Cryopreservation of the ovary by vitrification as an alternative to slow-cooling protocols

Blandine Courbiere, M.D.,[a] *Valentina Odagescu, Ph.D.,*[b] *Anne Baudot, Ph.D.,*[b] *Jérôme Massardier, M.D.,*[a] *Claire Mazoyer, M.S.,*[c] *Bruno Salle, M.D., Ph.D.,*[a,c] *and Jacqueline Lornage, M.D., Ph.D.*[a,c]

[a] Unité INSERM 418, Hôpital Debrousse, Lyon; [b] CRTBT, CNRS, Grenoble; and [c] Département de Médecine et de Biologie de la Reproduction et du Développement, Hôpital Edouard Herriot, Lyon, France

Objective: To study the thermal properties of a cryoprotectant solution, called VS4, and of VS4-impregnated whole sheep ovaries with pedicle.
Design: Physical and experimental animal study.
Setting: Academic research environment.
Animal(s): Five- to 6-month-old ewes.
Intervention(s): Thermal properties on cooling of a cryoprotectant solution called VS4 were measured by differential scanning calorimetry. VS4 contains 2.75 mol/L dimethyl sulfoxide, 2.76 mol/L formamide, and 1.97 mol/L propylene glycol. Whole sheep ovaries were collected at the slaughterhouse and prepared for a vitrification procedure. Cortex and vessels underwent histologic examination before and after vitrification, and the thermal properties of VS4-impregnated ovarian cortex were then studied.
Main Outcome Measure(s): Critical cooling rates (V_{ccr}), vitreous transition temperature (T_g), end-of-melting temperature (T_m), follicle viability assessment by trypan blue test, and histologic examination of ovary and vessel structure.
Result(s): The critical cooling rate (V_{ccr}) of VS4 solution was estimated to be 14.3 ± 1.1°C/min. Its vitreous transition temperature (T_g) was −125.2 ± 0.2°C, and its end-of-melting temperature (T_m) −34.3 ± 0.1°C. Following our vitrification procedure, immediate follicle viability was 61.4% ± 2.2%. The percentage of normal primordial follicles remaining after vitrification was 48% ± 3.8%. The V_{ccr} of VS4-impregnated cortex could not be determined because of the quantity of ice forming as of the top programmed cooling rate (−300°C/min). The mean ovarian cortex cooling rate actually attained experimentally during vitrification was −342.9 ± 49.6°C/min.
Conclusion(s): Vitrification of entire organs, such as ovaries, is a great challenge in cryobiology and reproductive medicine. Physical studies seem indispensable for progress with this technique. Thus, our ovarian perfusion procedure needs improving to enhance ovarian cortex impregnation and bring down the V_{ccr} rate in both tissue and VS4 solution. (Fertil Steril® 2006;86(Suppl 3):1243–51. ©2006 by American Society for Reproductive Medicine.)

Key Words: Vitrification, ovary cryopreservation, cryoprotectant, critical cooling rate, sheep

The freezing of human spermatozoids and embryos in reproductive biology now follows well-codified procedures. Although the first two human births following cryopreserved ovarian autograft have been reported recently (1, 2), the technique cannot be said yet to form part of current medical practice. Ovarian cryopreservation is, however, a particularly burning issue inasmuch as ongoing progress in cancer treatment means that more and more young women are surviving cancers occurring early in their genital life (3). The question of their future fertility needs addressing ahead of any chemotherapy or radiotherapy (4).

Yet there is no current consensus as to which cryopreservation technique to implement, what sized sample (cortex fragments or whole ovaries) to preserve, which cryoprotectants to use, or what happens after thawing. After freezing, it is vital that the immature ovocytes should mature to become fertilizable. Several lines of research are being conducted presently to this end (5): in vitro maturation of primordial follicles followed by IVF and ET; xenograft to an animal recipient for the maturation of human follicles; heterotopic ovarian autograft followed by ovarian stimulation and IVF (6); and orthotopic ovarian autograft, with a view to restoring spontaneous fertility (1).

There are two main ways of cryopreserving biologic tissue: either classic "slow" freezing or vitrification by rapid cooling (7–9). Slow cooling uses an optimal cooling rate specific to a given cell but produces extracellular ice crystals that are harmful to the surrounding tissue. Vitrification, on the other hand, traps all the aqueous solutions in an amorphous, so-called vitreous solid phase, preventing any ice crystal formation in the tissue (8). Thus, slow freezing may preserve single cells, such as spermatozoids, without damage, but vitrification seems to us to be preferable for cryo-

Received June 6, 2005; revised and accepted May 12, 2006.
Reprint requests: Jacqueline Lornage, M.D., Ph.D., 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).

preserving a more complex and heterogeneous system such as a whole ovary (10).

Vitrification requires rapid cooling, in combination with a high yet subtoxic concentration of cryoprotectants so as to give an efficient glass-forming mixture with water. Indeed, the main challenge in whole-organ cryopreservation is to find the best composition for a cryoprotectant solution having as low a biologic toxicity level as possible while still leading to vitrification on cooling (11).

The present study focused on studying the thermal properties of a cryoprotectant solution called VS4 and of VS4-impregnated whole sheep ovaries with pedicle. We sought first to describe the thermal properties of VS4, so as to be sure that it could, physically speaking, enable whole-ovary vitrification. The parameters in question were end-of-melting temperature (T_m)—the temperature below which ice may form during cooling—and vitreous transition temperature (T_g)—below which a solid phase without ice crystals can be obtained, particularly useful for cryopreserving biotissue. To achieve such a vitreous solid phase, cooling has to reach T_g at a speed in excess of the critical cooling rate of the cryoprotectant solution (V_{ccr}). V_{ccr} was determined by differential scanning calorimetry, as were T_m and T_g.

As a second stage, whole sheep ovaries were prepared with their vascular pedicle for a vitrification procedure. The cytotoxicity of VS4 was then assessed before and after cryopreservation at −196°C by direct plunging into liquid nitrogen.

Differential scanning calorimetry was then applied to VS4-impregnated ovarian cortex specimens so as to determine their thermal properties and the V_{ccr} required to achieve vitrification. To determine whether this critical cooling rate was reached during the ovarian vitrification procedure, cooling rates were measured in both cortex and vitrification solution: see the third and last parts of this article.

MATERIALS AND METHODS

Cryoprotectant Solution

The VS4 cryoprotectant solution, developed by Fahy et al. (12), comprises three cryoprotectant agents (CPAs) (Sigma-Aldrich, St. Louis, MO): 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 molar (480 g/L) CPA. The vehicle for VS4 was RPS-1 (290 mOsm/kg H_2O) with NaCl (10 mmol/L), KCl (28.3 mmol/L), $MgCl_2$ (0.40 mmol/L), $CaCl_2$ (0.05 mmol/L), dextrose (180 mmol/L), reduced glutathione (5 mmol/L), adenine HCl (1 mmol/L), and K_2HPO_4 (7.2 mmol/L).

Thermal Analysis of VS4 Solution Properties

Differential Scanning Calorimetric Analysis. To determine the thermal properties of aqueous VS4 solutions, we conducted a series of experiments using a DSC_7 differential scanning calorimeter from Perkin-Elmer, Wellesley, MA. DSC measurements enable observation of phase transitions under dynamic conditions. This leads to the evaluation of the critical cooling rate above which a given solution vitrifies entirely. The DSC_7 was calibrated for temperatures and heat flow from the melting of ice from deionized water (T = 0°C and ΔH = 333.8 J/g) and the crystallographic transition of cyclohexane in its solid phase (T = −87.1°C). Temperature values were found to be reproducible within ±0.5°C.

VS4 was studied during cooling at a constant rate that varied from one experiment to another from 2.5°C/min to 320°C/min, between −153°C (which is below the vitreous transition of the solutions) and 12°C. Heats of solidification were measured at selected programmed cooling rates of 2.5°, 5°, 10°, 20°, 40°, 80°, 160°, and 320°C/min. According to our previous studies on a DSC_2, we knew that actual cooling rates are identical to the indicated rates up to 80°C/min (13).

In the present study, we worked with weights of 5.73 ± 0.01 mg. These experiments were conducted with use of standard hermetically sealed aluminum pans (0219-0062; Perkin-Elmer) designed for volatile samples. To check insulation, the sealed pans were weighed at the end of the experiments and the weights compared with those before the DSC_2 measurements. VS4 was tested three times. Between measurements, the solutions were preserved in properly insulated flasks, because of their high hygroscopy levels. To ensure good reproducibility, a syringe was used to deposit the VS4 droplets on the side of the pan.

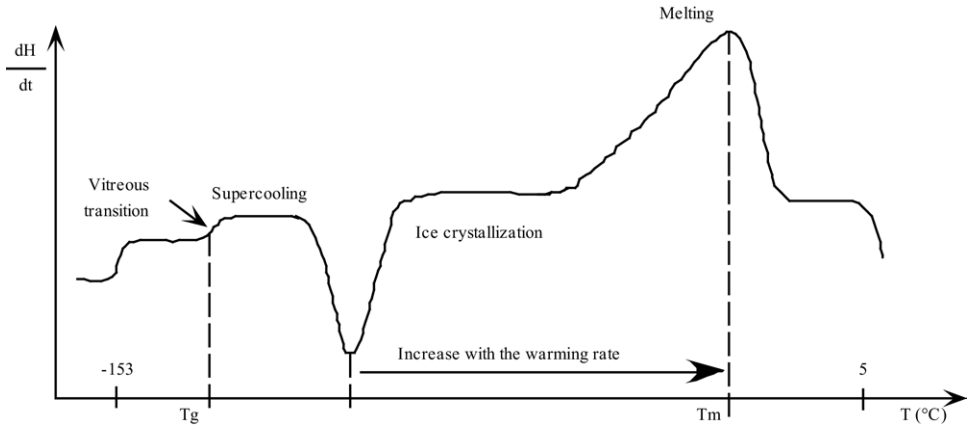
Determination of Critical Cooling Rates. The V_{ccr} value was calculated from recordings made at the various cooling rates, with use of the semiempirical model developed from Boutron's classical theory of crystallization (14). Its basic assumption states that ice crystals are spherical and have equal size, which has been verified cryomicroscopically for some polyalcohols (15). Growth is considered as a function of temperature, and the resulting equation includes a term to account for the reduction in growth rate because of crystal impingement (14, 16).

By measuring the thermogram area of the exothermic crystallization peak, the heat of ice solidification (q) can be determined for each cooling rate tested. Plotting these values against cooling rate, the model is used to fit a theoretical curve to the experimental data so as to estimate the critical cooling rate, defined as the rate above which <0.2% of the solution will crystallize. That is, a given solution's critical cooling rate is the lowest rate that allows it to vitrify.

T_m and T_g were measured during thawing, where the cryoprotectant solution first goes from a wholly amorphous state through the glass transition, where it becomes a supercooled liquid. If the warming rate is not fast enough here, the molecules acquire enough mobility to pass from the random arrangement of the amorphous state to an ordered crystalline structure, in which case, at a

FIGURE 1

Typical thermogram for VS4 solution on thawing. dH/dt is the derivative of the heat received by the sample versus the time; Tg = vitreous transition temperature; Tm = end-of-melting temperature.



Courbiere. Physical studies for ovary vitrification. Fertil Steril 2006.

higher temperature, molecular mobility increases further and the crystalline material passes from solid ice to the liquid phase, inducing a nonisothermal peak, corresponding to ice melting. All these transitions are presented in Figure 1.

The vitreous transition temperature, T_g, was determined at the inflexion point of the rapid increase in specific heat corresponding to the glass transition. The temperature T_m at the end of ice melting was defined as the temperature at the top of the melting peak.

Practical Application in Cryopreservation of Ovaries by Vitrification

Preparation of the Ovaries. Vitrification was attempted on five ovary pairs, with use of VS4. The two ovaries of each pair were taken from a single ewe at the slaughterhouse. One of each pair underwent vitrification, while the contralateral ovary served as control, exposed to VS4 but without vitrification. Ewes were from 5 to 6 months old (breed unknown). Ovaries were taken with their pedicle. The ovarian vessels were identified and cut so as to be as long as possible. Ovaries were removed via a pelvic incision made straight after slaughter.

On removal, 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. The time between removal in the slaughterhouse and initiation of perfusion and the total warm ischemia time between removal and immersion in liquid nitrogen were recorded.

Addition and Removal of Vitrification Solution. The perfusion rate was 0.35 mL/min, via a peristaltic pump (505DU; Watson Marlow, Dreux, France). The solution was introduced and removed in eight phases. Phases 1, 2, 3, and 4 involved increasing concentrations, to allow osmotic equilibration: 5 minutes with VS4 at 12.5% at room temperature, 5 minutes at 25% at room temperature, 15 minutes at 50% at 4°C, and 15 minutes at 100% at 4°C. Phases 5, 6, 7, and 8 consisted of gradual washout at room temperature: 5 minutes with VS4 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 mmol/L mannitol during phase 5, stepped down to 100 mmol/L at phases 6 and 7, and then to 0 mmol/L at phase 8. Previous trials had shown low toxicity for this impregnation protocol on the ovarian tissue (17).

Ovary Vitrification. After perfusion (phases 1, 2, 3, and 4), ovary and vessels were transferred to an ethyl vinyl acetate cryobag (Macopharma, Mouvaux, 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 thawing. To minimize physical stress, the ovary sample was immersed perfectly horizontally, by means of a hammock system. A dedicated suspension system prevented the vitrified sample hitting up against the liquid nitrogen tank walls.

After thawing and washout, the ovary was then cut in half sagittally: one half for the follicle viability study and the other, along with a fragment of pedicle, for histologic examination. The contralateral control ovary also was immersed in and perfused with VS4, so as to undergo the same full dehydration-rehydration-washout protocol, before being studied as described above.

Ovary Thawing. After storage in liquid nitrogen, the vitrified samples were warmed up to the vitreous transition temper-

ature (T_g) slowly to avoid fracture in the glassy state and then quickly above T_g (−125°C) to avoid ice crystallization. The first part of the warming was by forced hot air convection and the second by conduction and natural convection in water at 45°C. The T_g value had been predetermined by DSC study of VS4. Thermocouples were set in parallel to monitor thawing speed and estimate when samples reached the glass transition temperature.

The VS4 was removed by reversed concentration-gradient perfusion (phases 5, 6, and 7). The ovaries were then washed in BM1 medium (Eurobio, Les Ulis, France) 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.

Histologic Assessment of the Vitrification Procedure's Cytotoxicity

Blinded follicle viability and histologic assessment was performed by two different observers.

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). Fragments were supplemented with 1 mg/mL (200 IU/mL) type I collagenase (Sigma-Aldrich) 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 centrifuged 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). 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 saffron.

Follicular morphology was examined by microscope (magnification ×400). For each ovary, 100 primordial follicles were counted in sections where the oocyte nucleus was visible, and their morphology 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. Cytoplasm damage comprised cytoplasmic microvacuolation and/or retracted cytoplasm and/or granulosa cells disrupted from thecal cells. Nuclear anomalies were recorded in case of hypereosinophilic nucleic condensation.

Follicular density was estimated by counting primordial follicles in 10 contiguous microscopic fields (magnification ×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 used widely in anatomopathology for estimating cell density in heterogeneous tissue.

Histology of the ovarian artery and vein and of ovarian medulla blood vessels was evaluated 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 endothelial detachment, internal elastic membrane rupture, or smooth muscle cell bloating. In the case of the veins, only the vascular endothelium was examined.

Analysis of the Thermal Properties of VS4-impregnated Ovarian Cortex

Preparation of the Ovaries. Two ovaries were collected with their pedicle at the slaughterhouse. Ovaries and vessels were placed in X-Vivo medium (Bio Whittaker) and transported to the laboratory at 10°C. 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 VS4.

Ovarian Cortex VS4-impregnation Procedure. The perfusion rate was 0.35 mL/min, via a peristaltic pump (505DU; Watson Marlow). The solution was introduced in the first four phases, described above.

Differential Scanning Calorimetric Analysis. Differential scanning calorimetry of the VS4-impregnated ovarian cortex followed the procedure described above for VS4 itself. The aim was to determine the critical cooling rate for successful cortex vitrification. Two 14.1 ± 7.6 mg cortex fragments were taken from each ovary studied, and each placed in a sealed aluminum pan. VS4-impregnated cortex fragments from two ovaries were tested.

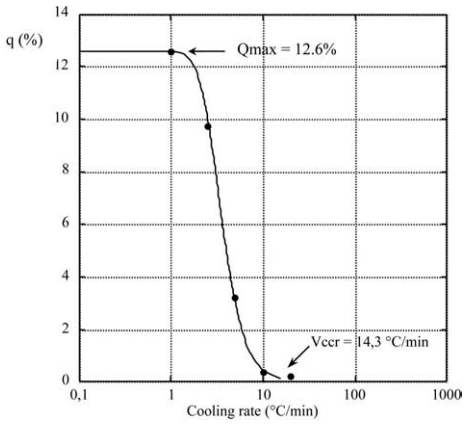
Measurement of Ovarian Cooling Rate and Thawing Temperatures

Fine 0.23 mm thermocouples (Physitemp Instruments Inc., Clifton, NJ) were placed on the samples to monitor temperature during cooling and thawing. They were connected to a Consort-T851 temperature scanner (Cole Palmer, Vernon Hills, IL), recording temperature every second. Data were then analyzed and the resultant speeds calculated with use of the Kaleidagraph software package (Synergy Software, Reading, PA).

Two thermocouples were placed on each ovary: [1] One series of ovaries (n = 4) had one thermocouple on the cortex and another in the CPA solution in which the ovary was

FIGURE 2

Variation in percentage crystalline ice (q) with cooling rate in VS4 solution. Isolated points are experimental points. The *solid line* is the theoretical curve according to the model. Vccr is the critical cooling rate of VS4 solution.



Courbiere. Physical studies for ovary vitrification. Fertil Steril 2006.

immersed. [2] Another series (n = 3) had one thermocouple on the ovarian medulla and another in the CPA solution. The same thermocouples served for monitoring temperature during thawing, so as to determine the vitreous transition temperature (T_g).

Statistical Analysis

Summary statistics are presented as means (±SEM). Transport and warm ischemia times were compared by Kruskal-Wallis test. The two groups of ovaries being paired, the nonparametric Wilcoxon test could be applied. Differences were considered statistically significant at $P<.05$. Unistat software (Unistat, London, United Kingdom) was used for statistical analysis.

RESULTS

Determination of VS4's Thermal Properties

The temperature T_m below which ice may crystallize in the VS4 solution at equilibrium was −34.3 ± 0.1°C. The vitreous transition temperature, T_g, below which VS4 is transformed into a glassy solid state is −125.2 ± 0.2°C.

The results obtained concerning VS4 V_{ccr} are presented in Figure 2. The heat of ice solidification is represented, as classically, by the number (q) of grams of ice of which the solidification at 0°C would liberate the same amount of heat as would 100 g of solution on crossing the corresponding peak. These units are chosen because they give a heat of solidification close to the quantity of ice crystallized in percentage (wt/wt) of solution when only ice forms (13). The heat in calories per 100 g of solution is obtained by multiplying q by 79.78.

With the DSC_7, it is possible to cool VS4 at its critical rate, estimated as V_{ccr} = −14.3° ± 1.1°C/min.

Cryopreservation of Ovaries by Vitrification

Ovary Preparation. The mean interval between removal in the slaughterhouse and initiation of perfusion was 74 ± 3.3 minutes. The mean warm ischemia time between removal and immersion in liquid nitrogen was 139 ± 4 minutes. There were no statistically significant differences between ovaries in terms of ischemia.

Ovarian Follicle Viability. Follicle viability was 70.6% ± 4.7% for ovaries exposed to VS4 without vitrification and 61.4% ± 2.2% for ovaries vitrified with VS4. This difference was not significant.

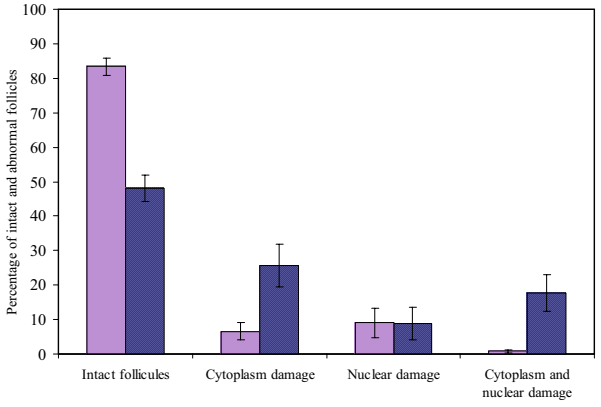
Ovarian Primordial Follicle Density. Follicle density was 51.6 ± 12.7 for ovaries exposed to VS4 without vitrification and 48 ± 12.4 for ovaries vitrified with VS4. Again, this difference was not significant.

Histologic Examination of Primordial Follicles. The percentage of normal primordial follicles fell from 83.4% ± 2.5% to 48% ± 3.8% after vitrification, a difference that was close to significance ($P=.05$). The increases in the rate of histologic cytoplasmic anomalies, from 6.6% ± 2.6% to 25.6% ± 6.2% after vitrification, and of total cell anomalies, from 0.8% ± 0.3% to 17.8% ± 5.3% after vitrification, were also close to significance ($P=.05$). On the other hand, there were not significantly more isolated nuclear anomalies before (9% ± 4.3%) than after vitrification (8.8% ± 4.7%) (Fig. 3).

Assessment of Vessel Tissue Quality After Vitrification (Fig. 4). No fractures occurred in vitrified vessels during warming. Pedicle vessels were consistently histologically

FIGURE 3

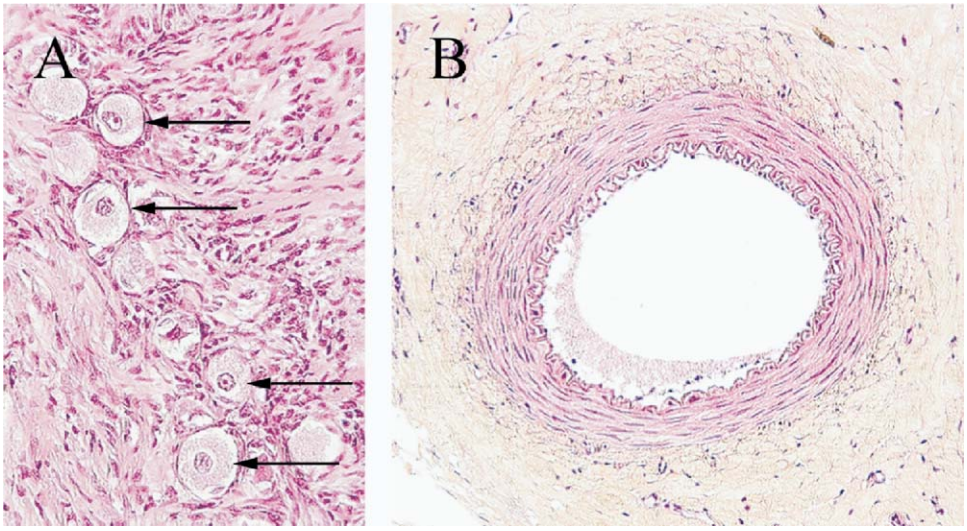
Histologic comparison of fresh ovaries (*pink bars*) and vitrified ovaries (*blue bars*): percentage of intact and abnormal follicles. *Bars* represent mean ± SEM.



Courbiere. Physical studies for ovary vitrification. Fertil Steril 2006.

FIGURE 4

Histology of vitrified-thawed sheep ovary (**A**) containing multiple intact primordial follicles (*arrows*) and of vitrified-thawed sheep ovarian pedicle (**B**). (Original magnification ×400.)



Courbiere. Physical studies for ovary vitrification. Fertil Steril 2006.

normal before vitrification despite 60 minutes VS4 perfusion.

After vitrification, the aspect of the ovarian vein was in all cases identical to that of its nonvitrified control. In three out of five cases, partial ovarian artery endothelial detachment was observed. Central medulla arteriole myocytes were often bloated (four out of five). Medulla veins showed better conservation, apart from two cases with conjunctive tissue edema.

Thermal Properties of VS4-impregnated Ovarian Cortex

Cortex fragment cooling data on one of the two ovaries subjected to differential scanning calorimetry were discarded for critical cooling rate calculation because of the amount of ice forming at the highest cooling rate available on the DSC_7: 16.4% ± 1.6% (w/w), which approximates the maximum (19.8% ± 0.4% [w/w]) found with the slowest cooling rates. This prevented the critical cooling rate for the impregnated tissue being calculated as such, although the results did show it to be in excess of 300°C/min.

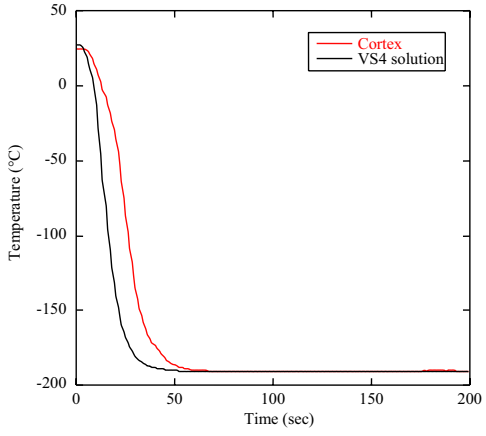
In the second perfused ovary, the amounts of ice forming in the cortex fragments at the highest programmed cooling rate (−300°C/min) were lower (9.1% ± 1.8% [w/w]), and the maximum amount crystallizing was 15.1% ± 0.2% (w/w). The theoretical model could be used in one of these two fragments to estimate V_{ccr}, which was about −340°C/min. This fourth fragment showed the best results for both cooling and thawing: T_g = −126.3°C and T_m = −26.5°C, as compared with T_g = −117.3 ± 0.3°C and T_m = −20.0 ± 1.5°C in the other three samples.

Measurement of Ovarian Cooling and Thawing Speeds

Figure 5 shows cooling rate data from the cortex vitrification sample. The mean tissue cooling speed was −342.9 ± 49.9°C/min, compared with −462.4 ± 92.3°C/min for the VS4 surrounding the ovary. Figure 6 shows the cooling rate data from the medulla vitrification sample. Here the mean tissue cooling speed was −333.6° ± 20°C/min compared with −498.3 ± 108.3°C/min for VS4. Mean cortex thawing

FIGURE 5

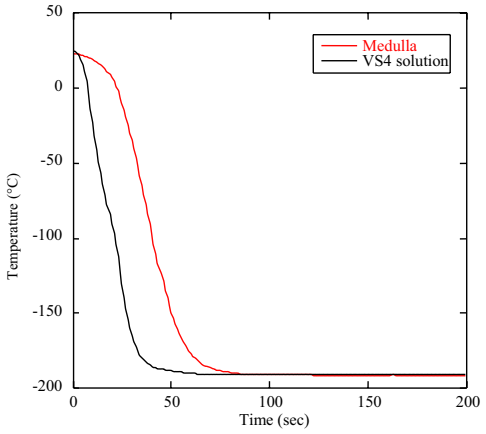
Temperature variation during sheep ovary cortex vitrification. *Red line*, cortex; *black line*, VS4 solution.



Courbiere. Physical studies for ovary vitrification. Fertil Steril 2006.

FIGURE 6

Temperature variation during sheep ovary medulla zone vitrification. *Red line*, medulla; *black line*, VS4 solution.



Courbiere. Physical studies for ovary vitrification. Fertil Steril 2006.

speeds were 22.8° ± 3.3°C/min during cool thawing, and 191° ± 85°C during rapid thawing, compared respectively with 20.6° ± 4.9°C/min and 380.5° ± 66.5°C/min for the VS4 surrounding the ovary.

DISCUSSION

Vitrification is an alternative to slow cooling procedures. For more than 20 years, vitrification has been described as the most promising approach to the cryopreservation of mammalian organs (18). Our study showed physical studies to be indispensable for the assessment and improvement of the vitrification, particularly in the case of whole ovaries, where the cell structure is complex.

Vitrification requires high concentrations of cryoprotectants to reduce the critical cooling rate. Even so, it still requires high cooling rates. To keep these rates down, the idea is to replace the water in the cells by a cryoprotectant solution. The cryoprotectant molecules hinder ice formation and limit crystallization by reducing water molecule mobility and hence limiting diffusion within tissue.

The critical cooling rate is the lowest rate that allows a given solution to vitrify. Water represents 60% to 80% of the organ's mass, but pure water requires a very high cooling rate (around 20,000°C/sec). The critical cooling rate decreases with increasing cryoprotectant concentration. Unfortunately, all the presently known cryoprotectants are toxic, and their toxicity increases with concentration.

Cytoprotectant agent toxicity also depends on the incubation temperature. Dimethyl sulfoxide penetrates human ovarian tissue more rapidly than PROH at 4°C. At 37°C, cell penetration is similar for the two cryoprotectants, but they are more cytotoxic at this temperature (19). Many vitrification solutions have been described. Fahy's team have developed several, including one they called VS4, which they tested on rabbit kidneys (11, 12).

Our own previous studies reported better results on ovarian tissue with VS4 than with another solution known as VS1, containing four cryoprotectants: 2.62 mol/L DMSO, 2.60 mol/L acetamide, 1.31 mol/L PROH, and 0.0075 mol/L polyethylene glycol. We reported more than 50% primordial follicle conservation after vitrification using VS4 (17). We therefore sought to go further in our study of this solution, so as to improve our vitrification protocol. In the future, other cryoprotectant solutions will be able to be studied in a similar manner, even if a cryoprotectant solution of very low toxicity with an amorphous state that remains highly stable has so far proved extremely difficult to obtain (10).

The DSC design used here had been validated previously for physical studies on other mammalian organ samples (20). Our VS4 study first confirmed that the critical cooling speed needed to make a success of vitrification, physically speaking, could be achieved under our experimental conditions with the VS4 cryoprotectant solution. Differential scanning calorimetry results showed that VS4 could be vitrified at speeds that are technically feasible (−14.3°C/min). Furthermore, the speed achieved in cooling was always greater than −300°C/min, that is, considerably higher than the VS4 critical cooling rate as determined by differential scanning calorimetry.

Under identical experimental conditions, the medulla region cooled just a little more slowly than the cortex, being more massive. It is also noteworthy that the VS4 solution cooled systematically faster than the tissues impregnated with it, no doubt because tissues have lower thermal diffusivity than liquids.

The study thus demonstrates that, from a strictly physical point of view, the VS4 solution was indeed vitrified, without ice crystals forming during cooling. However, the critical cooling rate for the impregnated ovarian cortex under this procedure exceeded −300°C/min, suggesting that, under the present experimental conditions, the ovarian tissue is unlikely to be totally vitrified at the end of cooling.

These results are probably due to defective impregnation, resulting in insufficient and uneven tissue penetration of the CPA, as suggested by the variable results between the four test fragments. The obtained T_m values were much higher in the cortex fragments than in the VS4 solution itself. Previous studies testing small pieces of mammalian organs (rabbit kidney, rat heart tissue) permeated with cryoprotectant agents by differential scanning calorimetry showed that the glass-forming tendency and the stability of the amorphous state were both greater in the tissue samples than in the cryoprotectant solution (20).

Confining the solution in the biologic tissue has the effect of reducing the critical cooling and thawing speeds (20).

Findings on ovary fragments point in the other direction, confirming our fears as to the difficulty of impregnating the ovarian cortex. This interpretation needs to be confirmed by nuclear magnetic resonance studies of VS4 diffusion and ovarian impregnation.

We have so far been using an empirical model of impregnation by ovarian perfusion via the ovarian artery, based on another team's previous studies in rabbit kidney (21). Dynamic nuclear magnetic resonance study of ovarian impregnation would enable the speed and quality of CPA diffusion in the ovary under our design to be assessed and optimized, and we hope to do this in future studies (19). This should enable specification of the necessary conditions for complete and homogeneous VS4 impregnation of the ovary.

In terms of biocompatibility, the cytotoxic impact of VS4 on primordial follicles and vessels was found to be acceptable. Immediate postvitrification follicle viability was over 60%. The technique thus conserves about half of the primordial follicle population in a whole ovary. No fall in ovarian tissue follicle density was observed.

Our histologic findings were, however, limited by the small number of tissue samples studied. Moreover, the trypan blue test for ovarian follicle viability is not an exhaustive ultrastructural study of ovocyte status, studying only small primordial follicles of <60 μm in diameter. We did not count partially or completely denuded oocytes, because we could not tell whether they resulted from histologic artifacts or from thermal damage or ischemia.

We do, however, intend to use other ovarian tissue exploration techniques (e.g., DNA fragmentation) once our vitrification protocol has been optimized. Nevertheless, it is obviously only the successful transplantation by vascular microanastomosis of a vitrified ovary that could confirm ovarian functions after such a vitrification procedure.

Fahy et al. have reported recently that it is difficult to achieve organ vitrification using VS4 (10). They have developed other solutions, called VM3 and M22, claimed to be preferable to VS4 for vitrification purposes. Before experimenting with these new solutions on ovarian tissue, we are waiting to have confirmation of their harmlessness with regard to human tissue. Meanwhile, we are continuing our research on VS4, which we have already shown to have low cytotoxicity for ovarian tissue and vessels (17). The know-how acquired in physical testing with our present vitrification protocol will stand us in good stead for future work with other vitrification solutions.

Many authors consider vitrification to be the best adapted cryopreservation technique for massive, heterogeneous, and complex biologic tissue, such as a whole organ (7, 8, 10). In vitrification, the disordered liquid state is brought to a standstill in an "amorphous" state without any crystallization, unlike in slow-freezing protocols, where the formation of extracellular ice causes disruption of the intercellular architecture.

There have been recent reports of attempted cryopreservation of whole ovaries in human subjects (22) and ewes (23, 24) from teams using a range of freezing techniques. Like us, they perfused the cryoprotectants through the ovarian artery, which seems essential for homogeneous impregnation of the ovary. None of the published studies, however, has gone into the physical parameters of their freezing technique with the cryoprotectant in question (DMSO) or into the possible functional consequences of extracellular ice crystals forming in the frozen ovaries.

Whole ovary vitrification has so far been described only in rats (25) and mice (26, 27). There have even been reports of births following vitrification in mice (27). In these small animals, the ovaries are small and were not given vascular cryoprotectant perfusion. Orthotopic grafting was facilitated by the presence of an ovarian bursa.

In human subjects, orthotopic grafting would have the advantage of restoring spontaneous fertility without recourse to IVF procedures. There are two orthotopic grafting techniques that could be envisaged after freezing-thawing: ovarian cortex (28, 29) or follicle (30) graft in an ovary that was not removed, or else whole ovary transplantation by vascular pedicle anastomosis (23).

It is now admitted that cortex grafting entails considerable follicle loss because of the posttransplantation ischemia before spontaneous revascularization of the graft can occur (31). It is this ischemia that limits the lifetime of the graft and consequently the long-term recovery of the ovary's endocrine and exocrine functions (32, 33).

We revised our thawing protocol in the light of too high a rate of vitrified vascular pedicle fracture observed with rapid thawing (17). We had been getting such fractures systematically, probably because of the brittleness of the VS4 glass formed during vitrification and of the tissue itself, which is very fine and is subjected to considerable mechanical stress by the presence of the catheter.

Fractures might appear in the vitrified sample between the liquid nitrogen temperature (-196°C) and the vitreous transition temperature (T_g), because of the brittleness of the glass. To avoid these fractures, one method consists of thawing the sample slowly up to T_g and then more quickly above T_g so as to avoid ice crystallization (34). This two-step thawing procedure was applied here and enabled us to avoid fracture.

Nevertheless, the final outstanding problem for organ vitrification remains the difficulty of thawing vitrified organs rapidly enough to outrun devitrification (10). This was not possible in our experiments, where ice formed during the second phase of thawing.

The ovarian pedicle vessels, however, were in a very satisfactory histologic condition after vitrification. The best proof of this would be successful anastomosis. Authors who have attempted ovarian pedicle vascular anastomosis in ewes

have been hampered by a high incidence of thrombosis in the anastomosed vessels (23, 24). The possible reasons for this include endothelial lesions caused by ischemia or by ice crystals, or again anatomic factors in these ovarian arteries that are so much narrower and more tortuous in ewes than in human subjects.

In conclusion, our study shows that vitrification of a whole organ such as a ewe's ovary is hard to achieve from the point of view of strict physical vitrification parameters. Ovary cryopreservation protocols can be helped by thermal characterization of the cryoprotectant. For techniques of ovarian cryopreservation by vitrification, such prior physical assessment indeed seems to us to be mandatory, so as to predetermine the critical cooling and thawing rates for the sample.

The design for whole ovary CPA impregnation does, however, need improving to achieve the technical preconditions for true vitrification free of ice crystal formation. An ovary can only be certified as having been vitrified if there is physical proof that a vitreous state has been attained and that the speeds reached did indeed prevent ice crystals forming.

Acknowledgments: The authors thank G. Bessard, M.D., the anatomopathology laboratory of Edouard Herriot Hospital, Lyon, France, and especially L. Frappart, M.D, and J.Y. Scoazec, M.D., for technical assistance.

REFERENCES

1. 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.
2. Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Zalel Y, et al. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. N Engl J Med 2005;353:318–21.
3. Linet MS, Ries LA, Smith MA, Tarone RE, Devesa SS. Cancer surveillance series: recent trends in childhood cancer incidence and mortality in the United States. J Natl Cancer Inst 1999;91:1051–8.
4. Aubard Y, Poirot C, Piver P, Galinat S, Teissier MP. Are there indications for ovarian tissue cryopreservation? Fertil Steril 2001;76: 414–5.
5. Gook DA, Edgar DH, Stern C. Cryopreservation of human ovarian tissue. Eur J Obstet Gynecol Reprod Biol 2004;113:S41–4.
6. 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.
7. Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. Fertil Steril 2002;78:449–54.
8. Pegg DE. The current status of tissue cryopreservation. Cryo Letters 2001;22:105–14.
9. Fahy GM. Vitrification: a new approach to organ cryopreservation. Prog Clin Biol Res 1986;224:305–35.
10. 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.
11. Fahy GM, Wowk B, Wu J, Paynter S. Improved vitrification solutions based on the predictability of vitrification solution toxicity. Cryobiology 2004;48:22–35.
12. 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.
13. Baudot A, Alger L, Boutron P. Glass-forming tendency in the system water-dimethyl sulfoxide. Cryobiology 2000;40:151–8.
14. Boutron P. Comparison with the theory of the kinetics and extent of ice crystallization and of the glass-forming tendency in aqueous cryoprotective solutions. Cryobiology 1986;23:88–102.
15. Mehl PM. Nucleation and crystal growth in a vitrification solution tested for organ cryopreservation by vitrification. Cryobiology 1993; 30:509–518.
16. Boutron P, Kaufmann A. Stability of the amorphous state in the system water–1,2-propanediol. Cryobiology 1979;16:557–68.
17. Courbiere B, Massardier J, Salle B, Mazoyer C, Guerin JF, Lornage J. Follicular viability and histological assessment after cryopreservation of whole sheep ovaries with vascular pedicle by vitrification. Fertil Steril 2005;84:1065–71.
18. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology 1984;21:407–26.
19. Newton H, Fisher J, Arnold JR, Pegg DE, Faddy MJ, Gosden RG. Permeation of human ovarian tissue with cryoprotective agents in preparation for cryopreservation. Hum Reprod 1998;13:376–80.
20. Peyridieu JF, Baudot A, Boutron P, Mazuer J, Odin J, Ray A, et al. Critical cooling and warming rates to avoid ice crystallization in small pieces of mammalian organs permeated with cryoprotective agents. Cryobiology 1996;33:436–46.
21. Kheirabadi BS, Fahy GM. Permanent life support by kidneys perfused with a vitrifiable (7.5 molar) cryoprotectant solution. Transplantation 2000;70:51–7.
22. Martinez-Madrid B, Dolmans MM, Van Langendonckt A, Defrere S, Donnez J. Freeze-thawing intact human ovary with its vascular pedicle with a passive cooling device. Fertil Steril 2004;82:1390–4.
23. 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.
24. Revel A, Elami A, Bor A, Yavin S, Natan Y, Arav A. Whole sheep ovary cryopreservation and transplantation. Fertil Steril 2004;82: 1714–5.
25. Sugimoto M, Maeda S, Manabe N, Miyamoto H. Development of infantile rat ovaries autotransplanted after cryopreservation by vitrification. Theriogenology 2000;53:1093–103.
26. Salehnia M. Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. Exp Anim 2002;51:509–12.
27. 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.
28. 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.
29. 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.
30. Almodin CG, Minguetti-Camara VC, Meister H, Ceschin AP, Kriger E, Ferreira JO. Recovery of natural fertility after grafting of cryopreserved germinative tissue in ewes subjected to radiotherapy. Fertil Steril 2004; 81:160–4.
31. Aubard Y, Newton H, Scheffer G, Gosden R. Conservation of the follicular population in irradiated rats by the cryopreservation and orthotopic autografting of ovarian tissue. Eur J Obstet Gynecol Reprod Biol 1998;79:83–7.
32. 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.
33. 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.
34. Pegg DE, Wusteman MC, Boylan S. Fractures in cryopreserved elastic arteries. Cryobiology 1997;34:183–92.