

Follicular viability and histological assessment after cryopreservation of whole sheep ovaries with vascular pedicle by vitrification

Blandine Courbiere, M.D.,^a Jérôme Massardier, M.D.,^a Bruno Salle, M.D., Ph.D.,^{a,b}
Claire Mazoyer, M.S.,^b Jean-François Guerin, M.D., Ph.D.,^{a,b} and
Jacqueline Lornage, M.D., Ph.D.^{a,b}

^aUnité INSERM 418, Hôpital Debrousse, and ^bDépartement de Médecine et de Biologie de la Reproduction et du Développement, Hôpital Edouard Herriot, Lyon, France

Objective: To evaluate a cryopreservation technique by vitrification of whole ovaries with their vascular pedicle in sheep, by using two cryoprotectant solutions.

Design: Animal study.

Setting: Fertility clinic in a university teaching hospital.

Animal(s): Five to 6-month-old ewes.

Intervention(s): Whole sheep ovaries with their vascular pedicles were collected at the slaughterhouse and prepared for cryoprotectant toxicity tests and freezing procedures.

Main Outcome Measure(s): Follicle viability assessment by trypan blue test and histological examination of ovary and vessel structure.

Result(s): No statistically significant difference in follicle viability or normal primordial follicle rates were observed between ovaries exposed or nonexposed to cryoprotectant solutions. Nor was any statistically significant difference observed before and after vitrification with the two cryoprotectant solutions. The decrease in the number of primordial follicles was smaller when frozen-thawed ovaries were treated with VS4 solution containing dimethyl sulfoxide, formamide, and propylene glycol. There were fewer nuclear anomalies and general follicular anomalies with the VS4 solution. Pedicle fractures occurred in most ovaries during thawing (11/15).

Conclusion(s): Cryopreservation of whole ovary by vitrification appears a promising technique in reproductive medicine. The best histologic results were obtained with the VS4 cryoprotectant. Further studies are required to overcome vitrified ovarian vessel fracture. (*Fertil Steril*® 2005;84(Suppl 2):1065–71. ©2005 by American Society for Reproductive Medicine.)

Key Words: Vitrification, ovarian cryopreservation, cryoprotectant agents, follicular viability, sheep

The cryopreservation of ovarian tissue is a promising technique for the preservation of fecundity in young female cancer patients after sterilization by chemotherapy and/or radiotherapy (1). It could enable ovarian functions to be restored by transplanting ovaries from young patients at risk of sterility or early menopause. Donnez et al. (2) recently announced for the first time that a human pregnancy could be obtained after heterotopic transplantation of cryopreserved ovarian cortical tissue.

Cryopreservation of whole ovaries could enable immature oocytes to be preserved, together with other ovarian cells necessary for the development and maturation of oocytes. The small primordial follicles are the main type of follicle that survives storage at -196°C (3). However, they are so immature that ovarian tissue has either to be grafted back to the donor (autograft) or to a histocompatible recipient (xeno-

graft), or else undergo prolonged in vitro culture, to produce mature oocytes (4–7).

In 1994, Gosden et al. (8) showed that the fertility of surgically castrated sheep could be restored by grafting frozen-thawed ovarian cortical strips onto the ovarian pedicle. Since then, many attempts have been made to cryopreserve ovarian tissue by slow-freezing techniques; only a few, however, have concerned the vitrification procedure.

Vitrification is an alternative approach to cryostorage (9–11). At sufficiently low temperatures, a highly concentrated aqueous cryoprotectant solution becomes so viscous that it solidifies without the formation of ice crystals. Aqueous solutions raise the problems of ice forming inside a biological organism. With slow-freezing protocols, the formation of extracellular ice causes disruption of the intercellular architecture.

For organs, vitrification is the best approach, given the complexity of the tissues and vascular system (12). In 1985, Rall and Fahy (13) reported the first ice-free cryopreservation of mouse embryos by vitrification. They used a cryoprotectant solution called VS1, containing 20.5% wt/vol

Received November 8, 2004; revised and accepted March 14, 2005.

Reprint requests: Jacqueline Lornage, M.D., PhD, Département de Médecine et de Biologie de la Reproduction et du Développement, Hôpital Edouard Herriot, 5 place d'Arsonval, 69 437 Lyon Cedex 03, France (FAX: 33-4-78-77-72-64; E-mail: jlornage@yahoo.fr).

dimethyl sulfoxide, 15.5% wt/vol acetamide, 10% wt/vol propylene glycol, and 6% wt/vol polyethylene glycol in a modified Dulbecco's saline (HB1).

Other investigators subsequently used this VS1 solution and found vitrification to be a useful strategy for the preservation of certain mammalian ovaries (14). Data on the cryopreservation of human ovarian tissue by vitrification are at present limited (15). Other vitrification solutions have been described since. Fahy's team (12) has developed several, including one called VS4, which they tested on rabbit kidneys (16). This solution contains 2.75 M dimethyl sulfoxide, 2.76 M formamide, and 1.97 M propylene glycol. A system of computer-assisted renal artery perfusion has been developed to enable the cryoprotectants to penetrate the kidney (17).

Sheep provide a good model for studying ovarian tissue preservation and transplantation (8). The aim of the present study was to demonstrate the feasibility of cryopreserving a whole ovary with vascular pedicles in sheep. First, two cryoprotectant solutions, VS1 and VS4, were compared for their toxicity for primordial follicles and vessels. As a preliminary step, a system of ovarian vessel perfusion was developed to ensure that cryoprotectants reached the center of the ovary. The second part of the study examined effects of ovarian vitrification using, respectively, VS1 and VS4.

MATERIALS AND METHODS

We collected ovaries with ovarian pedicle from 5- to 6-month-old lambs (breed unknown) at the slaughterhouse. The ovarian vessels were identified and cut so as to be as long as possible. Ovaries and vessels were placed in X-Vivo medium (Bio Whittaker, Walkersville, MD) and were transported to the laboratory at 10°C to minimize ischemia.

Cryoprotectant Toxicity

Vitrification Solutions. Each ovary was perfused via the ovarian artery with heparinized Ringer's solution, followed by perfusion and immersion in a bath containing various concentrations of cryoprotectant agents (CPAs). The VS1 solution comprises four cryoprotectants (Sigma Aldrich, St. Louis, MO): 2.62 M dimethyl sulfoxide, 2.60 M acetamide, 1.31 M propylene glycol, and 0.0075 M polyethylene glycol, diluted in BM1 medium (Eurobio, Les Ulis, France). The second solution, VS4, developed by Kheirabadi and Fahy (16), comprises three cryoprotectants: 2.75 M dimethyl sulfoxide, 2.76 M formamide, and 1.97 M propylene glycol, diluted in BM1 medium.

Addition and Removal of Vitrification Solution. The perfusion rate was 0.35 mL/min, by a peristaltic pump (Watson Marlow, Dreux, France). Cryoprotectant agent was introduced and removed in eight phases. Phases 1, 2, 3, and 4 involved increasing concentrations of cryoprotectants, to allow osmotic equilibration: 5 minutes with CPA at 12.5% at room temperature, 5 minutes with CPA at 25% at room

temperature, 15 minutes with CPA at 50% at 4°C, and 15 minutes with CPA at 100% at 4°C. Phases 5, 6, 7, and 8 consisted of gradual washout at room temperature: 5 minutes with CPA at 50%, 5 minutes at 25%, 5 minutes at 12.5%, and 5 minutes with BM1 medium. Such a stepwise procedure is essential to control CPA toxicity. To offset some of the osmotic stress associated with CPA washout, mannitol was incorporated into the VS4 used in phases 5, 6, and 7: 300 mM mannitol during phase 5, stepped down to 100 mM at phases 6 and 7, and then reduced to 0 mM at phase 8.

Experimental Design. In the first part of our study, there were four experimental groups of five ovaries each, randomly sampled at the slaughterhouse according to an optimal design. Two control groups were established: one group of nonperfused fresh ovaries and one group of ovaries incubated in X-Vivo medium and perfused for 60 minutes with heparinized Ringer's solution without cryoprotectant. The two other groups were exposed to VS1 and to VS4, respectively. After washout, one hemiovary and one piece of the vascular pedicle were fixed in Bouin liquid. The other hemiovary was used to determine the rate of follicle viability.

Cryopreservation of Ovaries by Vitrification

The second part of the study involved two groups. One group of five pairs of ovaries underwent vitrification with VS1; the other group of 10 pairs was vitrified using VS4. Each ovary pair was taken from a single ewe: one ovary was exposed to the CPA and then vitrified, and the controlateral ovary, exposed to CPA but without vitrification, served as control. After perfusion (phases 1, 2, 3, and 4), ovary and vessels were transferred into an ethyl vinyl acetate cryobag (Macopharma, Mouvoux, France) containing the cryoprotectant mix at a concentration of 100%. The samples were then plunged into liquid nitrogen, in which they were held for a minimum of 30 minutes until warming.

Ovary Thawing

After their storage in liquid nitrogen, the vitrified samples were warmed rapidly in a 37°C water bath. Cryoprotectant was removed by reversed concentration gradient perfusion (phases 5, 6, and 7). The ovaries then were washed in BM1 medium for 5 minutes (phase 8). One hemiovary and one piece of the vascular pedicle were fixed in Bouin liquid. The other hemiovary was used to determine the rate of follicle viability.

Isolation of Small Follicles and Ovarian Follicle Viability Assessment by Trypan Blue Test

One hemiovarian medulla was thinly sectioned in Leibovitz L-15 medium (Sigma Aldrich, St. Louis, MO). Fragments were supplemented with 1 mg/mL (200 IU/mL) type I collagenase (Sigma Aldrich, St. Louis, MO) and incubated at 37°C for 2 hours, pipetted every 30 minutes. Collagenase activity was inhibited by addition of 50% fetal calf serum

(Sigma Aldrich). The suspension was filtered through a 60- μm nylon filter (Bioblock Scientific, Illkirch, France) and processed by centrifuge at 400 g for 5 minutes. The precipitate was diluted with 50 μL of Leibovitz L-15 medium. Trypan blue (0.4%, Sigma Aldrich) was added to the suspension containing follicles (20 μL), which was deposited on a glass slide and examined under an inverted microscope (400 X). One hundred small follicles (<60 μm in diameter) were examined from each ovary fragment. Only intact follicles were examined: partially or completely denuded oocytes were excluded. Dead follicles were stained blue, and live ones not.

Histologic Examination

One hemiovary and a fragment of ovarian vessels were Bouin-fixed for 2 days. The fixed ovaries and vessels were embedded in paraffin, cut into serial 4- μm sections, then stained with hematoxylin, phloxin, and safran.

Follicular morphology was examined by microscope (magnification, $\times 400$). For each ovary, 100 primordial follicles were counted in sections where the oocyte nucleus was visible, and their morphology was recorded. Normal follicles had a complete layer of flattened granulosa cells, oocytes with cytoplasm, and a normal nucleus. Abnormal follicles were classified as follows: cytoplasm damage, pyknotic nucleus, and nucleus and cytoplasm damage combined.

Follicular density was estimated by counting primordial follicles in 10 contiguous microscopic fields (magnification, $\times 400$). The part of the ovarian cortex richest in primordial follicles was identified, and the primordial follicles in the 10 contiguous fields were counted. This technique has been validated and is widely used in anatomopathology for estimating cell density in heterogeneous tissue.

Histology of the ovarian artery and vein and of ovarian medulla blood vessels was performed to show the overall tissue architecture. The arterial wall was considered normal if the vascular endothelium, internal elastic membrane, and muscularis were whole. Anomalies were classified as follows: endothelial detachment, internal elastic membrane rup-

ture, or smooth muscle cell bloating. In the case of the veins, only the vascular endothelium was examined.

Statistical Analysis

Summary statistics are presented as means (\pm SEM). Non-parametric tests were used: Kruskal-Wallis test in the first part of the study to compare the four groups and Wilcoxon test in the second part. A Mann-Whitney test was applied to compare ovaries exposed to VS1 and VS4 solutions. Differences were considered statistically significant at $P < .05$. Unistat software (Unistat, London, UK) was used for statistical analysis.

RESULTS

Ovarian Cryoprotectant Toxicity

Ovarian Follicular Viability. Mean small follicle viability was $77.2\% \pm 1.9\%$ for fresh nonperfused ovaries, $77.6\% \pm 2.2\%$ for Ringer's-perfused ovaries, $78.4\% \pm 3.0\%$ for ovaries exposed to VS1, and $80.8\% \pm 1.6\%$ for ovaries exposed to VS4. Differences between the four groups were not significant.

Ovarian Density in Primordial Follicles. Mean small follicle density was 48.4 ± 11.4 for fresh nonperfused ovaries, 74.0 ± 6.9 for Ringer's-perfused ovaries, 52.0 ± 11.8 for ovaries exposed to VS1, and 58.4 ± 16.6 for ovaries exposed to VS4. Differences between the four groups were not significant.

Histologic Examination of the Primordial Follicles. Table 1 shows results of histologic examination. Among fresh ovary primordial follicles, $81.8\% \pm 2.7\%$ presented a normal aspect, compared with $80.4\% \pm 4.2\%$ of those exposed to Ringer's solution. After exposure to VS1 and VS4, the rates were $71.8\% \pm 3.9\%$ and $78.2\% \pm 1.8\%$, respectively. Differences between the four groups were not significant.

Cryoprotectant Toxicity on Vessels. No major histologic anomalies, whether of lumbo-ovarian pedicle vessels or ovarian medulla vessels, as compared with fresh ovary controls, were found secondary to perfusion with Ringer's solution or the vitrification solutions.

TABLE 1

Comparisons of histological examination of primordial follicles between control group and ovaries exposed to Ringer's solution and to two cryoprotectant solutions, VS1 and VS4.

Groups	Intact follicles (%)	Cytoplasm damage (%)	Nucleus damage (%)	Cytoplasm plus nucleus damage (%)
Fresh ovaries	81.8 ± 2.7	15.8 ± 3.0	1.2 ± 0.4	1.2 ± 0.4
Ringer's solution	80.4 ± 4.2	17.4 ± 4.1	0.6 ± 0.2	1.6 ± 0.6
VS1	71.8 ± 3.9	23.6 ± 3.0	1.6 ± 1.0	2.4 ± 0.2
VS4	78.2 ± 1.8	21.2 ± 4.4	0.6 ± 0.4	2.6 ± 0.6

Note: Values are mean \pm SEM.

Courbiere. Cryopreservation of sheep ovaries by vitrification. *Fertil Steril* 2005.

TABLE 2

Comparison of histological examination of primordial follicles with and without vitrification with two cryoprotectant solutions, VS1 and VS4.

Group	Intact follicles (%)		Cytoplasm damage (%)		Nucleus damage (%)		Cytoplasm plus nucleus damage (%)	
	FC	V	FC	V	FC	V	FC	V
VS1	74.0 ± 8.4	25.2 ± 7.0	14.4 ± 4.5	15.0 ± 8.4	10.4 ± 9.6	33.4 ± 12.1	1.2 ± 0.5	26.4 ± 7.4
VS4	84.3 ± 0.8	53.5 ± 3.2 ^a	10.8 ± 0.9	38.8 ± 2.8 ^a	5.2 ± 2.9	1.3 ± 0.5	3.3 ± 0.8	6.4 ± 1.09

Note: Values are mean ± SEM. FC = fresh control ovaries; V = vitrified ovaries.

^a $P < .05$.

Courbiere. Cryopreservation of sheep ovaries by vitrification. *Fertil Steril* 2005.

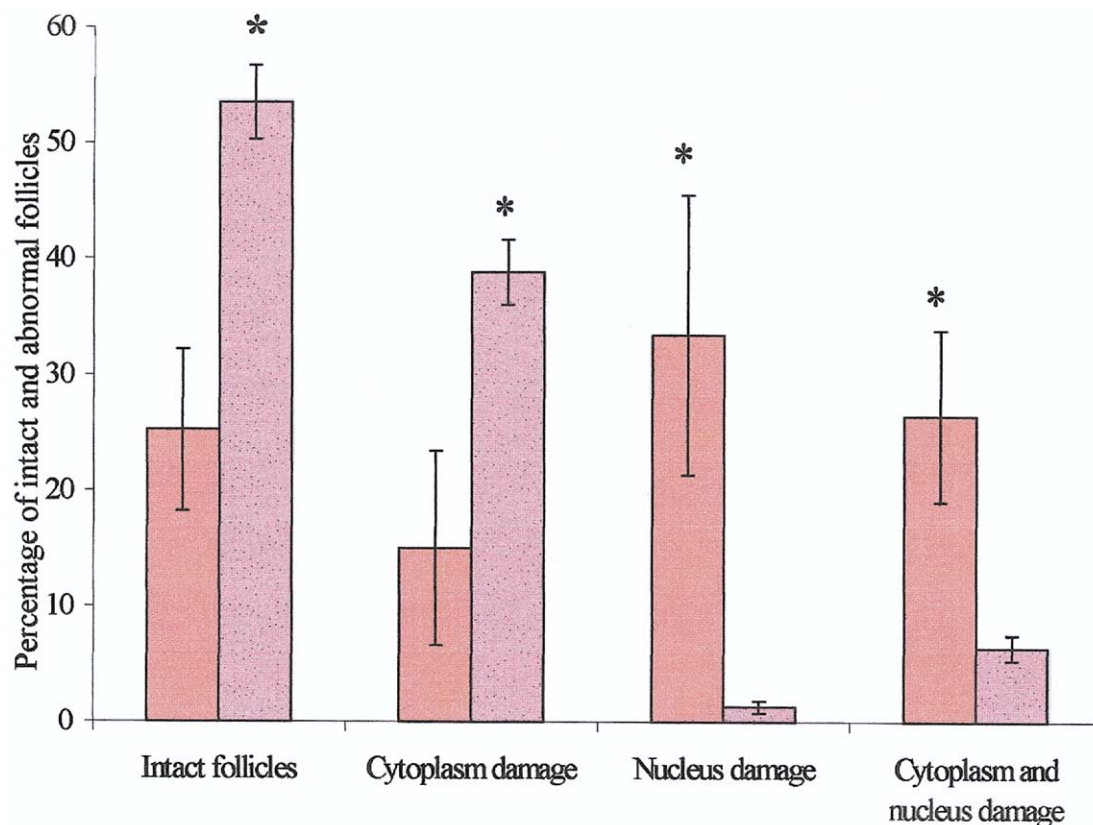
Cryopreservation of Ovaries by Vitrification

Ovarian Follicle Viability. Follicle viability fell from 75.6% ± 1.1% without vitrification to 68.2% ± 1.9% secondary to vitrification with VS1, and from 68.0% ± 3.8% to 60.7% ± 2.4% secondary to vitrification with VS4. The difference between the two groups was not significant.

Ovarian Primordial Follicle Density. Follicle density fell non-significantly from 40.6 ± 10.5 without vitrification to 23.0 ± 5.7 secondary to vitrification with VS1. With VS4, follicle density was 55.0 ± 9.2 without vitrification, and 51.5 ± 8.4 secondary to vitrification (non significant). Follicle density remained significantly higher secondary to vitrification with VS4 ($P < .05$).

FIGURE 1

Histological comparison of ovaries that were frozen-thawed with VS1 solution (red bars) and VS4 solution (purple bars): percentage of intact and abnormal follicles. Bars represent mean ± SEM. * $P < .05$.



Courbiere. Cryopreservation of sheep ovaries by vitrification. *Fertil Steril* 2005.

TABLE 3

Results of histological assessment of cryopreserved blood vessels with the VS1 solution.

Pair of ovaries	Medullary artery		Medullary vein		Ovarian artery		Ovarian vein	
	FC	V	FC	V	FC	V	FC	V
1	BM	BM, IEM	0	0	0	0	0	0
2	0	BM	0	ET	0	0	0	0
3	0	BM, ET	0	0	0	0 ^a	0	0 ^a
4	0	BM, ET	0	ET	0	0 ^a	0	0 ^a
5	0	BM	0	0	0	0 ^a	0	0 ^a

Note: FC = fresh control ovary; V = vitrified ovary; BM = bloated smooth muscle cells; ET = endothelial cell damage; IEM = rupture of internal elastic membrane; 0 = general histology considered normal.

^a Macroscopic fracture during thawing.

Courbiere. Cryopreservation of sheep ovaries by vitrification. *Fertil Steril* 2005.

Histologic Examination of the Primordial Follicles. As shown in Table 2, the percentage of normal primordial follicles fell after vitrification, with 25.2% ± 7.0% of follicles remaining normal secondary to vitrification with VS1 and 53.5% ± 3.2% remaining normal secondary to vitrification with VS4. As shown in Figure 1, there were more postvitrification cytoplasmic anomalies (38.8% ± 2.8%) with VS4 (*P* < .05) and were more nuclear (33.4% ± 12.1%) and combined anomalies (26.4% ± 7.4%) with VS1 (*P* < .05).

Assessment of Vessel Tissue Quality After Vitrification.

Fractures occurred in vessels during thawing in 3 of 5 cases with VS1 and in 8 of 10 cases with VS4.

The fractures ran perpendicular to the vessel wall and were invariably at right angles to the long axis of the vessels. They always extended around the vessel circumference and had clean-cut edges. Nevertheless, catheters to reperfuse the distal artery and ovary to wash out the cryoprotectants were able to be fitted without difficulty.

Tables 3 and 4 show that most vessels presented histologic anomalies secondary to vitrification. Bloated smooth muscle cells in the medulla arterioles of the ovarian core were found systematically with VS1, and in 80% of cases with VS4. Vascular endothelium lesions were found in 70% of ovarian arteries that were frozen-thawed with VS4.

TABLE 4

Results of histological assessment of cryopreserved blood vessels with the VS4 solution.

Pair of ovaries	Medullary artery		Medullary vein		Ovarian artery		Ovarian vein	
	FC	V	FC	V	FC	V	FC	V
1	0	BM, IEM	0	0	0	ET	0	ET
2	0	BM	0	ET	0	ET ^a	0	0 ^a
3	0	BM	0	ET	0	ET ^a	0	0 ^a
4	0	0	0	0	0	ET ^a	0	ET ^a
5	0	BM, ET, IEM	0	ET	0	0 ^a	0	0 ^a
6	0	BM, ET	0	0	0	ET	0	0
7	BM	BM	0	0	0	ET, IEM ^a	0	0 ^a
8	0	0	0	ET	0	0 ^a	0	0 ^a
9	0	BM, IEM	0	0	0	ET, IEM ^a	0	0 ^a
10	0	BM, ET	0	0	0	0 ^a	0	0 ^a

Note: FC = fresh control ovary; V = vitrified ovary; BM = bloated smooth muscle cells; ET = endothelial cell damage; IEM = rupture of internal elastic membrane; 0 = general histology considered normal.

^a Macroscopic fracture during thawing.

Courbiere. Cryopreservation of sheep ovaries by vitrification. *Fertil Steril* 2005.

DISCUSSION

The present study showed that intact primordial follicles can be preserved after vitrification of a whole ovary with vascular pedicle in sheep. To the best of our knowledge, there have been no previous reports of vitrification of a whole ovary from a large mammal such as a ewe: such whole-ovary cryopreservation so far has been described only in mice (18, 19) and rats (14). Vitrification has the advantage of preventing intracellular ice formation, because the cryoprotectant solution is highly concentrated. The very low temperatures make the solution extremely viscous, so that it solidifies without ice forming (9–11).

Sugimoto et al. (14) used VS1 and observed that rat ovary endocrine functions were preserved after vitrification and transplantation, but with a decreased number of small and large follicles. Migishima et al. (19) used DAP 213 (2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol) as a cryoprotectant for mouse ovaries. Maintained fecundity was observed after orthotopic transplantation, but with significantly lower fertility than controls. After transmission electron microscopy ultrastructural study, Salehnia et al. (20) showed that no noticeable changes occurred in the organelles of oocytes and follicular cells after vitrification of mouse ovaries.

The results of the first part of our study demonstrated that the ovary can tolerate perfusion with a vitrifiable concentration of cryoprotectant: no fall in follicle viability or in percentage of normal follicles was found with exposure to VS1 or VS4 cryoprotectants. Vascular system integrity also appears to be preserved, although a functional study with ovarian transplant would be needed to confirm this.

Two vitrification solutions were tested for optimal whole-ovary cryopreservation. After vitrification, the best combination of cryoprotectants was the VS4 solution. After an initial series of 5 ovary pairs, the VS4 group was enlarged to 10 pairs to confirm the first findings. In VS4, the acetamide of VS1 is replaced by formamide to speed up cryoprotectant permeation and avoid carcinogenicity. Propylene glycol is increased to compensate for the poor vitrifiability of formamide. High-molecular mass species such as polyethylene glycol are avoided so as to control viscosity and thus enhance perfusability (21, 22). Ovarian artery perfusion by peristaltic pump ensured uniform cryoprotectant penetration throughout the ovarian tissue.

Primordial follicle viability proved comparable in fresh and frozen-thawed ovaries (75.6% versus 68.2% with VS1 and 68.0% versus 60.7% with VS4). This shows that cryopreservation does not affect the immediate postthaw viability of the primordial follicles. The trypan blue test is a useful and quick method to attest the initial quality and viability of follicles but does not provide an exhaustive ultrastructural study of oocyte status. Moreover, it assesses only intact follicle viability and fails to take account of follicle loss by cellular lysis, for which it is important to associate concomitant study of follicle density.

After vitrification, histological follicle anomalies were principally cytoplasmic in the case of VS4 and nuclear in that of VS1. However, there remained $53.5\% \pm 3.2\%$ histologically normal follicles with VS4, as compared with $25.2\% \pm 7.0\%$ with VS1. Follicular viability assessment and histological study do not, however, enable any definitive conclusion as to oocyte status. A functional study admittedly will be needed in the future. The follicle density in VS1-vitrified ovaries was lower than in fresh controls ($P > .05$), whereas it was conserved after vitrification with VS4 ($P < .05$).

The unfavorable histological results observed with VS1 may be explained by osmotic injury during cryoprotectant washout. Samples vitrified with VS1 showed results with greater variances (SEM high, Table 2). With the VS4 protocol, a sugar (mannitol) was added during washout to reduce osmotic damage. Another possible cause of damage to cryopreserved ovaries is devitrification with ice formation that may occur during thawing.

The high incidence of postthaw fractures observed was attributed to stress during rapid warming. Gross circumferential fractures occurred in the vessel wall. Vessel fracture after vitrification has been widely reported in the literature (9, 23). Slower rewarming could be a means of avoiding fractures.

There were many vascular histological anomalies after vitrification. Arterial muscularis smooth muscle cell bloating was the most frequent anomaly in medulla vessels in the core of the ovary. It may be that the cooling rate was too slow for core cells, resulting in lesions caused by ice crystals forming. Among ovarian arteries, 70% showed endothelial lesions secondary to VS4 vitrification. The reason for this may have been mechanical, because of perfusing tissue that was rendered fragile by freezing. Such lesions were not encountered with the use of VS1, perhaps thanks to its greater viscosity. This histological study was insufficient for any firm conclusion to be drawn as to the viability of cryopreserved vessels: a functional study of the vessels will be needed.

Cryopreservation of a whole ovary plus vascular pedicle appears technically feasible. In sheep, reimplanted frozen-thawed cortical strips could result in normal estrous cycles for nearly 2 years despite a drastic reduction in the total number of follicles (24, 25). A high rate of follicle loss is attributed to postgraft ischemia (25). Transplantation of an intact ovary with microvascular anastomosis would minimize this postgraft ischemic follicle loss and conserve the ovarian graft functions. A few studies have attempted this after a slow-freezing protocol in animal models such as rats (26) or sheep (27). But most studies to date have been based on the cryopreservation of ovarian cortical strips with heterotopic (2, 28) or orthotopic graft (2, 28, 29). There already have been some reports of clinical application in humans (4, 5, 30).

In clinical practice, we envisage that one of the pair of ovaries would be left in situ with an intact pedicle available

so more easily to replace with the frozen–thawed organ once the patient has been treated with chemotherapy or radiotherapy. One major problem surrounding whole-ovary cryopreservation is the possibility that the ovary might be harboring malignant cells, with the risk of relapse after reimplantation (31). Ovarian biopsies could be examined by light microscopy or xenografted into immunodeficient mice (32). Nevertheless, zero risk cannot be guaranteed, and strict surveillance after reimplantation will be necessary.

One of the most serious concerns in our study was vascular injury. It is a fear that the endothelial lesions frequently encountered after freezing could cause vascular thromboses. Bedaiwy et al. (27) reported autotransplantation of 11 intact frozen–thawed sheep ovaries with microvascular anastomoses. Thrombosis occurred in the anastomosed vessels in 8 cases. The extreme tortuousness of ovarian vessels and the adverse effects of cryopreservation may explain these thromboses.

In conclusion, we have shown that a whole sheep ovary can survive vitrification with good immediate follicular viability and acceptable histological results. We have to extend this study to optimize our vitrification protocol. The cryopreservation of entire organs is one of the greatest challenges in cryobiology. Further studies along these lines are necessary to improve cryopreservation by vitrification for various organs, including the ovary.

Acknowledgments: The authors thank Lucien Frappart, M.D., Ph.D., for precious advice and the staff of the anatomopathology laboratory of Hôpital Edouard Herriot for technical assistance.

REFERENCES

- Aubard Y, Poirot C, Piver P, Galinat S, Teissier MP. Are there indications for ovarian tissue cryopreservation? *Fertil Steril* 2001;76:414–5.
- Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* 2004;364:1405–10.
- Shaw JM, Oranratnachai A, Trounson AO. Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology* 2000;53:59–72.
- Radford JA, Lieberman BA, Brison DR, Smith AR, Critchlow JD, Russell SA, et al. Orthotopic reimplantation of cryopreserved ovarian cortical strips after high-dose chemotherapy for Hodgkin's lymphoma. *Lancet* 2001;357:1172–5.
- Oktay K, Buyuk E, Veeck L, Zaninovic N, Xu K, Takeuchi T, et al. Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. *Lancet* 2004;363:837–40.
- 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.
- Wolfe BA, Wildt DE. Development to blastocysts of domestic cat oocytes matured and fertilized in vitro after prolonged cold storage. *J Reprod Fertil* 1996;106:135–41.
- Gosden RG, Baird DT, Wade JC, Webb R. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at –196 degrees C. *Hum Reprod* 1994;9:597–603.
- Baudot A, Odagescu V. Thermal properties of ethyleneglycol aqueous solutions. *Cryobiology* 2004;48:283–94.
- Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. *Fertil Steril* 2002;78:449–54.
- Liebermann J, Nawroth F, Isachenko V, Isachenko E, Rahimi G, Tucker MJ. Potential importance of vitrification in reproductive medicine. *Biol Reprod* 2002;67:1671–80.
- Fahy GM, Wowk B, Wu J, Phan J, Rasch C, Chang A, et al. Cryopreservation of organs by vitrification: perspectives and recent advances. *Cryobiology* 2004;48:157–78.
- Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at –196 degrees C by vitrification. *Nature* 1985;313:573–5.
- Sugimoto M, Maeda S, Manabe N, Miyamoto H. Development of infantile rat ovaries autotransplanted after cryopreservation by vitrification. *Theriogenology* 2000;53:1093–103.
- Isachenko E, Isachenko V, Rahimi G, Nawroth F. Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen. *Eur J Obstet Gynecol Reprod Biol* 2003;108:186–93.
- Kheirabadi BS, Fahy GM. Permanent life support by kidneys perfused with a vitrifiable (7.5 molar) cryoprotectant solution. *Transplantation* 2000;70:51–7.
- Fahy GM. Organ perfusion equipment for the introduction and removal of cryoprotectants. *Biomed Instrum Technol* 1994;28:87–100.
- Salehnia M. Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. *Exp Anim* 2002;51:509–12.
- Migishima F, Suzuki-Migishima R, Song SY, Kuramochi T, Azuma S, Nishijima M, et al. Successful cryopreservation of mouse ovaries by vitrification. *Biol Reprod* 2003;68:881–7.
- Salehnia M, Abbasian Moghadam E, Rezazadeh Velojerdi M. Ultrastructure of follicles after vitrification of mouse ovarian tissue. *Fertil Steril* 2002;78:644–5.
- Fahy GM, Wowk B, Wu J, Paynter S. Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology* 2004;48:22–35.
- Fahy GM, Ali SE. Cryopreservation of the mammalian kidney. II. Demonstration of immediate ex vivo function after introduction and removal of 7.5 M cryoprotectant. *Cryobiology* 1997;35:114–31.
- Pegg DE, Wusteman MC, Boylan S. Fractures in cryopreserved elastic arteries. *Cryobiology* 1997;34:183–92.
- Salle B, Demirci B, Franck M, Berthollet C, Lornage J. Long-term follow-up of cryopreserved hemi-ovary autografts in ewes: pregnancies, births, and histologic assessment. *Fertil Steril* 2003;80:172–7.
- Baird DT, Webb R, Campbell BK, Harkness LM, Gosden RG. Long-term ovarian function in sheep after ovariectomy and transplantation of autografts stored at –196 C. *Endocrinology* 1999;140:462–71.
- Yin H, Wang X, Kim SS, Chen H, Tan SL, Gosden RG. Transplantation of intact rat gonads using vascular anastomosis: effects of cryopreservation, ischaemia and genotype. *Hum Reprod* 2003;18:1165–72.
- Bedaiwy MA, Jeremias E, Gurunluoglu R, Hussein MR, Siemianow M, Biscotti C, et al. Restoration of ovarian function after autotransplantation of intact frozen-thawed sheep ovaries with microvascular anastomosis. *Fertil Steril* 2003;79:594–602.
- Oktay K, Buyuk E, Rosenwaks Z, Rucinski J. A technique for transplantation of ovarian cortical strips to the forearm. *Fertil Steril* 2003;80:193–8.
- Salle B, Demirci B, Franck M, Rudigoz RC, Guerin JF, Lornage J. Normal pregnancies and live births after autograft of frozen-thawed hemi-ovaries into ewes. *Fertil Steril* 2002;77:403–8.
- Oktay K, Economos K, Kan M, Rucinski J, Veeck L, Rosenwaks Z. Endocrine function and oocyte retrieval after autologous transplantation of ovarian cortical strips to the forearm. *JAMA* 2001;286:1490–3.
- Shaw JM, Bowles J, Koopman P, Wood EC, Trounson AO. Fresh and cryopreserved ovarian tissue samples from donors with lymphoma transmit the cancer to graft recipients. *Hum Reprod* 1996;11:1668–73.
- Kim SS, Radford J, Harris M, Varley J, Rutherford AJ, Lieberman B, et al. Ovarian tissue harvested from lymphoma patients to preserve fertility may be safe for autotransplantation. *Hum Reprod* 2001;16:2056–60.