from the patent vessel group that demonstrated the presence of partial transmural necrosis, indicating cryoinjury. Although our study did not evaluate the long-term vascular patency and viability of ovarian tissue, based on some evidence in the literature that cryopreserved vessels can completely reendothelialize within 1 to 2 months postoperatively, it can be speculated that the damaged vessels observed in our study would normally heal and further contribute to ovarian viability (33).

In conclusion, we have shown that an intact ovary of a sheep can survive cryopreservation-thawing insults with reasonable tissue viability. This may be due, in part, to the perfusion of the cryoprotectant through the vascular channel. Autotransplantation of an intact, frozen-thawed ovary with microvascular anastomosis is technically feasible with good short-term results; however, long-term survival and function of these ovaries should be investigated.

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