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Towards whole sheep ovary cryopreservation ☆

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Abstract

Cryopreservation of ovarian tissue aims to assist young women who require treatments that may lead to sterility or infertility. Cryopreservation procedures should therefore be as simple and efficient as possible. This study investigates rapid cooling outcomes for whole sheep ovaries. Ovaries were perfused with VS4 via the ovarian artery, and cooled by quenching in liquid nitrogen in less than a minute (estimated cooling rate above 300 °C/min till the vitreous transition temperature). The ovaries were rewarmed in two stages: slow warming (12–16 °C/min from −196 to −133 °C) in liquid nitrogen vapour, followed by rapid thawing in a 45 °C water bath at about 200 °C/min. DSC measurements showed that under these cryopreservation conditions VS4 would vitrify, but that VS4 perfused ovarian cortex fragments did not vitrify, but formed ice (around 18.4%). Immediately following rewarming, a dye exclusion test indicated that 61.4 ± 2.2% of small follicles were viable while histological analysis showed that 48 ± 3.8% of the primordial follicles were normal. It remains to be clarified whether follicle survival rates will increase if conditions allowing complete tissue vitrification were used.

Keywords: Vitrification; Ovary cryopreservation; VS4; Differential scanning calorimetry; Sheep

Advances in clinical oncology have dramatically enhanced survival rates. One US study estimated that, by 2010, about one in 250 individuals will be long-term survivors of childhood cancer [13], and the issue of infertility caused by gonadotoxic drugs arises for survivors that reach adulthood [12]. Ovaries are highly sensitive to cytotoxic treatment, and especially to chemotherapy with alkylating agents, which can cause a loss of both endocrine and reproductive function. Ovarian tissue transplantation is receiving attention, as it could be both a means of preserving fertility for women with malignant disease, and a solution for young women treated for non-malignant pathologies by bone-marrow transplantation, that are currently treated with high doses of chemo- and/or radio-therapy [22].

There are many ways of preserving ovarian function ahead of gonadotoxic treatment. Surgical transposition is simple, but provides only partial protection against radiotherapy, and none against chemotherapy [46]. Mature oocytes can be collected for IVF. However oocyte cryopreservation outcomes are not yet consistent [49] and fertilizing the eggs to obtain embryos (which can be successfully cryopreserved) requires a partner. Freezing mature oocytes (or embryos) after induced ovulation delays initiation of treatment, and exposes the individual to raised hormone levels. Ovarian tissue cryopreservation ahead of any cytotoxic treatment, in comparison [4,60], is a promising option to preserve fertility. This approach offers several advantages: the ovarian cortex is rich in primordial follicles, enabling straightforward laparoscopic harvesting at any age and at any point in the menstrual cycle. Both

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primordial and growing follicles can be cryopreserved, but at present most groups cryopreserve cortical tissue as this contains the individuals lifetime store of primordial follicles. Several ovarian cortex autografts have already been reported for human subjects [21–23,48,50]. Although three human births following cryopreserved ovarian autograft have been recently reported [20,21,43], the technique does not yet form part of the current medical practice. Evidence indicates that ovarian tissue cryopreservation is a very forgiving technique, success is possible with isolated follicles [1], cortical fragments [20,21,43] and whole ovaries [2,33], a range of cryoprotective agents and a range of cryopreservation strategies (slow cooling, rapid cooling and vitrification [14]). Following warming follicles mature successfully when returned to the original site, other sites within the original donor animal, other histocompatible recipients, and immunocompromised animals of other species (xenografting). Cortex collection, cryopreservation and grafting, while technically simple, is associated with severe long-term follicle loss due to ischemic reperfusion injury [3,5,37,57]. Ischemic tissue damage is clearly correlated with duration of ischemia [37]. One concern that applies to all forms of ovarian grafting (for patients with systemic or metastatic cancers) is the potential risk of cancer cell transmission [21,36] with the graft. The biggest problem is however the finding that ovarian cortical grafts appear to only restore short-term fertility, possibly because the grafting perturbs primordial follicle arrest. This may be overcome if whole ovaries are cryopreserved. This study's long-term goal is therefore to develop a whole ovarian autotransplantation procedure that permits vascular anastomosis to the orthotropic site. This is possible as it has been performed for both normal and cryopreserved ovaries in rats [62], sheep [11] and humans [44].

The two main cryopreservation techniques used for biological tissue are slow and rapid cooling [25,39,51]. Both require the cells and tissues to undergo severe water loss (dehydration) and cryoprotectant uptake before they can be stored at low sub-zero temperatures. With slow cooling this water loss and cryoprotectant uptake take place gradually, while the materials are cooled, as it is driven by gradual extracellular ice crystal growth. With rapid procedures the majority of this water loss and cryoprotectant uptake has to be completed before cooling commences because the cooling step is too quick for major changes and is not aided by ice formation. The advantages of slow cooling are that cells are only exposed to high cryoprotectant concentrations and severe dehydration at low temperatures at which little metabolic activity takes place. The advantage of rapid cooling is that physical damage associated with ice formation and osmotic damage caused by melting ice are reduced (e.g. in non-vitrifying or devitrifying conditions) or avoided (e.g. in vitrifying conditions). However rapid cooling does expose the material to dehydration and high, potentially toxic, concentrations of cryoprotectants (penetrating and non-penetrating) at temperatures at which cell metabolism is likely (above −20 °C). Many workers favor the use of solutions which form an amorphous, so-called "vitreous" solid phase, without ice crystals. This usually requires particularly high solute concentrations in combination with both rapid cooling and rapid warming, which enhances any problems associated with cryoprotectant toxicity or dehydration [51]. Both rapid and slow cooling are easy to implement and both have been used for preserving isolated cells, tissues and organs but it is not yet clear which is best for large, complex, tissues such as ovaries. Cryopreservation of whole ovaries has been attempted in human subjects [41], ewes [2,11,55], rats [61,62] and mice [45,56]. There have been births following grafting of both slowly and rapidly cooled ovarian pieces, and whole ovaries, but it remains to be determined which is best.

From the physical point of view, vitrification requires rapid cooling, in combination with a high yet sub-toxic concentration of cryoprotectants so as to give an effective glass-forming mixture with water. Indeed, the main challenge in whole-organ cryopreservation is to find the best composition for a cryoprotective solution having as low a biological toxicity level as possible while still leading to vitrification on cooling under technically feasible conditions [29]. A lot of vitrification solutions have been described. Fahy's team has developed several, including VS1 and later VS4, which they tested on rabbit kidneys [26,29]. Our own previous studies reported better results on ovarian tissue with VS4 than with VS1 [18]. The VS4 we used is a 7.5 M vitrification solution basically comprising 3 cryoprotectants (2.75 M dimethyl sulfoxide, 2.76 M formamide, and 1.97 M propylene glycol) diluted in RPS (Ringer–Phosphate–Sucrose) solution [26], which vitrifies as a bulk solution under pressure. More recently, 21st Century Medicine published some remarkable findings on new and highly promising cryoprotectants [28,30]. Some of these, such as the VM3 and M22 solutions, display properties that would be especially suited to ovary vitrification. Before going on to experiment with them on ovarian tissue, however, we are awaiting further confirmation of their harmlessness for human tissue. Meanwhile, we sought to pursue our VS4 studies, so as to develop the vitrification protocol. As soon as possible, we shall adapt it to use the VM3 or M22 families of cryoprotective solutions.

The present study seeks to demonstrate the feasibility of whole ovary cryopreservation in ewes and the interest of additional physical studies to develop a vitrification procedure. This ewe model was selected due to its similarities to human ovaries, such as dense fibrous stroma and relatively high primordial follicle density in the ovarian cortex [2]. In this paper, we will describe how whole sheep ovaries were prepared with their vascular pedicle for a vitrification procedure, and how the cytotoxicity of the process was assessed before and after cryopreservation at −196 °C by direct plunging into liquid nitrogen followed by warming up to 4 °C. Temperature was measured continuously to

determine the cooling and rewarming rates reached during the procedure. At the same time, differential scanning calorimetry (DSC) was applied to measure the thermal properties of VS4 and of VS4-impregnated fragments such as ovarian cortex and ovarian lumbar pedicle. DSC enables phase transitions to be observed under dynamic conditions in a sample, measuring several thermal parameters such as the end-of-melting temperature (T_m), which is the temperature below which ice may form during cooling, and the vitreous transition temperature (T_g) below which a solid phase without any ice crystals can be obtained. To achieve such a "vitreous" solid phase, cooling has to reach T_g at a speed in excess of the critical cooling rate (V_{ccr}) of the cryoprotective solution. Moreover, the sample must afterwards be rewarmed sufficiently quickly to avoid ice crystallization on rewarming. Warming must exceed the critical warming rate (V_{cwr}) of the cryoprotective solution. These critical rates above which no ice forms are calculated from DSC experiments [6,54]. The method we use will be explained below.

Materials and methods

Ovary preparation

Ovaries were removed from 5 to 6 month-old ewes (breed unknown) via a pelvic incision made straight after slaughter at a slaughterhouse. The ovaries (about 1.5 cm long, 1 cm wide, 0.5 cm thick, and weighing around 0.7 g) were removed together with as much pedicle as possible to maximize the length of associated ovarian vessels and placed in X-Vivo medium (Bio Whittaker, Walkersville, MD). They were transported at 10 °C (tightly-controlled by a thermometer) to the laboratory. After cleaning and rinsing in heparinized saline, the ovaries were perfused with VS4 solution via the ovarian artery at a flow-rate around 0.15 ml/min per gram, kept constant by a peristaltic pump (Watson Marlow 505DU, Dreux, France) with a mean perfusion pressure of 39 mmHg. The perfusate was not oxygenated. The VS4 (composition shown in Table 1), adjusted to pH 7.6, was introduced in 4 steps with increasing concentration: 12.5% for 5 min at room temperature; 25% for 5 min at room temperature; 50% for 15 min at 4 °C; and 100% for 15 min at 4 °C. The VS4 concentration was the same in the perfusate and in the bath surrounding the perfused ovary. The temperature was measured with a thermocouple. To enhance penetration, clips were applied to the ovarian artery efferent arterioles (shown by white arrows in Fig. 1) that did not directly vascularize the ovary. The time between removal in the slaughterhouse and initiation of perfusion, and the total ischemic time between removal and immersion in liquid nitrogen were recorded. The VS4 solution was prepared twice during testing. Each VS4 preparation was kept at 4 °C in properly insulated flasks, due to its highly hygroscopic nature.

Table 1
VS4 composition used in this whole ewe-ovary vitrification study

Component	Quantity
NaCl	10.00 mM
KCl	28.30 mM
$MgCl_2$	0.40 mM
$CaCl_2$	0.05 mM
K_2HPO_4	7.20 mM
Dextrose	180.00 mM
Reduced glutathion	5.00 mM
Adenine HCl	1.00 mM
Dimethyl sulfoxide	2.75 M
Formamide	2.76 M
Propylene glycol	1.97 M

Cooling and rewarming

After perfusion with VS4, one ovary from each sheep was immediately re-warmed and re-perfused (solution control ovary) while the contralateral control ovary, from the same sheep was cooled and placed in liquid nitrogen before being re-warmed and perfused. The cryopreservation step involved placing the ovary and vessels into a liquid-nitrogen-proof ethyl vinyl acetate cryobag (Macopharma, Mouvaux, France) with about 10 mL of 100% VS4. The bag was sealed by welding with particular care being taken to ensure the removal of all air from the bag ahead of cooling. Three thermocouples (Physitemp Instruments Inc., Clifton, NJ), connected up to a Consort-T851 temperature-scanner were fitted, one to the follicle-rich cortex, one to the medulla and one to a region of the bag containing only VS4 solution (Fig. 1). They recorded the temperature every second. The thermocouple leads entered the bag via cuffed medical inlets to prevent leakage. Data was analyzed using Kaleidagraph software package (Synergy Software, version 3.5—2000).

The cooling step involved placing the cryobag between two metal-framed lattices of waxed string fixed on a tripod stand (Fig. 2) on which the position of the upper frame was adjusted vertically so as to secure the cryobag in a horizontal position while minimizing vibrations and mechanical stress. This system minimized contact between the bag and metal while maximizing specimen visibility. The samples were held for a minimum of 30 min in the liquid nitrogen.

Rewarming was in two steps: slow rewarming by natural convection in liquid nitrogen vapour up to −133 °C (lying between the vitreous transition temperature of pure water −140 °C [32] and the vitreous transition temperature of VS4 −125 °C) to avoid fracture in the glassy state [40] followed by rapid rewarming above T_g by conduction and convection in a 45 °C water bath to reduce ice crystallization. Once the temperature in the ovary reached −5 °C, the cryobag was placed on a bed of crushed ice to complete warming gently and avoid overheating. The system of bag and lattices, developed especially for the present study, enabled cooling/warming to be conducted under technically simple conditions.

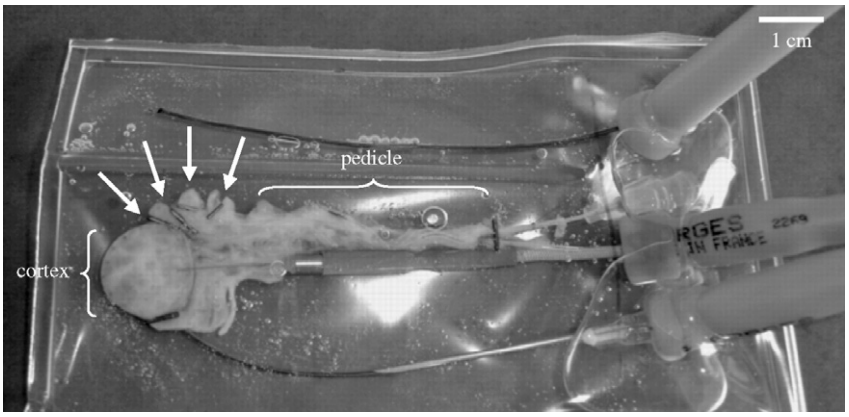

Fig. 1. Preparation of the ovary for vitrification, with thermocouples fitted in the tissue (thermocouple with implantable needle at cortex and thermocouple introduced into the medullar region via a catheter) and in the VS4 solution, via watertight-cuffed teats, before closing the bag by ethyl-vinyl-acetate welding. The white arrows indicate the clips applied on the ovarian artery efferent arterioles. The medulla is not indicated on this picture because it is the central part of the ovary, lying within the cortex.

Washout

VS4 was washed out of the solution control and cryopreserved ovaries both by perfusion and immersion, in four 5-min 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 (the given concentrations in mannitol are the final concentrations of this compound in the perfused solution); and finally 10 min washout in BM1 solution (Eurobio, Les Ulis, France) containing 10 g/L of human albumin. The mannitol was added to reduce osmotic stress during CPA washout [26]. A shrinking of the ovary was observed during the washout. After washout, each ovary was cut in half sagittally: One half of each ovary was used to determine the follicle dye exclusion, and the other hemi-ovary, along with a fragment of pedicle, was fixed for 24 h in Bouins for histology.

Cytotoxicity testing

In order to evaluate cytotoxicity follicle membrane integrity assessment by trypan blue exclusion and histological examination of the ovary and vessel structure were undertaken on the paired solution control and cryopreserved ovaries. Follicular membrane integrity and histologic assessments were performed by two independent blinded observers.

Isolation of small follicles and dye exclusion assessment of ovarian follicle by trypan blue test

The trypan blue test was performed by thinly sectioning each non-fixed half ovarian cortex in Leibovitz L-15 medium (Sigma Aldrich, St. Louis, MO) supplemented with 1 mg/mL (200 IU/mL) type I collagenase (Sigma Aldrich, St. Louis, MO). Cortex fragments were incubated at 37 °C for 2 h and pipetted every 30 min. Collagenase activity was then inhibited by addition of 1:1 fetal calf serum 100% (Sigma Aldrich, St. Louis, MO). The suspension was filtered through a 60 μm nylon filter (Bioblock Scientific, Illkirch, France) and centrifuged at 400*g* for 5 min. The pellet was resuspended in 50 μL Leibovitz L-15 medium. Trypan blue (0.4%; Sigma Aldrich, St. Louis, MO) was added to a suspension containing the follicles (20 μL), 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. It was assumed that dead follicles were those that stained blue and that living follicles were those that did not.

Histologic examination

After 24 h in Bouins, the fixed hemi-ovaries and ovarian vessels were dehydrated in 70% alcohol, before being embedded with paraffin, cut into serial 4 μm sections, then stained, hematoxylin (RAL Reagent, Bordeaux, France), phloxin (RAL Reagent, Bordeaux, France) and saffron (Microm Microtech, Francheville, France). This technique sheds light on follicle morphology and density and on the condition of the ovary vessels:

- 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.
- Follicular density was estimated by counting primordial follicles in 10 contiguous microscopic fields (magnification 400×). The ovarian cortex (which is richest in primordial follicles) was identified and the primordial follicles in 10 contiguous fields were counted. (This tech-

Fig. 2. Sliding lattice frames, designed for cooling by plunging and two-step rewarming the cryobag containing the ovary. The entire assembly dimensions are: 17 cm × 10 cm × 38 cm. The cryobag is placed on the lower lattice and the upper one is lowered on the bag and locked in place to ensure a horizontal position. On cooling, the entire assembly is plunged in liquid nitrogen. It avoids mechanical vibration and stress that could weaken the glass. On rewarming, this assembly is fixed in liquid nitrogen vapour during the slow rewarming, in a position corresponding to −23 °C (this temperature will slightly increase during the procedure due to liquid nitrogen evaporation) before being plunged in a water bath during the rapid rewarming.

nique 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 medullary blood vessels was performed to assess overall tissue architecture. The arterial wall was considered normal if the vascular endothelium, internal elastic membrane and muscularis were whole, or abnormal if there was evidence of: endothelial detachment, internal elastic membrane rupture, or smooth muscle cell bloating. In veins, only the vascular endothelium was examined.

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 non-parametric Wilcoxon test could be applied. Differences were considered statistically significant at $P < 0.05$. Unistat software (Unistat, London, UK) was used for statistical analysis.

Thermal analysis by DSC

The VS4 solution and small pieces of equilibrated tissue, were evaluated using a DSC_7 differential scanning calorimeter from Perkin-Elmer (940 Winter Street, Waltham, Massachusetts 02451 USA) [47], with Pyris software. The temperatures and heat flow in the DSC_7 were calibrated using melting of ice from deionized water ($T = 0$ °C and $\Delta H = 333.8$ J/g) and the crystallographic transition from the monoclinal to the cubic form of cyclohexane in its solid phase ($T = -87.1$ °C). Temperature values were reproducible to within ±0.5 °C. Samples were cooled and rewarmed between −153 °C (which is below the vitreous transition of the samples) and 5 °C. Thermograms were drawn on cooling with the programmed cooling rates of 2.5, 5, 10, 20, 40, 80, 160 and 300 °C/min. The actual cooling rates are equal to the indicated ones up to 80 °C/min. For a programmed cooling rate of 300 °C/min, the real cooling rate decreases progressively from 300 °C/min at −25 °C to 160 °C/min at −95 °C and 80 °C/min at −130 °C [6,16]. Thermograms were drawn on warming to determine the transitions at 2.5, 5, 10, 20, 40 and 80 after quenching, that is cooling at the programmed cooling rate of 300 °C/min. These experiments were conducted using standard hermetically sealed aluminum pans (Perkin-Elmer, 0219-0062) designed for volatile samples. The pans were weighed at the start and end of the experiments and data discarded for any incorrectly sealed pan (any detectable weight change).

DSC measurements were performed on VS4 and on impregnated ovarian tissue fragments to establish cooling criteria preventing ice-crystal formation in tissue, as compared to in the VS4 itself. We conducted these experiments on a DSC_7 differential scanning calorimeter. V_{ccr} was calculated using the semi-empirical model developed from Boutron's classical theory of crystallization [15]. Its basic assumptions state that ice crystals are spherical and have equal size, which has been verified cryomicroscopically for some polyalcohols [42]. Growth is considered as a function of temperature, and the resulting equation includes a term to account for the reduction in growth rate due to crystal impingement [15]. By measuring the thermogram area of the exothermal crystallization peak, the heat of crystallization can be determined for each cooling rate tested. From these results, a value q (%) can be derived, representing the percentage of the sample that has crystallized as pure ice, and corresponding to the amount of energy per unit mass of the sample released during crystallization over the heat of crystallization of pure water that

freezes at 0 °C. 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 less than 0.2% of the solution will crystallize. This value was chosen by Boutron for practical reasons, assuming that this minimum amount of ice would not damage cells [15]. T_m and T_g were measured during the rewarming after the highest cooling rate. During this thawing, the cryoprotective solution first goes from a wholly (or sometimes partially) amorphous state through the glass transition, where the amorphous phase 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 higher temperature, the crystalline material passes from solid ice to the liquid phase, inducing a nonisothermal peak corresponding to melting. As usual, the vitreous transition temperature T_g, was determined at the inflection point of the rapid increase in specific heat corresponding to the glass transition. The top of the devitrification peak was called T_d [6,7,9,10] and the temperature T_m of the end of melting was defined as the temperature at the top of the melting peak. The temperature corresponding to the peak of devitrification (T_d) rises as the warming rate increases. The critical warming rate is reached when T_d approaches the melting temperature T_m, and ice crystallization is avoided. V_{cwr} is estimated by extrapolating the experimental values of T_m/T_d to 1.05, which corresponds to about 0.5% crystallization [7,17].

The pans contained droplets of VS4 (weights of 4.5 ± 1.2 mg) or small pieces of cortex or vessel (11.5 ± 2.4 mg). Only the critical cooling rate was studied in the case of the ovary fragments as this avoided testing tissue too long after perfusion (which would have biased the results in terms of impregnation) or having fragments damaged by successive freezing and ice melting over several freezing/thawing cycles (which would have perturbed the results in terms of ice crystallization kinetics). The ovaries for the DSC tests were prepared and impregnated with VS4 in the same way as the ovaries intended for cryopreservation. The behavior of the cryoprotectant in the ovarian tissue at the end of the impregnation procedures, and the theoretical conditions required to vitrify the parts of the ovary from which the fragments had been taken, were thereby determined. Mean values are presented as mean ± SEM, as for the histology data.

Results

VS4 calorimetry

The VS4 solution was prepared twice during testing, and each preparation was analyzed by DSC (Table 2). The critical cooling rate of the VS4 was estimated from the mean values of q (%) as 11.7 °C/min (Fig. 3) and the mean critical warming rate was evaluated from the mean values of T_m/T_d as 340 °C/min (Fig. 4). The mean vitreous transition temperature and the end-of-melting temperature were, respectively, estimated as $T_g = -124.3$ °C and $T_m = -37.3$ °C (to within 0.5 °C).

Table 2
Critical cooling and warming rates in the samples of VS4 used for ovary perfusion

	V_{ccr} (°C/min)	V_{cwr} (°C/min)	T_m (°C)	T_g (°C)
Preparation no. 1	13.5	700	-36.5 ± 0.5	-124.5 ± 0.5
Preparation no. 2	11	119	-38 ± 0.5	-124 ± 0.5

Ovary cooling

Seven ovaries were vitrified using VS4, as described above. The mean interval between removal in the slaughterhouse and initiation of perfusion was 74 ± 3.3 min. The mean ischemic time between removal and immersion in liquid nitrogen was 139 ± 4 min (as was the mean ischemic time between removal and the start of reperfusion for the solution control ovaries). There were no statistically significant differences between ovaries in terms of ischemia. Fig. 5 shows the evolution of the temperature recorded during cooling in the medullary and cortical areas. The mean tissue cooling speed was -407 ± 66 °C/min in the

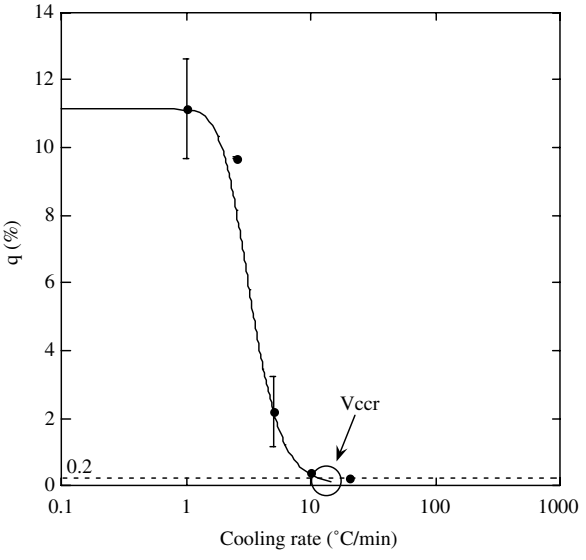

Fig. 3. Variation with cooling rate of the heat of ice formation q on cooling the VS4 solution. Isolated points are experimental points (mean ± SEM). The solid line is the semi-empirical curve calculated according to the model. V_{ccr} is the critical cooling rate of the VS4 solution. The latent heat of ice formation is represented, as usual in similar studies [5,8,50], 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 were chosen as they give a heat of solidification close to the quantity of ice crystallized in percentage (w/w) of solution when only ice forms. The heat in joules per kilogram of solution is obtained by multiplying q by $4.18 \times 79.78 \times 10$.

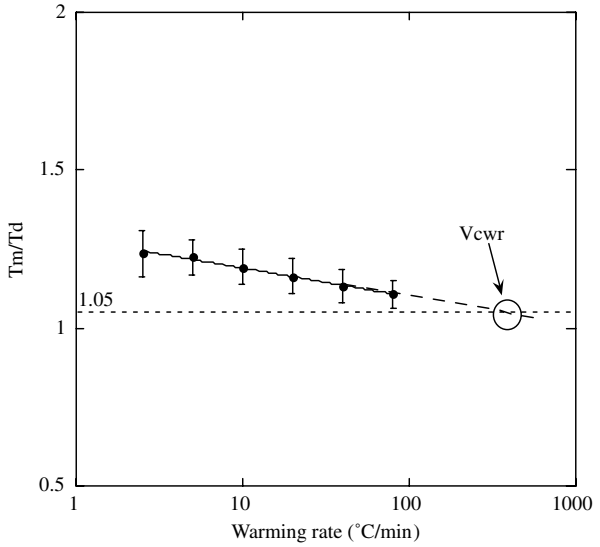

Fig. 4. Variations in the experimental values of T_m/T_d with warming rate. Isolated points are the mean experimental points with SEM as error bars. The critical warming rate is defined at the warming rate corresponding to $T_m/T_d = 1.05$. As usual, T_m/T_d varies linearly with the warming rate within a very good approximation. The solid straight line is an interpolation by the least square method.

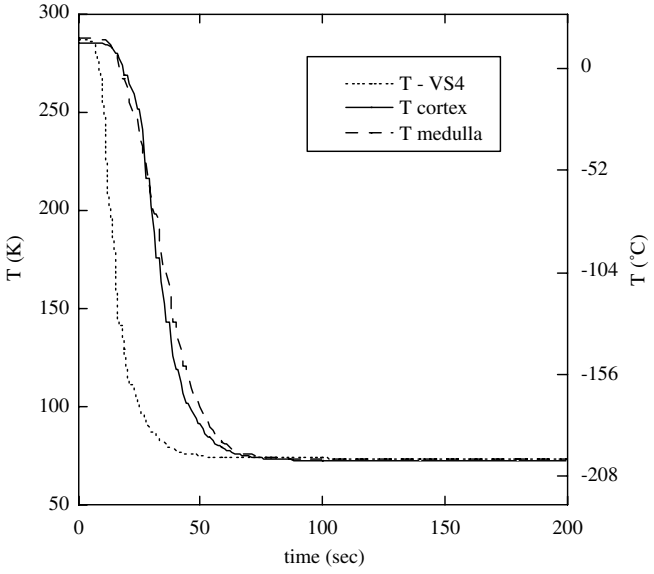

Fig. 5. Plot of temperature in the ovary medulla and cortex upon plunging into liquid nitrogen. The solid line plots cortical temperature, the broken line medullar temperature, and the dotted line VS4 temperature. The cooling rates were estimated from the time when cooling really starts till a temperature around −150 °C (that is a temperature below the vitreous temperature of the sample), while the curve is still in its almost linear part. The goal of this calculation is to know at which cooling rate each part of the sample is passing through the vitreous transition into a complete or partially amorphous state.

cortex, and -358 ± 42 °C/min in the medulla, compared to -557 ± 55 °C/min for the VS4 surrounding the ovary.

Fig. 6 shows the appearance of the ovary after cooling. Fractures were present around the thermocouples and leads. Areas of ice formation (white) form between the leads and in the cortex. The ice near the thermocouple inlet teats was limited to the surface of the VS4 menisci in that area (Fig. 1), and thus harmless for the tissue. The white area seen in the cortex, on the other hand, shows that this part of the tissue had not vitrified but frozen.

Rewarming

Fig. 7 shows the temperature changes during rewarming. The mean tissue thawing rates were 16 ± 4 °C/min in the cortex and 12 ± 4 °C/min in the medulla during the first step, and 237 ± 37 °C/min and 218 ± 47 °C/min, respectively, during the second step, compared to 23 ± 6 °C/min and 384 ± 39 °C/min, respectively, for the VS4 surrounding the ovary. Here again, broad standard deviations are to be explained as for those found in cooling.

During the rapid warming step the VS4 surrounding the ovary devitrified (turned white and opaque), but no macroscopic fractures occurred in vitrified vessels during warming (Fig. 8). Nor was any leakage observed during the subsequent perfusion. Differences in the index of refraction of unmixed solutions and micro-bubbles present in the circuit enabled the progress of the perfusate and its venous efflux to be monitored.

Cytotoxicity of the cooling and rewarming procedure with VS4

Follicle membrane integrity was $70.6 \pm 4.7\%$ without cryopreservation and $61.4 \pm 2.2\%$ after cryopreservation with VS4. This difference was not significant ($P = 0.1$). Ovarian primordial follicle density was 51.6 ± 12.7 without cryopreservation and 48 ± 12.4 after cryopreservation with VS4. Again, this difference was not significant ($P = 0.22$). However, histologic examination showed that the percentage of normal primordial follicles fell from $83.4 \pm 2.5\%$ to $48 \pm 3.8\%$ after cryopreservation ($n = 5$), a difference that was close to significance ($P = 0.06$). Intracytoplasmic vacuoles and/or deformed oocytes with basal membrane detachment were taken to indicate cytoplasm anomaly (cf. Fig. 9b). Oocyte nuclei were considered pyknotic in case of hypereosinophilic nucleic condensation (Fig. 9c). The rate of histologic cytoplasmic anomalies increased from $6.6 \pm 2.6\%$ to $25.6 \pm 6.2\%$ after cryopreservation, while the rate of total cell anomalies increased from $0.8 \pm 0.3\%$ to $17.8 \pm 5.3\%$ after cryopreservation—both differences being also close to significance ($P = 0.05$). However, there were not significantly more isolated nuclear anomalies before ($9 \pm 4.3\%$) than after cryopreservation ($8.8 \pm 4.7\%$).

Pedicle vessels were consistently histologically normal prior to cryopreservation, despite 40 min perfusion with VS4. After cryopreservation, the aspect of the ovarian vein was in all cases identical to that of its non-cryopreserved control. In three out of five cases, partial ovarian artery endothelial detachment was observed. Central medullary

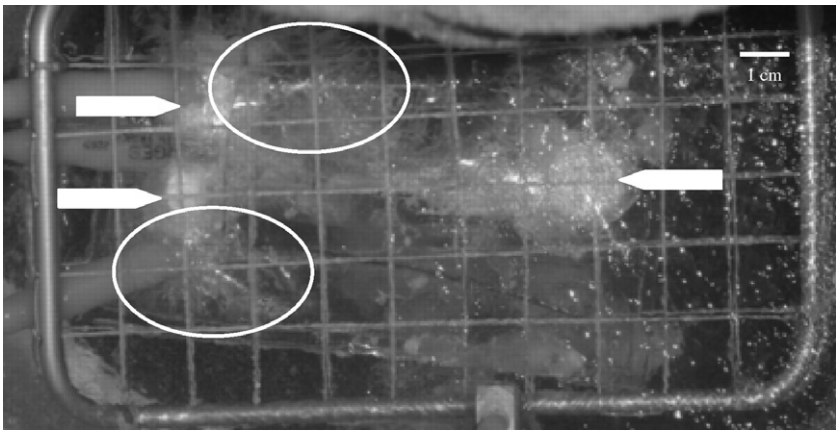

Fig. 6. Ovary aspect after freezing by plunging in liquid nitrogen. A certain number of fractures can be seen against the lights where the solid bodies (thermocouples and teats) have been introduced in the cryobag. They are pointed out by white ellipses. Characteristically white areas of ice formation can clearly be seen at the cortex and close to the teats, exactly where the VS4 menisci form between the teats. They are shown by white arrows.

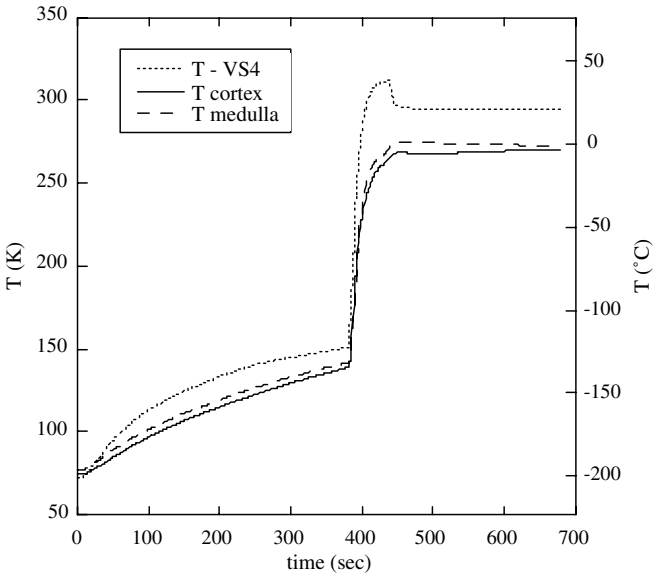

Fig. 7. Evolution of temperature in the medulla and cortex during 2-step rewarming of the ovary previously cooled by plunging in liquid nitrogen. The solid line plots cortical temperature, the broken line medullar temperature, and the dotted line VS4 temperature.

arteriolar myocytes were often bloated (four out of five cases). Medullary veins showed two cases with conjunctive tissue edema.

Ovarian fragment calorimetry

Six ovaries were used for DSC experiments on ovarian tissue. Only tissue from the cortex and ovarian lumbar pedicle vessels was tested. To minimize the consequences of repeated cooling/thawing cycles, maximal cooling and rewarming speeds, at which the least quantity of ice could be expected to form, were tested first followed by slower rates reduced in a step-wise fashion. A possible repetition-effect on crystallization was examined by repeating a cycle of cooling and rewarming at 300 °C/min several times: no significant change in crystallization peak size was observed (data not shown).

Measurements in cortex

In 3 of the 4 cortex samples (cf. Table 3), the critical cooling speed could not be calculated, as the amount of ice crystallizing at the highest and lowest cooling rates were comparable ($18.4 \pm 1.1\%$ vs. $18.8 \pm 1\%$ (Q_{max}), respectively). In ovary no. 2, less ice (10%) formed at high cooling rates—significantly below the 15% Q_{max} value for this sample. The theoretical model could then be used to estimate V_{ccr}, which was about −340 °C/min. Results for thawing of this fragment were: $T_m = -26.4$ °C and $T_g = -126.3$ °C, as compared to the melting temperature and the glass transition temperature of the others fragments (cf. Table 3).

Measurements in vessels

At the highest cooling speeds ice formed in 1 of the 5 samples. The other four samples had a mean critical cooling rate of 123 ± 33 °C/min and a mean maximum quantity of ice Q_{max} of $12.0 \pm 0.3\%$ (in comparison, the mean Q_{max} in the VS4 was $11.1 \pm 1.5\%$). The standard deviations were broad, perhaps due to the intrinsically heterogeneous biological characteristics of the samples, even for a given anatomical area. Table 3 presents the DSC data for the ovary samples.

Discussion

In the light of the various failures encountered using freezing for whole-organ cryopreservation [34,59], vitrification currently appears to be more adapted to complex heterogeneous [31,35] massive and vascularized [25,39,51] tissue. While slow freezing induces ice formation outside the cells, vitrification, by rapid cooling, has indeed the potential to convert liquids into an amorphous solid phase, free of ice crystals. To be feasible under technically manageable conditions for large amounts of tissue such as with whole organs,

Fig. 8. Photograph of the ovary after rewarming. The thermocouples remain in place, and no macroscopic tissue fracture is visible. Bubbles may indicate that fractures developed during cryopreservation.

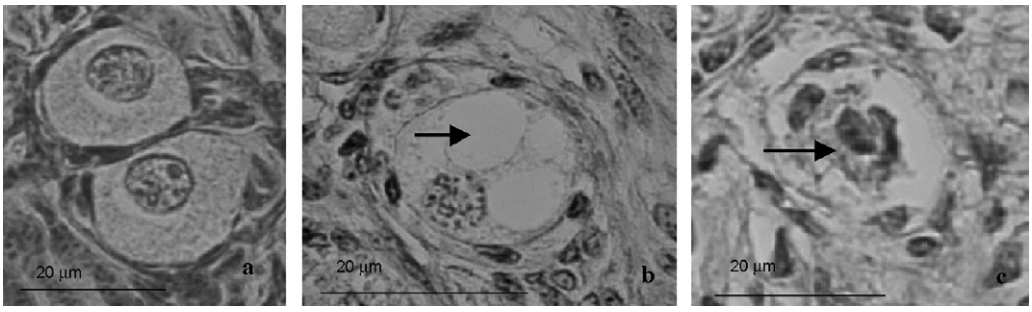

Fig. 9. Primordial follicle histology (a); normal primordial follicles; (b) primordial follicles with intracytoplasmic vacuoles (black arrow) (c); primordial follicles with mixed nuclear/cytoplasmic anomaly (black arrow).

Table 3
Characteristic values measured by, or calculated from DSC experiments in the tested ovary fragments

Type of tissue	Sample	Q_{max}[a] (% w/w)	V_{ccr} (°C/min)	T_m (°C)	T_g (°C)
Cortex	Ovary no. 1	16.8	—	−21.7	−117.5
Cortex	Ovary no. 2	15	340	−26.4	−126.3
Cortex	Ovary no. 3	20	—	−19.5	−117.5
Cortex	Ovary no. 4	19.5	—	−18.8	−117.5
Vessels (artery + vein)	Ovary no. 5	30	—	−15.7	−116.9
Vessels (artery + vein)	Ovary no. 5	12.5	33	−34.5	−124
Vessel (artery)	Ovary no. 6	11	190	−27	−108.8
Vessel (artery)	Ovary no. 6	12	144	−26.2	−108.6
Vessel (vein)	Ovary no. 6	12.4	125	−27.2	−108.6

[a] Q_{max} is the maximum quantity of ice crystallized in the sample, measured at a cooling rate of −2.5 °C/min, and expressed as % w/w.

vitrification requires high cryoprotectant concentrations, to hinder nucleation and crystal growth by interacting with the water molecules. A certain number of solutions combining various cryoprotective agents have been reported, mainly developed by Fahy's team [26,28,30]. One such, VS4, has been tested on rabbit kidney [26]. The present study sought to test cryopreservation of a whole ewe ovary impregnated with VS4. Although the use of slaughterhouse material may raise questions about the tissue behavior after warm and cold ischemic treatment, such ovaries are still alive during the harvesting. Schmidt et al. [58] have shown that transport of isolated ovarian cortex cooled on ice for a period of up to 4 h allows survival of primordial follicles following cryopreservation and transplantation to immunodeficient mice. Thus we considered that this model could allow us to establish some boundary conditions on the problem of large ovaries cryopreservation.

DSC measurements were performed on VS4 and on impregnated ovarian tissue fragments to establish cooling criteria preventing ice-crystal formation in tissue, as compared to in the VS4 itself. The vitrification conditions for VS4 itself were first determined. The DSC data indicated a mean critical cooling rate of 11.7 °C/min, in agreement with the 10 °C/min (range: 4–12 °C/min) reported by De Graaf et al., using the same semi-empirical model [19]. This value, determined under atmospheric pressure, matches that of Kheirabadi and Fahy [38] under high hydrostatic pressure; De Graaf et al. account for this on methodological grounds [19], Fahy using a macroscopic approach with 10 ml samples, whereas ours were around 5 μl. As regards rewarming, on the other hand, our present V_{cwr} values were significantly lower than those of De Graaf et al. (between 1570 and 6991 °C/min), although—as they likewise reported—with a variability verging on a factor of 4 (cf.

Table 2). We doubt that this discrepancy was due to the measurements as such or to the calculations, which were double-checked several times. More likely, our use of pure substances and filters may have eliminated impurities liable to induce nucleation, and thus devitrification during rewarming. In addition, in the second preparation, not mentioned above some slight but critical dehydration may have occurred between the moment when the solution was prepared and when it underwent DSC experiments (indeed, organizational questions of equipment availability delayed DSC testing after the cooling and rewarming experiments and required transport, under deficient refrigeration, to a different site).

During the cooling phase of our procedure, calculation of the cooling speeds applied to the ovary first of all shows that overall cooling was faster than the critical cooling rate of VS4. From Fig. 5, it can be seen that the cooling speed in the cortex was slightly higher than in the medulla, due to the external position of the cortex around the medulla. However, the difference is slight because of the density of the cortical tissue, giving it a greater heat capacity. Both types of tissue were also found to cool more slowly than the surrounding VS4 solution itself. The thermal conductivity of biological tissue (between 0.4 and 0.2 W/mK) slightly lower than for water (0.6 W/mK) might explain this difference in the cooling rates. In addition, the fact that the thermocouple placed in VS4 solution was closer to the cryobag surface than the two other thermocouples located in the tissue might also explain this difference, as it delays the onset of cooling in the ovary compared to the VS4 solution (as it can be observed on Fig. 5). However, it must be generally noticed that the standard deviations found were considerable, no doubt due to the variations of the thermocouple positions from run to run in a cryobag whose volume and geometry are not perfectly reproducible (due to physiological fluctuation in the ovary pedicle size), and the variations in the speed of plunging in the liquid nitrogen—a parameter that could be standardized in future by automating the plunging procedure.

From the point of view of vitrification, the DSC study showed the critical cooling rate in the cortex fragments (cf. Table 2) to be at best equal to or else higher than the mean cooling speed applied to the whole-cortex tissue. This finding explains the white area observed in the cortex (cf. Fig. 6), corresponding to ice forming under a cooling rate too slow to achieve vitrification. And yet the ovary had been perfused with VS4, and the surrounding VS4 had vitrified (as the numerous fractures to be seen in Fig. 6 bear witness). The impregnation of the cortical tissue by the VS4 must therefore have been non-homogeneous and insufficient, further shown by the variation in T_m values found within the cortex (the more thoroughly the sample is impregnated with cryoprotectant, the lower the expected value of T_m). The findings for the ovarian lumbar pedicle vessel fragments (cf. Table 3) show the same pattern. Being in direct contact with the VS4 solution, which was perfused via the ovarian artery, this tissue should have been especially well impregnated, containing more CPA, and thus shows critical rates closer to that of VS4 itself. And yet impregnation turned out to be far from complete, varying from sample to sample (compare the values for ovaries no. 5 and no. 6 in Table 3). The perfusion procedure thus needs to be improved to enhance impregnation, especially of the ovarian cortex, and ensure vitrification of the entire ovary.

Heat transfert limits are set by the low thermal diffusivity of the tissue. The problems of heat and mass transfer in such large tissue samples may require longer exposure to the cryoprotectant and/or a different perfusion rate to achieve adequate penetration of the most deep-lying ovary cells. We are currently planning a dynamic NMR study of ovarian impregnation to examine the speed and quality of cryoprotectant diffusion under our perfusion conditions, with a view to determining the parameters to be optimized. Complete homogeneous VS4 impregnation should facilitate tissue vitrification by reducing the speeds required to prevent ice formation according to the DSC studies we performed a few years ago on small organ (rat heart and rabbit kidney) fragments impregnated with cryoprotective solutions [54]. These studies found lower critical rates in tissue than in the impregnating cryoprotective solutions. Such an improvement in vitrification conditions was attributed to cryoprotectant confinement within the tissue, partitioning of liquids increasing the tendency to supercool. The present results for cortex fragments, however, differ from the above, confirming that this particularly follicle-rich tissue would be hard to impregnate. But this report presents a first approach, starting the study of the vitrification procedure and especially the development of the cooling and rewarming protocol with dedicated equipment. In this work, we used an empirical model of impregnation based on several results: Bedaiwy et al. [11] and Martinez-Madrid et al. [41] for the perfusion via the ovarian artery, and Kheirabadi et al. [38] for the beneficial decrease of the temperature when cryoprotective solution concentration is increased. In our procedure, the VS4 was introduced in 4 steps of increasing concentration, while temperature was reduced for the latest two steps to 4 °C. The aim was to reduce cryoprotectant toxicity, which increases with rising concentration but tends to diminish with falling temperature. Fahy et al. recommended a perfusion temperature lower than 4 °C for VS4 in rabbit kidney [38], but toxic impact varies with the type and composition of the biological tissue [24] and the low toxicity of the present protocol had been previously checked on sheep ovaries [18] using controls not exposed to VS4. In addition, in the present experiments, the perfusate was not oxygenated, but good histological results in our previous report [18] have shown that primordial follicles survive when whole ovary is perfused during 1 h at room temperature. Like Schmidt et al. [58], we think that primordial follicles

seem to be resistant to ischemia prior to the vitrification process. In the future, we would like to be able to develop and adjust a home-made perfusion device designed to change continuously temperature and cryoprotective solution concentration, on the basis of the works done by other teams in that way [38,52], after adaptation to the ovarian vitrification.

During rewarming, the mean speeds applied (cf. Fig. 7) show that the cortex warms slightly more quickly than the medulla, and tissue as a whole slightly more slowly than the VS4 solution. This accelerated cortical rewarming in itself testifies to the presence in this tissue of ice, with a thermal conductivity (2.2 W/mK) greater than that of water or biological tissue, and a heat capacity (~2.1 J/g K) lower than that of the solution (~4.18 J/g K for liquid water). Even so, from Fig. 8 it can be seen that the thawed ovary never showed any macroscopic fracture, unlike in our preliminary studies, where there had always been a fracture around the ovarian attachment, which is very fine and subject to considerable mechanical stress by the presence of the catheter. Improved cryobag preparation, the use of the sliding frames and slow rewarming from the vitreous phase doubtless helped minimize stress within the glass, which would be liable to damage the tissue [53] especially around the ovarian attachment. Moreover, after rewarming, perfusion was achieved without leakage, confirming the absence of macroscopic fracture and the harmlessness of the fractures found in the surrounding VS4—which we would, nevertheless, like to be able to minimize. Improved impregnation would allow cooling speeds to be reduced without risk, thereby reducing the fracture rate [27]. Cooling with annealing would then allow the glass to be relaxed, further reducing the stress that causes fracture [8].

At the outcome of our cooling and rewarming procedure, the immediate follicle membrane integrity assessed by trypan blue test was 61%, with 48% of normal primordial follicles remaining - indicating an acceptable cytotoxicity level. Our histological findings concerned, however, only small primordial follicles of less than 60 μm diameter, and did not count partially or completely denuded oocytes, as we could not tell whether they resulted from histological artifacts or from thermal damage or ischemia. Moreover, they were limited by the small number of tissue samples, and that probably explains why we do not have a significant finding despite a big fall in the number of normal-appearing primordial follicles after attempted vitrification. Dye exclusion tests and histological observations are not exhaustive ultrastructural studies of oocyte status. That is why, we do intend to use other ovarian tissue exploration techniques (e.g. DNA fragmentation), and to repeat the experiments on larger groups, once our vitrification protocol has been optimized. The indices we used here to test ovaries after attempting vitrification and rewarming provide preliminary evaluations. They give information on follicle integrity immediately after thawing, but may not have much to do with actual capacity to recover, and then grow into healthy antral follicle. It is obviously only the successful transplantation by microvascular anastomosis [33] of a vitrified ovary that could confirm ovarian functions following the vitrification procedure. The medulla vessels were slightly affected by the procedure, presenting an edematous aspect of the wall and adjacent conjunctive tissue, probably due to ice crystals forming in the centre of the ovary, although the tissue as a whole appeared histologically satisfactory.

Conclusion

The current study showed that an experimental ovarian cryopreservation technique for whole sheep ovaries—ovaries perfused with VS4 and cooled to −196 °C in less than a minute when plunged into liquid nitrogen—appears feasible. Differential scanning calorimetric measurements showed that the critical cooling rate required to vitrify VS4 cryoprotective solution was about −12 °C/min. This rate can be achieved under our experimental conditions, since the speed reached on cooling in the ovary was in the range of a few hundred degrees per minute, i.e. considerably higher than the critical VS4 cooling rate. Cryopreservation with VS4 left more than 50% of primordial follicles intact and immediately post rewarming follicle membrane integrity was over 60%, no fall in ovarian tissue follicle density being observed. Nevertheless, it is obviously only the successful transplantation by vascular micro-anastomosis of a vitrified ovary that could confirm ovarian functions following such a cooling and rewarming procedure.

This work demonstrated also the interest of physical studies for the assessment and improvement of the vitrification approach. Differential scanning calorimetry, undertaken to study the bulk cryoprotective solution and small pieces of impregnated ovary, helped determine the critical cooling rates required for the samples and demonstrated 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 insufficient and uneven tissue penetration, as suggested by the variability of results between the ovarian fragments. This shows that our perfusion procedure must be improved to enhance ovarian cortex impregnation and bring down the critical rates in tissue. This is necessary to achieve complete vitrification of all the tissue, as well as to rewarm without ice crystal formation. Nevertheless, our measurements indicate an acceptable cytotoxicity level at the outcome of our cryopreservation procedure, although ovarian tissue is not totally vitrified at the end of cooling. Maybe this means that successful cryopreservation of the whole ovary does not need complete vitrification of the cortex.

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