Difficulties improving ovarian functional recovery by microvascular transplantation and whole ovary vitrification

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Objective: To evaluate recovery of endocrine function and fertility after transplantation and vitrification of whole ovaries.

Design: Animal study.

Setting: Lyon Veterinary School, France.

Animal(s): Ewes.

Intervention(s): In group 1 (n = 5), the left ovary was removed with its vascular pedicle and was transplanted onto the contralateral pedicle. In group 2 (n = 5), the left ovary with its pedicle was cryopreserved after a vitrification procedure. After thawing, transplantation was performed by microvascular anastomosis to the contralateral ovarian pedicle.

Main Outcome Measure(s): Median ischemia time, progesterone levels, histologic examination.

Result(s): Successful microsurgical transplantation was performed in both groups. The median ischemia time was statistically significantly longer in group 2 (287 minutes, range: 226 to 349) versus 129 minutes (range: 125 to 130) in group 1. In group 1, four sheep recovered spontaneous ovarian endocrine function about 2.5 (range: 2.00 to 3.75) months after transplantation. Two ewes gave healthy live births at 12 and 25 months, respectively, after transplantation. In group 2, one ewe recovered ovarian endocrine function 6 months after transplantation. However, histologic evaluation showed a follicular survival rate of 6% in group 1, and total follicle loss in group 2.

Conclusion(s): Autograft of whole sheep ovaries with microvascular anastomosis seems technically feasible but resulted in a very poor follicle survival rate (6%), in spite of endocrine function recovery and birth of two lambs. Attempts at cryopreservation with vitrification resulted in no follicle survival at all. (Fertil Steril 2008; ––: –––. ©2008 by American Society for Reproductive Medicine.)

Key Words: Whole ovary, fertility, vitrification, cryopreservation, transplantation, orthotopic, sheep

Although advances in oncology have extended life expectancy after cancer, treatments may be toxic for the gonads, entailing a major risk of early ovarian failure and sterility (1). Thus, when medical management is liable to endanger the fertility of a girl or young woman, it is important to find a means of protecting her gametes with a view to restoring fertility on remission (2). Ovary transposition or ovario-pexy may be performed before pelvic radiotherapy, but this will reduce rather than prevent radiation damage to the ovary (3). Some investigators therefore have recommended transposing just one ovary and cryopreserving the other (4).

Under French law, when a woman of reproductive age is living as part of a couple, in vitro fertilization (IVF) may be suggested ahead of any gonadotoxic treatment so as to freeze embryos in anticipation of recovery. When, however, emergency anticancer treatment has to be initiated, there may be no time to perform a gonadotropin ovarian stimulation cycle. Ovarian stimulation, moreover, is contraindicated in hormone-sensitive tumors such as breast cancer (5). Also, after one or two chemotherapy cycles, IVF shows impaired ovarian response to gonadotropin stimulation (6). Metaphase II mature oocyte cryopreservation is not presently recommended as a first-intention fertility conservation.
strategy before cancer treatment, although some teams have included it in their medical routine (7–9). La Sala et al. (10) reported only a 4.2% clinical pregnancy rate with cryopreserved-oocyte IVF. These rather limited results may have been due to the sensitivity of the metaphase II mature oocyte cytoskeleton and nucleus to ice crystals during freezing (11). Freezing can damage the meiotic spindle microtubules, with a consequent high risk of aneuploidy (12, 13). In the near future, however, mature oocyte vitrification can be expected to develop rapidly in view of the excellent results obtained in oocyte survival after rewarming and the ever increasing number of live births worldwide (14–16).

Immature germinal-vesicle stage oocytes survive freezing better than mature ones at metaphase II (17). After freezing, immature oocytes require in vitro maturation (IVM) techniques to achieve metaphase II and become “fertilizable.” Only one birth has as yet been reported from a frozen germinal-vesicle stage oocyte that was matured and fertilized in vitro (18). However attractive in theory, this strategy cannot be adopted for routine practice in all teams because of the poor results obtained in humans (19). Vitrification is presently enabling the development of a strategy based on cryopreservation of oocytes harvested when immature and matured in vitro (20–22). Kagawa et al. (23) recently described an alternative method for obtaining live births in mice without autografts after ovarian vitrification: in vivo growth of preantral follicles of cryopreserved ovarian tissue, grafted beneath the kidney capsule of SCID mice, followed by in vitro oocyte maturation and IVF.

Cryopreservation in view of subsequent ovarian tissue graft is one alternative approach to conserving fertility in prepubescent girls or young women without partners who are at high risk of early ovarian failure and sterility. This technique preserves a large reserve of primordial follicles, which are less cryosensitive than when in growth phase (24, 25). Primordial follicles are smaller than more mature follicles and show less metabolic activity. They are, moreover, relatively non–species-specific, making them very suitable for research model purposes.

Surgical harvesting of ovarian cortical tissue for cryopreservation is currently proposed by many teams worldwide. Silber et al. (26, 27) showed, after ovarian transplantation between monoygotic twins, that fresh ovarian cortical grafts over the ovarian recipient’s medulla can completely restore endocrine function within 3 months after surgery and can enable subsequent spontaneous fertility. However, only four live births have been reported in humans after orthotopic transplantation of cryopreserved ovarian tissue (28–31). After cryopreservation, only nonvascular cortex fragments have as yet been transplanted in humans (5), and the time needed for graft revascularization entails massive follicle loss by ischemia with a consequently reduced graft lifespan and transitory recovery of ovarian function. Recovery of endocrine function after ovarian cortex transplantation is never immediate; it varies from case to case, with a minimum latent period of 3 months (26) and a mean of 4 to 5 months, according to the literature (5, 32).

Vascular transplantation of whole ovaries aims to reduce the period of posttransplantation ischemia by minimizing ischemic follicular loss (33). Our hypothesis was that whole ovary transplantation, by analogy to other organs such as the heart or kidney, should enable rapid revascularization with immediate and lasting endocrine and exocrine functional recovery. A few cases of success of microvascular allotransplantation of human whole fresh ovaries have been described (34, 35), but never after whole ovary cryopreservation.

We previously described a whole ovary vitrification procedure in sheep using the VS4 solution (36, 37). Our present study aimed at developing an orthotopic transplantation technique with microvascular anastomosis of a whole ewe ovary plus vascular pedicle, and assessing restored spontaneous fertility after transplantation of vitrified and fresh whole ovaries. As in our previous research, we chose the ewe as a study model because of the histologic resemblance to the human ovary.

MATERIALS AND METHODS

Ten female ewes, 6 months to 1 year of age, were used for experiments. The study was approved by the ethics committee of the Lyon Veterinary School. Figure 1 schematizes our experimental protocol.

Surgical Procedure

For organizational reasons, all the “fresh” (group 1) ovaries were transplanted first and the vitrified (group 2) ovaries later.

**Group 1** In group 1 (n = 5), all ewes underwent a first midline laparotomy: the left ovary was dissected, isolated, and then harvested with its vascular pedicle, as proximally as possible. Likewise, the right ovary and pedicle were dissected, isolated, and then clamped. The right ovary was placed in X-Vivo survival medium (Lonza, Verviers, Belgium) at 10°C, then fixed in formaldehyde when the pedicle anastomosis was unclamped. The right transplantation site was prepared microsurgically by separating artery and vein. The left lumbo-ovarian pedicle was then clamped and sectioned. At this point, a flash of 5000 IU unfractionated heparin (heparin choay; Sanofi-Synthelabo, le Plessis-Robinson, France) was delivered and repeated once hourly. The transplantation site was orthotopic, by vascular anastomoses to the right pedicle stump. The vascular anastomoses were achieved under 3.5 × 420 mm binocular loupes (Heine, Hersching, Germany) by experienced microsurgeons. End-to-end arterial anastomoses were achieved by separate nonresorbable 9/0 Ethilon sutures (Ethicon, Johnson & Johnson, Somerville, NJ), and end-to-end venous anastomoses by two semi-continuous PDS 6/0 sutures (Ethicon). Anastomosis was facilitated by suturing after placing the vessels in double vascular microclamps (Teleflex, Le Faget, France). Arteries were dilated locally by 4%...
Papaverine (SERB, Paris, France). Pedicles were instilled with 40 IU/2 mL of heparin sodium (Dakota Pharm, Sanofi-Synthelabo, Le Plessis-Robinson, France) via a microcannula (Moria, Antony, France).

After anastomosis, the vessels were unclamped. Ischemia time was defined as the time between ovary removal and pedicle unclamping. Pedicle and ovary were then put back in place and orthotopically fixed in the pelvis and at uterus level as anatomically as possible. One month after laparotomy, the sheep underwent laparoscopy to assess transplant viability and adhesion formation.

Group 2 In group 2 (n = 5), for each sheep the left ovary was harvested with its vascular pedicle and cryopreserved according to our previously described vitrification procedure (36). The right ovary was left in situ to provide a vascular pedicle for retransplantation. One month after initial laparotomy, the sheep underwent a second laparotomy for transplantation after thawing of the vitrified ovary. For group 2, ischemia time was defined as the time between ovary removal and transplantation, including cryopreservation and thawing time.

After awakening and extubation, the ewes were placed under 24-hour surveillance in a closed box before feeding was resumed. Systematic postoperative prophylactic antibiotic therapy was administered with 1 mL/10 kg of intramuscular amoxicillin (Clamoxyl LA, Pfizer Santé Animale, Paris, France). Postoperative analgesia was ensured by an intramuscular nonsteroidal anti-inflammatory injection (Ketofen, Merial, Lyon, France).

Prophylactic anticoagulation by subcutaneous low-molecular-weight heparin injection (40 mg/0.4 mL of enoxaparin sodium; Sanofi-Aventis, Paris, France) was ensured for both groups for 10 days after transplantation.

Vitrification and Thawing Procedure

In group 2, the ovarian artery was cannulated immediately after removal with a 24-gauge catheter anchored with a 5.0 prolene suture. After cleaning and rinsing in heparinized saline, the ovaries were impregnated with VS4 solution (Sigma-Aldrich, St. Louis, MO) via the ovarian artery at a flow-rate of 0.35 mL/minute, kept constant by a peristaltic pump (Watson Marlow 505DU, Dreux, France).

The VS4 solution comprises three cryoprotectant agents: 2.75 mol/L dimethyl sulfoxide (DMSO), 2.76 mol/L formamide, and 1.97 mol/L propylene glycol (PROH) diluted in a carrier solution RPS-1; VS4 contains a total of 7.5 moles of solute (480 g/L). To ensure osmotic balance, the VS4 was introduced in four steps of increasing concentration: 12.5% for 5 minutes at room temperature, 25% for 5 minutes at room temperature, 50% for 15 minutes at 4°C, and 100% for 15 minutes at 4°C. After perfusion, the ovary and vessels were transferred into a liquid-nitrogen–proof ethyl vinyl acetate cryobag (Macopharma, Mouvaux, France) containing pure VS4. The ovary was vitrified by plunging it in liquid nitrogen.

Thawing was performed in two steps: slow rewarming by natural convection in liquid nitrogen vapor up to the
vitreous transition temperature \( (T_g) \) to avoid fracture in the glassy state, followed by rapid rewarming above \( T_g \) by conduction and convection in a 45°C bain-marie to avoid ice crystallization. The changeover from liquid nitrogen vapor to bain-marie was made at approximately −133°C. Because −140°C is the vitreous transition temperature of pure water and −125°C that of the VS4 solution as measured by the DSC \(_7\) differential scanning calorimeter (37), we presumed that the liquid in the tissue would have a vitreous transition temperature lying between these two.

The VS4 washout comprised four 5-minute steps: first, at 4°C with 50% VS4 plus 0.3 M mannitol; then 25% VS4 plus 0.1 M mannitol, at room temperature; then 12.5% VS4 plus 0.1 M mannitol, at room temperature; and finally 10 minutes’ washout in BM1 solution (Eurobio, Les Ulis, France).

**Hormone Assays**

Blood samples were collected from the jugular vein before the operation and at weekly intervals for hormone assay. The serum progesterone concentration was measured with the Vidas Progesterone kit (Biomerieux, Marcy-l’Etoile, France). The assay sensitivity was 0.25 ng/mL. The intra-assay coefficients of variation were 5.7% for 2.2 ng/mL. Progesterone >1 ng/mL was considered as evidence of a functional corpus luteum (38).

**Histology**

The ovary and vessels were fixed for 2 days in formalin and embedded in paraffin. Each ovary was completely cut into serial 4-μm sections and stained with hematoxylin-eosin-saffron. All sections were examined microscopically at ×400 magnification. All normal follicles were counted and ranked as per Turnbull (39). Histologically atretic follicles were also counted and classified as cytoplasm anomaly, nuclear anomaly, or global anomaly (i.e., combined cytoplasm and nuclear anomaly). Follicle number ranking and anomaly percentages were compared between untransplanted control and transplanted ovaries. Control fresh (group 1) and vitrified ovaries (group 2) were also compared.

**Statistical Analysis**

All statistical analysis was performed with GraphPad Prism 4.01 (GraphPad Software, San Diego, CA). Results were subjected to Kruskal-Wallis nonparametric tests. For analyses concerning only two groups, an unpaired Mann-Whitney \( U \) test or paired Wilcoxon test were used. \( P<.05 \) was considered statistically significant. Results are presented as medians for small-sample group 1 and 2 results, as mean ± standard deviation for large quantitative variables (i.e., follicles) and as number (percentage) for qualitative variables.

**RESULTS**

**Evaluation of the Surgery Protocol**

For group 1, the median arterial and venous anastomosis times were, respectively, 45 minutes (range: 25 to 75) and 45 minutes (range: 40 to 45). The median ovarian warm ischemia time was 129 minutes (range: 125 to 130). Immediate per-operative anastomosis quality was excellent, with good arterial pulsatility and venous return (Fig. 2).

For group 2, the median arterial and venous anastomosis times were, respectively, 30 minutes (range: 25 to 31) and 32 minutes (range: 30 to 33). There was no statistically significant intergroup difference in anastomosis time. The median ovarian warm ischemia time was 287 minutes (range: 226 to 349). After thawing, four cases showed no macroscopic fracture. A small proximal arterial fracture in one ewe was patent around the catheter fixation wire; the pedicle was long, however, and caused no subsequent problem for reperfusion or transplantation.

For group 1, 1 month after transplantation, laparoscopy showed no ovarian pedicle thrombosis. Considerable abdominopelvic and especially epiploon to parietal peritoneum adhesions, however, hindered laparoscopic visualization of the transplant and diagnosis. It was therefore decided not to perform control laparoscopy in group 2 either; the ewes had already undergone two laparotomies, making a third general anesthetic excessive with respect to our study interest.

**FIGURE 2**

Detail of downstream lumbo-ovarian pedicle 14 months after ovarian cryopreservation and transplantation. A: Lumbo-ovarian artery, B: Lumbo-ovarian vein.
Assessment of Ovarian Function Recovery

In group 1, ovarian endocrine recovery was observed in four out of five ewes at a median of 2.5 months (range: 2.00 to 3.75). The first ewe to be transplanted failed to recover ovarian function. One ewe was killed after endocrine arrest at 10 months after transplantation. In the other three ewes, endocrine function persisted up to death at 26, 16, and 17 months after transplantation, respectively. Two ewes presented spontaneous pregnancy; one gave birth to a healthy 4100 g female lamb 12 months after transplantation, and the other gave birth at 25 months to a 3000 g female lamb that died from deficient nurture (a commonly described complication in laboratory animals). Autopsy revealed no visceral anomaly indicating any other cause of death.

In group 2, only one ewe recovered ovarian endocrine function 6 months after transplantation, with a progesterone-mia rate of 3.79 ng/mL. This ewe, however, died of pneumopathy 10 months after transplantation. The transplant was not removed until a few hours after death, and histologic examination found no primordial follicles.

Long-term Evaluation of Vascular Anastomosis Quality

Long-term vascular anastomosis quality was assessed macroscopically after death. In group 1, vascular anastomoses were conserved in the four ewes that had recovered endocrine function. In the ewe killed at 10 months, a nonobstructive stenosis of the anastomosis was observed, with conserved upstream and downstream blood flow.

In group 2, anastomosis was normal in one case, with macroscopically normal cryopreserved and anastomosized vessels. In two cases, the lumbo-ovarian pedicle showed thrombosis as of the anastomosis, with atrophied ovary. A fourth ewe showed arterial thrombosis with normal venous return, probably due to the development of collateral circulation. Anastomosis quality data for the ewe that showed ovarian function recovery are unfortunately lacking; the ewe having died of infectious pneumopathy, and the pedicle was nonanalyzable on autopsy.

Histologic Findings

Group 1 ewes were killed at a median 16.5 months (range: 14.5 to 19.25) after transplantation: laparotomy performed to observe and recover the transplants consistently found fine velar periovian and tubo-ovarian adherences, even in the two ewes that had developed spontaneous pregnancies. Four out of five ovaries were macroscopically normal, the other being atrophied downstream of a nonobstructive vascular stenosis.

In group 1, the mean total follicle count in the control group was 6326.8 ± 2161.2 with a mean total primordial follicle count of 3751.6 ± 1672.3. After transplantation, the mean total follicle count fell significantly to just 375 ± 685.7, and the mean total primordial follicle count decreased to 216.8 ± 407.3. The mean posttransplantation follicle survival was 6%. In the two ewes with gestation, follicle survival was 0.5% for one and 24.6% for the other at 25 and 16 months after transplantation, respectively (Table 1).

Group 2 ewes were killed at a median 12 months (range: 10 to 13) after transplantation. The ovary of ewe that died accidentally was atrophied but could not be removed until 12 hours after death. Except in one case, the other three ovaries were atrophied, no more than about 0.5 cm in diameter. In group 2, the mean total follicle count in the control group was 4066.6 ± 2100.8 with a mean total primordial follicle count of 2501.2 ± 1358.1. There was no statistically significant intergroup difference in total follicle number in nontransplanted controls. After cryopreservation, thawing, and transplantation, follicle loss was total in group 2 (Table 2). Microscopic examination found an atrophied ovarian cortex with a few atretic follicles (Fig. 3).

DISCUSSION

We hypothesized that ovarian transplantation would be a well-adapted technique to restore long-term ovarian function and spontaneous fertility. However, although our surgical microvascular technique of whole ovary transplantation seemed technically feasible, in the long term it resulted in

| TABLE 1 |

Ovarian function following transplantation by vascular micro-anastomosis of noncryopreserved ovaries.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Ovarian function recovery MAT</th>
<th>Birth MAT</th>
<th>Sacrifice MAT</th>
<th>Total follicle loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ewe 1</td>
<td>No</td>
<td>—</td>
<td>17</td>
<td>100</td>
</tr>
<tr>
<td>Ewe 2</td>
<td>6</td>
<td>25</td>
<td>26</td>
<td>99.5</td>
</tr>
<tr>
<td>Ewe 3</td>
<td>3</td>
<td>12</td>
<td>16</td>
<td>75.4</td>
</tr>
<tr>
<td>Ewe 4</td>
<td>2</td>
<td>—</td>
<td>17</td>
<td>99.8</td>
</tr>
<tr>
<td>Ewe 5</td>
<td>2</td>
<td>—</td>
<td>10</td>
<td>99.8</td>
</tr>
</tbody>
</table>

Note: MAT = months after transplantation.

only a 6% follicle survival rate in the fresh transplant (group 1), and led to a total follicle loss after vitrification (group 2).

For organizational reasons, the fresh ovaries were transplanted before the cryopreserved series. This explains why the vascular anastomosis operative times appear shorter (although not significantly so) in group 2. The learning curve for lumbo-ovarian pedicle anastomosis microsurgery was further demonstrated to be short.

In previous studies, our team focused on freezing ovarian cortex fragments for orthotopic graft (40–42). In cortex ovarian autograft, it is necessary to wait for graft neovascularization, entailing considerable follicle loss through ischemia. Baird et al. (43) estimated follicle loss due to cryopreservation at 7% and due to posttransplantation ischemia at 60% to 70%. In one of our previous studies of vitrification and orthotopic autograft of hemi-ovarian cortex, normal gestations and live births were obtained (42). Histologic examination of the grafted vitrified ovaries, however, showed considerable long-term follicle loss. We therefore sought to develop a technique that involved transplanting the ovary with its vascular pedicle so as to limit warm ischemia after thawing. Vascular anastomosis theoretically enables virtually immediate revascularization, although our findings actually show a significant warm ischemia period, with a median surgery-linked duration of 129 minutes (range: 125 to 130) in group 1.

**TABLE 2**

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Ovarian function recovery MAT</th>
<th>Birth MAT</th>
<th>Sacrifice MAT</th>
<th>Total follicle loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ewe 1</td>
<td>No</td>
<td>—</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>Ewe 2</td>
<td>No</td>
<td>—</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>Ewe 3</td>
<td>No</td>
<td>—</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Ewe 4</td>
<td>6</td>
<td>—</td>
<td>10 (accidental)</td>
<td>100</td>
</tr>
<tr>
<td>Ewe 5</td>
<td>No</td>
<td>—</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

*Note: MAT = months after transplantation.


**FIGURE 3**

Histological comparison of primordial follicles between group 1 and group 2 (original magnification X 400). (A), Ovarian cortex aspect 16 months after transplantation (group 1). Black arrow indicates intact primordial follicle. (B), Ovarian cortex aspect 10 months after cryopreservation and transplantation (groupe 2). White arrow indicates atretic primordial follicle.

lengthened to 287 minutes (range: 226 to 349) in group 2 due to the time spent in the cryopreservation procedure.

This very long median ischemia time in the cryopreservation group was more due to technical and/or organizational factors than to the difficulty of surgery as such. Our work requires two perfectly synchronized teams, one on the surgical side doing transplantation, and the other busy with cryopreservation and thawing. Furthermore, a purely technical problem meant that vascular anastomosis was performed with only 3.5 × 420 mm binocular loupes instead of a microscope. In future, we intend to use microscopy, both to facilitate surgery and to reduce preoperative ischemia time. The ischemia times recorded for group 1, on the other hand, were comparable with those in our previous studies (36) for transport from the slaughterhouse and preparation (unpublished data).

The ovarian cortex and primordial follicles seem fairly well resistant to ischemia. Schmidt et al. (44) found human ovarian cortex fragments were able to support approximately 4 hours of warm ischemia. The fragments were transported in an icebox, although the temperature was not mentioned. In our study, samples were transported in an icebox at 10°C, on the assumption that cold slows down ovarian tissue metabolic activity. Our own results from the development of our ovarian cryopreservation research protocol indicated that a transportation temperature of 10°C was preferable to 4°C in terms of follicle survival (unpublished data).

The recovery of endocrine activity in the transplant was related to both ovarian warm ischemia time and the surgical quality of the anastomosis and, in group 2, to the quality of the cryopreservation procedure. In human grafts after ovarian cortex cryopreservation, ovarian function recovers about 4 to 5 months after transplantation (28, 31, 45–47). This interval corresponds to the physiologic timing of follicle development from the primordial stage. In ovarian tissue cryopreservation, primordial follicles are the least cryosensitive (25). This explains why it takes several months to recover normal endocrine functioning if cryopreservation has destroyed growth-stage follicles. In human fresh cortical grafts, ovarian endocrine recovery took only 3 months, probably because follicles were conserved at growth stages more advanced than the primary follicle (26, 27).

The folliculogenesis cycle into ewes is around 180 days (48). For Imhof et al. (49), follicle-stimulating hormone (FSH) reached physiologic levels 6 months or more after transplantation. In the present study, in group 1, the median time to endocrine secretion recovery was 2.5 months, which is less than one full folliculogenesis cycle. It can be deduced from this that the 129-minute median warm ischemia time experienced by the group 1 ovaries damaged the terminal growth-phase but not primordial follicles.

In group 1, three out of four ewes conserved ovarian endocrine function over a period of at least 16 months. Two had gestation with live birth of a healthy lamb. One of the two lambs died when its mother failed to feed it, but autopsy definitively ruled out any visceral anomaly as being responsible for this death. The temporary recovery of ovarian function for just 7 months in one ewe can be explained by partial posttransplantation ovarian ischemia: autopsy found a vascular anastomosis stenosis although upstream and downstream blood flow was conserved. Our follow-up evaluation stopped at 26 months when it was decided to kill the ewes for histologic assessment.

In group 1, mean posttransplantation follicular survival was just 6%. In the first part of our research, we previously reported variation in follicle count not only between two different ewes of the same age but also between the two ovaries of a single ewe (36). This accounts for the wide standard deviation in follicle number between ovaries. Even so, the difference in the follicle survival rate—0.5% versus 24.6%—between the two ewes that had gestations is surprising. Ovarian endocrine function also persisted in these two ewes up to death. Finally, only one ewe, with 100% follicle loss, logically failed to recover ovarian function. Imhof et al. (49) reported between 1.7% and 7.6% follicle survival at 18 to 19 months after transplantation of whole cryopreserved ovine ovaries (n = 8 ewes). Despite this low rate, one ewe conceived spontaneously, and four showed posttransplantation ovarian function. These results suggest ewes can recover ovarian endocrine function and spontaneous fertility even with a much reduced stock of primordial follicles.

In group 1, only warm ischemia and/or poor ovarian vascularization can be implicated in this very severe follicle loss. After these preliminary trials, our surgical technique needs to be improved so as to reduce micro-anastomosis time: the ischemia time due purely to surgery was around 2 hours (129 minutes [range: 125 to 130]). The ovarian artery gets particularly twisted near the ovarian hilum in ewes. Rinsing the pedicle with heparinized saline may not prevent capillary microthrombi arising in the ovarian medulla, causing local ischemia. For this reason, we delivered a 10-day regime of preventive postoperative anticoagulants.

In group 2, only one ewe recovered ovarian endocrine function, 6 months after transplantation. This interval, identical to that reported in other whole-ovary transplant studies (49), shows that the cryopreservation and transplantation procedure fails to conserve growth-phase follicles. Our present procedure did not offer any progress in terms of ovarian function recovery compared with our previously described cortex vitrification and orthotopic graft protocol (42). Rapid recovery of ovarian function requires growth-phase follicles to be conserved after freezing and thawing, and the surgical procedure not to involve too long a period of warm ischemia.

Group 2 also showed a higher rate (3 out of 4) of pedicle thrombosis than group 1. Bedaiwy et al. (50) likewise reported thrombosis in most (8 out of 11) ovarian vessels in a series of cryopreserved and transplanted ewe ovaries. They explained this by the extreme twistedness of ewe ovary vessels and by damage to vessel intima through cryopreservation. As vascular pedicles did not show thrombosis in
group 1, the vitrification procedure may be assumed to have induced the procoagulant lesions.

In group 1, diagnostic laparoscopy was performed between 1 and 2 months after transplantation. The amount of adherence caused by surgery was surprising. This can be explained partly by the length of the laparotomy operation and partly by the fact that on awakening the ewes stand up, causing the epiploon and small intestine to come up against the transplantation site. Even so, two pregnancies were obtained despite all these adherences. If the technique were eventually to be applied in humans, postoperative antiadherence barriers might be deployed (51).

In our previous studies, ovary and pedicle were thawed in a single step (36). Rapid thawing in bain-marie at 37°C very often led to pedicle fracturing. Our technique was refined in the light of our previous findings on the physical properties of vitrified samples (37). Briefly, the vitreous transition temperature (Tg) is defined as the temperature below which a solid phase without ice crystals can be obtained. Vitrification is successful if cooling reaches Tg at a speed in excess of the critical cooling rate of the cryoprotectant solution (Vc,cr). Fractures often occur when rewarming the vitrified sample between the liquid nitrogen temperature (−196°C) and the vitreous transition temperature (Tg) because of the brittleness of the glass. To avoid fractures, our revised method consisted of a two-step procedure: the sample is thawed slowly up to Tg and then quickly above Tg to avoid ice crystallization. Pegg et al. (52) also demonstrated the usefulness of two-step thawing to avoid fractures. After vitrification and thawing, ovarian vessels presented a macroscopically normal aspect, with no technical difficulty for surgical reanastomosis. Arterial pulsatility after reanastomosis would seem to be an interesting index of arterial function.

We previously had reported that ovarian cortex vitrification with VS4 cryoprotectant was incomplete in the strict physical sense (37, 53). Even so, our first results with the VS4 design were histologically satisfactory (36). Postvitrification follicular viability was 61.6% ± 2.6%, and the percentage of histologically intact primordial follicles was 53.4% ± 6.3%. Although the rate of ice crystals in the cortex on differential calorimetry was nearly 20%, the incidence of ice crystals is not indicative of the ovary’s ability to recover endocrine and exocrine functioning after thawing and transplantation.

Several teams use slow-freezing protocols with whole ovaries (49, 50, 54–57). By definition, these induce extracellular ice crystal formation (58), and yet initial results have nevertheless been encouraging. Bedaiwy et al. (54) compared applications of the same slow-freezing protocol in ewes with whole ovaries with their vascular pedicle (n = 11) and ovarian cortical strips (n = 6). After thawing, no difference in apoptosis or follicular viability was found. Imhof et al. (49), in a series of eight ewes, reported the first birth following slow freezing, thawing, and orthotopic transplantation. Some 19 months after transplantation, however, histologic examination of the transplanted ovaries found a follicle survival rate of no more than 1.7% to 7.6%. It is difficult to say whether this damage to the primordial follicles was due to warm ischemia ahead of reanastomosis, to the freezing technique, or to the ovarian capillary thrombosis that affected nearly 30% of the ovary. Arav et al. (56), with a very slow freezing protocol, showed primordial follicle survival of 97.7% on fluorescent stains test. Martinez-Madrid et al. (59) observed no signs of apoptosis with the TUNEL method and immunohistochemistry for active caspase-3 after cryopreservation of three whole human ovaries with their vascular pedicle. Twenty-nine out of 30 primordial and primary follicles observed on electron microscopy showed no ultrastructural anomalies.

Choi et al. (60) were less optimistic after their observation of cryopreservation impact on mouse ovaries: in both slow-freezing and vitrification protocols, apoptosis and necrosis inhibited primordial follicle development and growth.

Future whole-ovary transplantation indications will be excellent, in cases, for example, of conditioning therapy before bone marrow transplantation for benign hematologic diseases such as sickle cell anemia, thalassemia major, or aplastic anemia. On the other hand, clinical application of whole ovary cryopreservation for transplantation seems not to be indicated in cases of high risk of residual ovarian medullar pathology, where residual cancer cells are liable to be reintroduced by transplantation (61, 62). Shaw et al. (63) showed that frozen ovarian tissue grafts from a mice with lymphoma could cause transmission to recipient mice. Sanchez et al. (64) found immunohistochemical evidence of ovarian cortex micrometastases in cases of infiltrating breast cancer.

**CONCLUSION**

Autotransplantation of a whole ovary with microvascular Anastomosis seems technically feasible. In theory, transplantation seeks to achieve rapid revascularization of the ovary and virtually immediate and prolonged functional recovery, avoiding the destruction of growth-phase and primordial follicles. Our present study unfortunately failed to bear out this hypothesis, although ovarian transplantation does seem to be a well-adapted technique for restoration of ovarian function and spontaneous fertility (27). We showed that fresh orthotopic transplantation enables natural spontaneous conception, despite the incidence of pelvic adhesion and despite a long-term follicular survival rate of no more than 6%.

Our results, however, do not highlight any advantage of whole ovary autograft with microvascular anastomosis over reports on ovarian cortex autograft (42). Despite what seemed to us to be a satisfactory surgical procedure, and despite two live births being obtained, fresh ovarian microvascular transplantation resulted in very poor (6%) long-term follicle survival, well below that reported with cortical grafting (43, 65). Our surgical procedure thus first needs improving technically, both to reduce ischemia time and to minimize the
incidence of thrombotic events so frequent in the twisted vessels of ewe ovaries after transplantation (50).

Attempted cryopreservation with vitrification, on the other hand, resulted in no follicle survival at all. Even so, despite the better results obtained with slow whole-ovary freezing compared with vitrification (55, 59), we still believe that vitrifying whole organs such as ovaries remains an important line of research in cryobiology, avoiding ice crystal formation within the organ (66, 67). Our present results, alongside those previously described (37), lead us to think that VS4 may not be the best adapted solution for whole-ovary vitrification. Other cryoprotectant solutions developed by Fahy et al. (67, 68) must be tested with a view to achieving complete whole-ovary vitrification and improving posttransplantation results.

The present report is of a preliminary development and feasibility study for a technique of whole ovary and vascular pedicle transplantation and cryopreservation by vitrification. Our results were disappointing, but we feel it is important to report “negative” findings and describe failed techniques, sharing our experience with other teams working in the field of ovarian transplantation and whole-ovary cryopreservation. Moreover, although more and more ovarian transplants are performed in humans and live births have been reported, we consider animal models are still useful to evaluate and improve cryopreservation and transplantation procedures.

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