Effects of different freezing parameters on the morphology and viability of preantral follicles after cryopreservation of doe rabbit ovarian tissue

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Objective: To compare the effects of dimethyl sulfoxide (DMSO), 1,2-propanediol (PROH), sucrose, trehalose, concentration of cryoprotectants, equilibration method, and postseeding freezing rate on doe rabbit ovarian tissue preservation after freezing, using fractional experimental design.
Design: Experimental prospective study.
Setting: Research institute in veterinary and agronomic colleges.
Animal(s): Californian doe rabbits.
Intervention(s): Ovarian cortices were prepared from ovaries collected in slaughterhouse. Fractional experimental design was used to evaluate simultaneously five chemophysical factors influencing the cryopreservation of ovarian tissue.
Main Outcome Measure(s): Follicle viability by Live/Dead® viability/cytotoxicity kit and histologic evaluation of the ovarian tissue.
Result(s): Experimental design suggests that equilibration method and cryoprotectant concentration have no effect on the proportion of normal follicles. Penetrating and nonpenetrating cryoprotectants seems to influence the preservation of the follicles with advantage for PROH and trehalose. The follicular preservation seems to be highly influenced by the postseeding freezing rate. Freezing rate of 0.3°C/min seems to be less deleterious than 2°C/min. Morphologic preservation ratio reaches 85% using PROH and trehalose.
Conclusion(s): Cryopreservation of doe rabbit ovarian tissue using conventional cryoprotectant and 0.3°C/min as freezing rate seems to be a promising technique and could be used as a model for women. (Fertil Steril® 2008;89: 1348–56. ©2008 by American Society for Reproductive Medicine.)

Key Words: Ovarian tissue, cryopreservation, preantral follicles, cryoprotectant, doe rabbit, freezing rate, follicular morphology, follicular viability

The preservation of female gametes has important medical and veterinary applications. The cryopreservation of the ovarian tissue is a promising technique which allows the simultaneous preservation of thousands of follicles located in the ovarian stock, up to the primary stage. Because no stimulation is necessary, it could be used as an emergency preservation method. Immature follicles are better suited to cryopreservation because of their small size and the absence of zona pellucida and because they are metabolically quiescent and undifferentiated (1). By these properties, frozen ovarian tissue takes advantage over cryopreservation of mature oocytes.

The cryopreservation of ovarian tissue has been developed in women to preserve the fertility of young cancer patients before a gonadotoxic treatment (e.g., chemotherapy or radiotherapy). After cancer remission, it may restore the natural reproductive function of these women after autografting. Even though ovarian cryopreservation is still experimental, it is today performed for young patients who risk becoming sterile because of gonadotoxic treatments (2). Moreover, the cryopreservation of ovarian tissue could be applied to the preservation of animal genetic resources: endangered wild species, domestic breeds, transgenic animals, or biomedical models. Doe rabbits may constitute a model for the human and the animal applications of the ovarian tissue cryopreservation, because of their biologic and breeding characteristics. Because its prolificity is high (∼10 kits per litter, every 42 days) and its generation interval is reduced (5–6 months), the rabbit would allow rapid assessment of the long-term deleterious effects of the cryopreservation process over successive generations. The rabbit is also the most used among the nonrodent species for regulatory teratology studies. Finally, the rabbit model was chosen because of its cheap breeding management and its easy manipulation. Gene banks have been established to collect and to store the genetic material of such animals (3). This method could represent a new tool for genetic resources storage by the female pathway.

After cryopreservation, mature oocytes could be obtained from the ovarian tissue by grafting or in vitro folliculogenesis.

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Live offspring have been born from cryopreserved ovarian tissue autografted or allografted in mice (4–7), rabbits (8), ewes (9–12), and women (13, 14). Large antral follicles developed after xenograft of frozen ovarian tissue from different animal species into immunodeficient mice (15, 16) and also in humans (17–21). Although offspring were obtained from cryopreserved ovarian tissue, it is necessary to determine the critical steps of the freezing protocol by in vitro evaluation before grafting.

Cryopreservation process is influenced by several chemophysical parameters affecting directly or not the equilibration time, the freezing up to the storage temperature, and the thawing. The equilibration in cryoprotective solutions partly replaces the inner cell water by cryoprotective agents (CPA). However, the CPAs can be damaging to cells, especially when used at high concentrations. The toxicity can be reduced by decreasing the time or the temperature of the equilibration step (22). But equilibration at low temperatures requires increasing the exposure time to freezing solution. Furthermore, CPAs may have dramatic osmotic effects upon cells during their addition and removal. Consequently, the use of several steps of increasing CPA concentrations during the equilibration allows reducing the osmotic gradient. Cells exposed to such penetrating CPAs undergo initial dehydration followed by rehydration and potential gross swelling upon removal. This osmotic shock may generate membrane damage by mechanical means and predisposition of the cell to injuries during the other steps of cryopreservation or even cell death (23). During the freezing step, follicular preservation depends on the nature and concentration of CPA. Freezing rate and temperature of seeding also influence the ice properties. Finally, thawing and removal of CPA depend on temperature and on presence of nonpenetrating CPA limiting the osmotic swelling during rinsing.

The objective of the present work was to distinguish the effects of five chemophysical factors which may influence the morphology and the viability of preantral follicles after cryopreservation of doe rabbit ovarian tissue. Each factor was evaluated according to two modalities. Two penetrating CPAs—dimethylsulfoxide (DMSO) and 1,2-propanediol (PROH)—were evaluated at two concentrations (1.5 mol/L and 2 mol/L). Two nonpenetrating CPAs (sucrose and trehalose), two freezing rates (2°C/min and 0.3°C/min, postseeding), and two equilibration methods (1 step and 3 steps) were evaluated also. In a second part, the most adaptive modalities of these chemophysical factors were evaluated in a wider population.

MATERIALS AND METHODS

Collection of Ovaries and Preparation of the Ovarian Tissue

The ovaries from young (12–15 weeks old) female Californian rabbits were collected at the slaughterhouse and placed in TCM 199 immediately after the death of the animal. The ovaries were transfered to the laboratory within 1.5 hours. According to previous results (24), the transport was performed at 10°C to limit the effects of the ischemia. The ovaries were dissected free of fat and mesentery into TCM 199. Any remaining stromal tissue was gently removed until the cortex had a thickness of 1 mm. For each ovary, the ovarian cortex was divided into two to three equal fragments (around 0.3 to 0.6 cm^2 each) according to the experiment. For each experiment, one fragment per female was fixed as control in 4% paraformaldehyde before treatment. Unless otherwise indicated, all chemicals were obtained from Sigma (St. Louis, MO).

Evaluation of Cryopreserved Ovarian Tissue

Histologic protocol and morphologic evaluation Ovarian fragments were fixed in 4% paraformaldehyde for 24 hours at room temperature. After dehydration and embedding in paraffin wax, fragments were cut (semiserial sections 4 μm thick every 10 μm) and stained with hematoxylin and eosin. Primordial to primary follicles—from oocytes surrounded by flattened granulosa cells to oocytes surrounded by one layer of cuboidal granulosa cells (25)—were classified into four types of morphologic defects:

Type I: follicle without any morphologic defect. Follicle is regular, with joined follicular cells. Cytoplasm of the oocyte is homogeneous and chromatin is diffused and regular.

Type II: follicle with cytoplasmic defect. Cytoplasm of the oocyte is vacuolized or eosinophilic.

Type III: follicle with nuclear defect. Nucleus of the oocyte is pycnotic, without apparent nuclear membrane or with an irregular nuclear membrane.

Type IV: degenerated follicle. Oocyte with combined cytoplasmic and nuclear defects or follicle with irregular shape or with disjoined follicular cells or with swollen follicular cells.

One hundred primordial to primary follicles with a visible nucleus were examined for each ovarian fragment. Morphologic preservation ratio was defined as percentage of type I follicles after preservation/percentage of type I follicles before preservation.

Follicular viability assay Viability of follicles was evaluated on isolated follicles by live/dead test using calcein AM and ethidium homodimer I stains (Live/Dead® Viability/Cytotoxicity Kit (L-3224); Molecular Probes, Leiden, The Netherlands).

Isolation of small follicles. Ovarian fragments were finely dissected in TCM 199 at room temperature and incubated with collagenase type I (0.5 mg/mL) at 37°C for 1.5 hours and gently pipetted every 30 min. Collagenase action was blocked by addition of fetal bovine serum (FBS). The suspension was filtered through a 60-μm nylon filter (Fisher Bioblock Scientific, Illkirch, France) to recover the small follicles and centrifuged at 400*g* for 5 minutes. The pelleted cells were resuspended with 30 μL Euroflush medium (IMV, L'Aigle, France).

Viability test using calcein AM and ethidium homodimer I. Calcein AM and ethidium homodimer I were used for the observation of living and dead cells, respectively. Calcein AM is a nonfluorescent dye that is hydrolyzed by intracellular esterases and generates intense green fluorescence in living cells (excitation/emission = 494/517 nm). Ethidium homodimer I passes through damaged cell membranes and binds to DNA, thereby producing a bright red fluorescence in dead cells (excitation/emission = 528/617 nm).

Calcein (2 μmol/L)/ethidium (4 μmol/L) solution was added to the cellular suspension. After 10 minutes of incubation in darkness, cell suspension was examined under an inverted microscope (IX-71; Olympus, Rungis, France) equipped for epifluorescence examination.

Use of a conventional fluorescein long-pass filter allowed simultaneous visualization of calcein AM metabolite and ethidium homodimer. Follicles were preselected under direct light before epifluorescence observation (26, 27).

One hundred follicles were observed for each ovarian fragment and classified as live or dead follicles (Fig. 1). Follicles presenting a live oocyte but more than one half of dead follicular cells were considered to be dead. Follicles presenting a dead oocyte but live follicular cells were also considered to be dead.

Viability ratio was defined as percentage of viable follicles after preservation/percentage of viable follicles before preservation.

Experiment I: Discrimination Between Freezing Parameters

Experimental design and statistical analysis A fractional experimental design $2^{(5-2)}$ (The nomenclature $2^{(k-p)}$ is related to a two-level factorial design that is run for the evaluation of k factors and a resolution of p. The experimental design allows simultaneous evaluation of all main effects and all interaction effects. The resolution is related to the expected alias pattern. A resolution p = 2 indicates that main effects may be aliased with 2 factor interactions. The value $2^{(k-p)}$ is also related to the final number of evaluated combinations of each parameters.) was elaborated (Table 1) to evaluate the combined effect of five different factors according to two modalities for each of them (Table 2) (28). This experimental design allowed discriminating between five factors influencing the cryopreservation process and the interactions between penetrating CPA and concentration of penetrating CPA.

Eight combinations of factors were performed. For each of them, the ratio of morphologic preservation and the ratio of viability of the preantral follicles were recorded. The linearity (structural and estimated model) of the experimental model was evaluated by an analysis of variance (ANOVA) test. One randomly chosen assay was replicated three times to estimate the experimental error. Ten experiments were randomly performed.

Multilinear regression was performed using all the variables to evaluate experimental results according to this model:

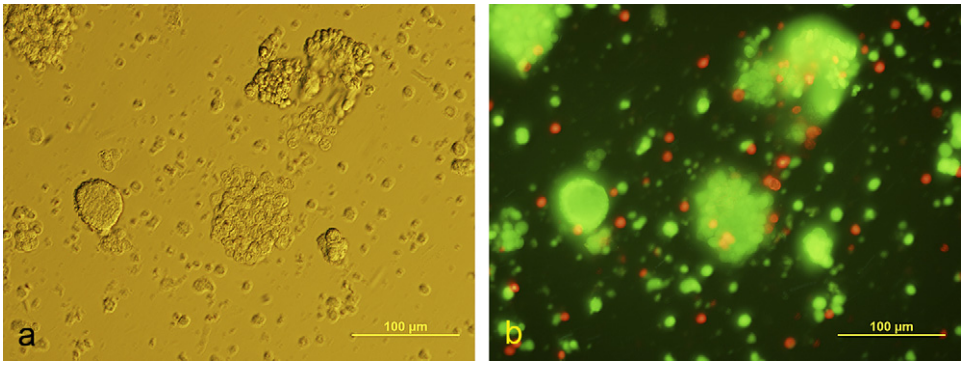
$$\hat{y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{12} X_1 X_2 + E$$

Differences were considered to be statistically significant when $P<.05$.

Cryopreservation process After isolation, ovarian cortices were equilibrated in the freezing media at room temperature. According to the experimental design (Tables 1 and 2), the equilibration was performed in one step (directly in the final freezing solution for 10 minutes) or in three steps (variable ×5). For the three-step equilibration, ovarian cortices were immersed in successive baths of increasing concentration

FIGURE 1

View of viability evaluation of isolated preantral follicles. **(a)** Direct observation of isolated ovarian follicles. **(b)** Fluorescent staining with calcein AM and ethidium homodimer I. No dead follicle is shown in this view.



Neto. Cryopreservation of ovarian tissue. Fertil Steril 2008.

TABLE 1

Fractional experimental design $2^{(5-2)}$ used to evaluate the cryopreservation protocols.

	I	X1	X2	X3	X4	X5	X1.X2
1	+1	−1	−1	−1	+1	−1	+1
2	+1	+1	−1	−1	+1	+1	−1
3	+1	−1	+1	−1	−1	+1	−1
4	+1	+1	+1	−1	−1	−1	+1
5	+1	−1	−1	+1	−1	+1	+1
6	+1	+1	−1	+1	−1	−1	−1
7	+1	−1	+1	+1	+1	−1	−1
8	+1	+1	+1	+1	+1	+1	+1

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of penetrating CPA (0.5, 1.0, and 1.5 or 2.0 mol/L) for 5 minutes each. Freezing solutions were composed of TCM 199, supplemented with 10% FBS and 1.5 mol/L or 2.0 mol/L (variable ×2) of penetrating CPA (variable ×1; DMSO or PROH) and 0.2 mol/L sucrose or trehalose (variable ×3). Sucrose or trehalose was added in the last equilibration step.

Freezing of ovarian fragments was performed in 1 mL CBS straw (IMV, L'Aigle, France), thermowelded at the two extremities. Cryopreservation was performed with a programmable freezer (Freeze Control; Cryologic, Victoria, Australia). Ovarian cortices were cooled from 21°C up to the seeding temperature at the rate of 2°C/min and hold for 2 minutes at this temperature before seeding. Manual seeding was performed by touching the straw with a cotton swab precooled in liquid nitrogen up to the start of crystallization. Seeding was performed at −7°C or −9°C for freezing media composed of 1.5 mol/L or 2 mol/L penetrating CPA, respectively. Five minutes after seeding, samples were frozen via two freezing rates (variable ×4; 2°C/min vs. 0.3°C/min) to −35°C followed by a rapid decreasing in temperature to −140°C (mean of 3.4°C/min) before immersion in liquid nitrogen. Histologic observation and viability evaluation were performed as control before freezing.

TABLE 2

Dependent variable list.

Variable	Level −1	Level +1
X1: Penetrating CPA	DMSO	PROH
X2: Concentration of penetrating CPA	1.5 mol/L	2 mol/L
X3: Nonpenetrating CPA	trehalose	sucrose
X4: Freezing rate	0.3°C/min	2°C/min
X5: Equilibration	1 step	3 steps

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Thawing process Thawing was performed by immersion of straws in a water bath at 37°C until the ice melted. The CPAs were removed step by step in four successive baths of TCM 199 + 10% FBS, supplemented by 0.2 mol/L sucrose and decreasing concentration of penetrating CPA (1.0, 0.5, and 0 mol/L); final bath was TCM 199 + 10% FBS alone. Each step was performed at room temperature for 5 minutes. Each ovarian cortex was then divided into two fragments for the subsequent histologic observation and the viability evaluation.

Experiment II: Evaluation of Freezing Protocols

Thirty ovarian pieces from 10 females were treated according to the results obtained in experiment I. Ovarian cortices were equilibrated (three steps) in the freezing media based on TCM 199 and 10% FBS at room temperature. The freezing media was supplemented with 1.5 mol/L DMSO or PROH and 0.2 mol/L sucrose or trehalose. Freezing of ovarian fragments was performed as described in experiment I. Seeding was performed at −7°C and freezing up to −35°C was performed at a 0.3°C/min rate. Thawing, histology, and viability tests were performed before and after cryopreservation as described previously.

Statistical Analysis

One-way ANOVA for equally sized groups was used to compare the proportion of normal or viable follicles before and after cryopreservation and the morphologic and viability ratios (ANOVA I, Stat View for Windows; SAS Institute, Cary, NC). Treatments were considered to be significantly different when $P<.05$.

RESULTS

Experiment I: Discrimination Between Freezing Parameters

The experimental variability expressed as the repetition of one single combination shows that both the structural and the estimated models of this experimental design are valid when considering the morphologic preservation ratio of the follicles. The concentration of the penetrating CPA ($P=.67$) and the number of equilibration steps ($P=.19$) seem to have no significant effect on the morphologic preservation ratio of ovarian follicles. The nature of the penetrating and nonpenetrating CPA seems to influence the morphologic preservation ratio of the follicles ($P=.08$ and $P=.07$, respectively) though nonsignificantly. Dimethylsulfoxide tends to reduce the morphologic preservation ratio compared with PROH. Morphologic preservation ratio is increased in the presence of trehalose compared with sucrose. The freezing rate seems to be the factor that has the greatest impact on the morphologic preservation ratio of the follicles. At a freezing rate of 0.3°C/min we observed a significant increase of the follicular morphologic preservation ratio compared with 2°C/min ($P<.01$). No significant interaction was observed between the nature of the penetrating CPA and its concentration.

Considering the viability ratio of the follicles, the experimental variability expressed as the repetition of one combination shows that this model of experimental design is not valid. None of the evaluated chemophysical factors can be discriminated when considering the viability ratio.

Experiment II: Comparison of Cryopreservation Processes

According to the results of experiment I, the precise evaluation of the best combination of factors influencing positively the morphologic preservation ratio (three-step equilibration protocol, 1.5 mol/L DMSO or 1.5 mol/L PROH, medium supplemented with sucrose or trehalose, 0.3°C/min freezing rate) was performed as described in Material and Methods.

Results are summarized in Table 3 and Figure 2. In control fragments, we observed 72.6 ± 2.8% and 77.7 ± 3.9% type I follicles (no significant difference). Proportion of viable follicles for sucrose control groups (76.4 ± 2.5%) was significantly lower than for trehalose control group (85.9 ± 1.9%; $P<.05$; Table 3). Consequently, comparison between the four cryopreserved groups can be realised on the basis of the viability ratio (Fig. 2).

No statistical difference of the proportions of type I follicles is found between sucrose and trehalose (50.2 ± 4.1% vs. 51.1 ± 1.8%, respectively) when using DMSO for cryopreservation. When using PROH as penetrating CPA, the proportion of type I follicles was lower after cryopreservation with sucrose compared with trehalose (55.0 ± 3.8% vs. 65.0 ± 3.3%, respectively; $P<.05$). When freezing with trehalose, the proportion of type I follicles was higher with PROH compared with DMSO (65.0 ± 3.3% vs. 51.1 ± 1.8%, respectively; $P<.01$). Nevertheless, the proportions of type I follicles were significantly reduced after cryopreservation, whatever the penetrating and the nonpenetrating CPAs were (from $P\leq.01$ to $P<.001$; Table 3). No significant difference was observed between the different groups of frozen ovarian cortices when considering the morphologic preservation ratio (Fig. 2).

Before freezing, type II follicles represented the most important part of follicles with morphologic defect (19.1 ± 2.9% and 16.1 ± 3.2% in sucrose and trehalose groups, respectively). After cryopreservation, follicular defect of type IV (degenerated follicles) was the most important type of morphologic defect: 32.5 ± 4.8% and 24.0 ± 1.9% after freezing using DMSO with sucrose and trehalose, respectively, versus 27.2 ± 5.6% and 18.1 ± 3.0% after freezing using PROH with sucrose and trehalose, respectively.

No significant difference was observed between the different groups when considering the viability ratio, except for the combination DMSO/trehalose, where viability ratio was lower than with DMSO/sucrose ($P<.05$; Fig. 2).

The general aspect of ovarian tissue before and after cryopreservation shows a good preservation of structural architecture (follicular structure and connective tissue). Spaces are observed in some cases in the ovarian stroma and the albuginea (Fig. 3). Epithelial cells are often absent compared with the fresh ovarian tissue.

TABLE 3

Morphologic and viability evaluation of frozen/thawed ovarian tissue before and after cryopreservation.

	Morphologic evaluation (% type I)	Viability evaluation (% viable)
Control Sucrose	72.6 ± 2.8%[a,c]	76.4 ± 2.5%[a]
DMSO	50.2 ± 4.1%[b]	79.8 ± 3.1%[a]
PROH	55.0 ± 3.8%[b]	74.7 ± 2.7%[a]
Control Trehalose	77.7 ± 3.9%[a]	85.9 ± 1.9%[b]
DMSO	51.1 ± 1.8%[b]	78.0 ± 2.3%[c]
PROH	65.0 ± 3.3%[c]	79.8 ± 2.7%[b,c]

Note: Results are expressed as mean ± SEM. In each column, different superscripts represent a significant difference ($P<.05$).

Neto. Cryopreservation of ovarian tissue. Fertil Steril 2008.

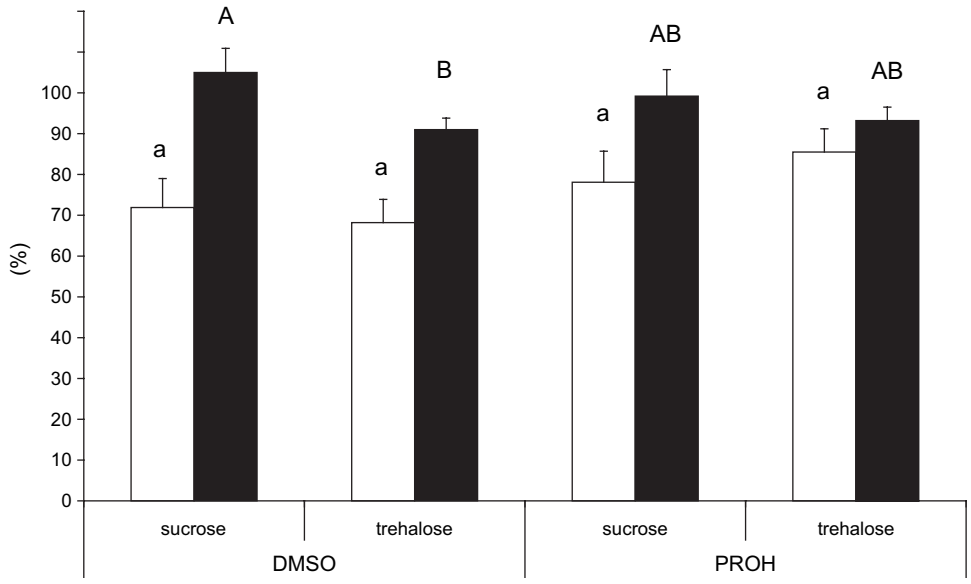
DISCUSSION

The use of a fractional experimental design allowed evaluating simultaneously five chemophysical factors influencing the cryopreservation of the ovarian tissue. The experimental design gives a general view of the results and distinguishes the most valuable factors. Finally, the factors which seemed to have a discriminating effect on follicular morphologic preservation were evaluated in a wider population.

The results of the experimental design show that the most important chemophysical factor influencing the morphology of ovarian follicles is the freezing rate after seeding. A slow freezing rate (0.3°C/min) seems to be more appropriate for the cryopreservation of the doe rabbit ovarian tissue. Most authors use a similar slow freezing rate, which is derived from embryo freezing protocols. However, few studies show the importance of this freezing parameter. In contrast to the present study, Demirci et al. (29) observed a high (but nonsignificant) proportion of follicles without any morphologic defect after a postseeding freezing rate of 2°C/min in the ewe. Nevertheless, Gook et al. (30) also observed a better follicular preservation when using a slow freezing rate (0.3°C/min) with human ovarian tissue. Whereas Cleary et al. (31) observed no difference in terms of follicular growth after grafting, between a conventional embryo freezing protocol (0.3°C/min) and a passive cooling at 1°C/min from

FIGURE 2

Morphologic preservation ratio (*white bars*) and viability ratio (*black bars*), mean ± SEM, after freezing with DMSO or PROH associated with sucrose or trehalose. For each group, different superscripts represent a significant difference ($P<.05$).



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0°C to −84°C on the mouse ovarian tissue. Although both of these cooling rates (0.3°C/min and 2°C/min) could be considered slow, the results may be explained by a difference in cell dehydration during the postseeding step. With rapid cooling rates, we can hypothesize that time required for the exosmosis of the cell water is insufficient and consequently promotes the formation of lethal intracellular ice. Although at very slow cooling rates, a high level of dehydration occurs with concomitant increase in solute concentration (salting out).

The present experimental design revealed a crucial role of the penetrating and the nonpenetrating CPA, despite no significant evidence. Among the various freezing protocols described in the literature, those using DMSO or PROH as penetrating CPA seems to be more efficient, whatever the species. Experiment II allowed us to evaluate them in detail. The results suggest that PROH and trehalose improve the follicular morphologic quality after freezing. Cryopreservation with sucrose seems to improve the viability of follicles. And viability ratio of follicles is significantly higher using sucrose plus DMSO than using trehalose plus DMSO. Nevertheless, concerning the morphologic preservation ratio, no significant difference is observed. So we may hypothesize that the combinations PROH/trehalose or DMSO/sucrose have similar protective effect against the freezing damages during the cryopreservation process. Sugars are not systematically associated with penetrating CPA, but Marsella et al. (32) showed the advantageous effect of sucrose. Trehalose has been frequently used in embryo cryopreservation but not in ovarian tissue cryopreservation.

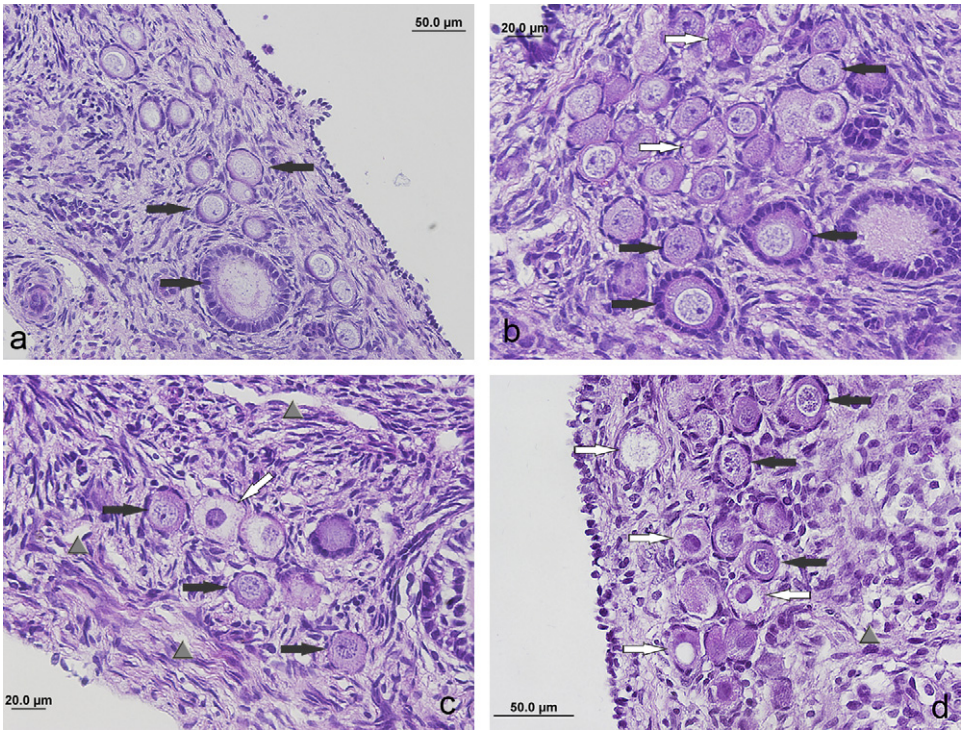
Sucrose and trehalose share the property to stabilize cellular membranes and proteins via the formation of hydrogen bonds with polar residues of phospholipidic membrane. This property allows preserving the membrane integrity under anhydrous conditions. Moreover, it modifies the temperature at which the separation of lipid phase occurs during cooling (33–35). Compared with other sugars, trehalose seems to have a higher capacity to preserve biomolecules, cellular membrane, and cells in a drying or in a freezing state (36–40).

Few comparable studies have been reported in the cryopreservation of doe rabbit ovarian tissue (41). The difference between morphologic preservation ratio and viability ratio for each treatment may be explained by the capacity of damaged follicle to survive. Little follicular growth after autografting of cryopreserved hemiovary was obtained by Daniel et al. (42) using glycerol as CPA. Recently, Almodin et al. (8) obtained live offspring after grafting of small fragments of cryopreserved rabbit ovarian tissue using 1.5 mol/L DMSO and a very slow postseeding freezing rate.

Most authors have observed a reduction of normal follicles in frozen/thawed ovarian tissue compared with fresh control when using similar freezing protocols in the mouse (43), the goat (44), the cow (45), and the ewe (29). But no morphologic difference was observed in human follicles before and after cryopreservation (46, 47). Newton et al. (1) observed similar

FIGURE 3

Morphologic view of doe rabbit ovarian cortex before and after cryopreservation. **(a)** Control group. **(b)** After freezing with PROH and trehalose. **(c)** After freezing with DMSO and trehalose. **(d)** After freezing with PROH and sucrose. *Black arrows:* type I primordial to primary follicles; *white arrows:* type IV follicles; *gray triangles:* spaces in ovarian tissue after freezing.



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proportions of "viable" follicles after freezing when using DMSO, ethylene glycol, or PROH and xenografting. Finally, Schubert et al. (48) observed a high proportion of morphologically normal follicles (79%) after tissue cryopreservation in a serum-free media composed of PROH and raffinose supplemented with taurine and L-glutamine.

The Live/Dead® test seems to be a good complementary method for the evaluation of the ovarian follicles' viability and for completing the morphologic assessment allowed by histology. It is easy and faster to perform and seems to be more discriminating than trypan blue staining. It could also be used directly in fresh tissue section, but detection of dead follicles seems to be more difficult (48).

For the first time, a complete evaluation process of important factors influencing the morphology and the viability of preantral follicles has been performed after equilibration process and freezing. The present results suggest that cryopreservation of doe rabbit ovarian tissue is a promising technique. More than 85% of initially normal follicles are preserved after cryopreservation using 1.5 mol/L PROH and 0.2 mol/L trehalose. The present results suggest that doe rabbit could be used as a biomedical model to investigate the long-term consequences of cryopreservation on ovarian follicles and the future birth of offspring. Moreover, these results are sufficient to hope for the application of this technique to preserve the animals' genetic resources by the female pathway. This technique could be used as a complementary tool to embryo and semen cryopreservation techniques. But live births after allograft will objectively confirm the good results obtained after cryopreservation of doe rabbit ovarian tissue which will be used to complete cryobanks. Moreover, allograft is feasible in this species, according to the study of Petroianu et al. (49). We have therefore commenced to study autograft in this species and hope to demonstrate the appropriateness of this technique in the future.

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