Improving ovarian tissue cryopreservation for oncologic patients: slow freezing versus vitrification, effect of different procedures and devices

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Objective: To compare slow freezing (SF) with four vitrification techniques (VT) for cryopreservation of ovarian tissue (OT) and to evaluate the best protocol for human OT in a xenograft model.
Design: Experimental study.
Setting: University hospital.
Patient(s): Patients undergoing fertility preservation.
Animal(s): Ovariectomized nude mice.
Intervention(s): Cryopreservation of bovine OT after SF and four VTs (VT1, VT2, VT3, and VT4) by combining two cryoprotectant vitrification solutions (VS1 and VS2) and two devices (metallic grid and ethyl vinyl acetate bag), after which the cryopreservation of human OT by SF and VT1 and xenograft into nude mice.
Main Outcome Measure(s): Follicular densities, proliferation, vascularization, fibrosis, apoptosis, tissue viability.
Result(s): The in vitro study in bovine OT showed a lower percentage of quiescent follicles in the SF group but not in the vitrification groups (VT1–VT4). Apoptosis increased and cell proliferation decreased in all the experimental groups except VT1 (20% ethylene glycol, 20% dimethyl sulfoxide, 0.5 M sucrose, and 20% synthetic serum substitute in HEPES-buffered M199 culture media with Cryotissue metallic grids). Tissue viability was diminished in VT3, and the SF-xenografted human samples showed reduced primordial and secondary densities and unbalanced follicular populations when compared with fresh and VT1 tissue.
Conclusion(s): VT1 offers similar conditions to fresh tissue for follicular density, proliferation, viability, and cell death and preserves a larger population of quiescent follicles than SF after transplantation, thus ensuring the maintenance of graft potential fertility. (Fertil Steril® 2014;101:775–84. ©2014 by American Society for Reproductive Medicine.)
Key Words: Fertility preservation, ovarian cortex, slow freezing, vitrification

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Treatment with high-dose chemotherapy and/or radiotherapy in young female cancer patients can have deleterious effects on both the ovary and the follicular pool, leading to premature ovarian failure (1–6) and loss of fertility (2, 3). Several options have been developed to preserve the fertility of these patients, including the cryopreservation of oocytes (7–12), embryos (10, 11, 13, 14), or ovarian

tissue (OT) (15–17). Although cryopreservation of the ovarian cortex is still considered an experimental procedure, to date, 24 children have been born after orthotopic autotransplantation of cryopreserved tissue (18). In future, the increasing number of cancer patients who require fertility preservation procedures should lead us to expect an increase in the number of births using this technique.

Cryopreservation of the ovarian cortex can be performed in two ways: conventional slow freezing (SF) or vitrification. Slow freezing is the conventional technique currently used for cryopreserving human ovarian cortex (18–21), and it was the method applied in the 24 pregnancies previously mentioned. This system has been used since 2005 by our group at the Valencia Programme for Fertility Preservation; to date, we have cryopreserved the ovarian cortex of more than 650 patients, among whom 22 were transplanted with their own cryopreserved ovarian cortex and four healthy children have been born (18, 21, 22).

The main problems associated with SF are that several hours and expensive equipment are required to complete the process, so it is not possible to perform the technique in any laboratory. To resolve these problems, over the last decade, vitrification (or the fast-freezing method), one of the main advances in cryobiology, has been developed (23, 24). In principle, the vitrification technique (VT) is a simple, potentially faster protocol than SF (25), requires no special equipment, and may be performed even in an operating theater during oophorectomy. Such positive features and the encouraging results obtained when VT has been tested with embryos (26, 27) and oocytes (7) have led many researchers to conduct further studies into VT for OT from both humans and animals, which have shown the advantages that VT offers over SF (28–33).

Among the many studies of SF and VT effects, fundamental aspects of cryopreservation of ovarian cortex have yet to be systematically reviewed. Furthermore, the results obtained in previous studies in both human and animal models have shown high variability, with sometimes discordant conclusions owing to the heterogeneity of the methodology employed and the variables studied. To find the best method for cryopreservation of the ovarian cortex, we compared SF with several VT that have previously described by others as being successful, employing ethylene glycol (EG) and dimethyl sulfoxide (DMSO) at varying concentrations with different cryopreservation devices. We performed a preliminary study in bovine OT on the effects of SF and four different VT procedures, obtained by combining two vitrification solutions (VS) with two cryopreservation devices. After we had evaluated stroma and follicle morphology, densities and percentages of follicular populations, proliferation, apoptosis, and tissue viability in the preliminary study, we chose the protocol that had the best results for additional testing in human ovarian cortex, which was cryopreserved and xenografted in an in vivo study.

MATERIALS AND METHODS

Chemicals

All the chemicals, culture media, and reagents used in this study were purchased from Sigma-Aldrich unless otherwise stated.

Study Design

In vitro study. Given the limited amount of human OT available for research purposes, our preliminary in vitro study was performed in bovine OT. Ovarian cortex from each animal was divided into six pieces and randomly allocated to six experimental groups; see *Cryopreservation Protocols* for the vitrification solution (VS) contents:

1. Slow freezing (SF) group: slow-freezing cryoprotectant solution with an ethyl vinyl acetate (EVA) bag.
2. Vitrification technique 1 (VT1) group: VS1 with metallic grids (MG).
3. Vitrification technique 2 (VT2) group: VS2 with MG.
4. Vitrification technique 3 (VT3) group: VS1 with an EVA bag.
5. Vitrification technique 4 (VT4) group: VS2 with an EVA bag.
6. Fresh tissue (F) group: another strip of each ovary as fresh control tissue was immediately fixed in 4% neutral buffered formalin; a small portion of each ovarian strip was frozen at −80°C for the RNA studies.

In vivo study. After we had performed different cryopreservation procedures on the bovine tissues conditions, we analyzed them with the same criteria and experimental variables. We selected the best-scoring vitrification system from the in vitro study to be compared with SF techniques in human OT in an in vivo study using xenotransplantation of cryopreserved/thawed human samples in a nude mouse model. Supplemental Figure 1 (available online) shows a schema of the experimental design.

In Vitro Study

Tissue samples. Bovine ovaries (n = 16) were obtained from cows slaughtered at a local abattoir, transported to the laboratory in saline solution supplemented with 10% antibiotic-antimycotic solution at 4°C, and processed within 6 hours. The ovaries were rinsed in 70% alcohol and washed with fresh saline solution. We sliced 1-mm thick sections of the outer cortex from each ovary into 1 × 1 cm squares approximately; one strip of each ovary was allocated to each experimental group.

Cryopreservation protocols. Three different cryopreservation solutions and their respective protocols were tested.

1. Slow-freezing protocol. The SF cryoprotectant solution contained 5% of human serum albumin (HSA) and 10% of DMSO in M199 culture medium. The cryoprotectant was added by sequential dilution in two steps, as previously described elsewhere (21, 22), with minor modifications. The tissue fragments embedded in 5% HSA-supplemented M199 media were exposed first to 5% DMSO for 15 minutes and second to 10% DMSO for 15 minutes. Both incubations were performed at cold temperatures (2°–10°C). After the cryoprotectant loading protocol had been completed, the tissue fragments were submerged in cryoprotective solution and distributed in EVA bags (Cryocyte; Baxter Healthcare). The sealed bags were placed in the freezing chamber of a