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).