Direct comparative analysis of conventional and directional freezing for the cryopreservation of whole ovaries

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Objective: To compare conventional slow equilibrium cooling and directional freezing for cryopreservation of whole ovaries.
Design: Experimental animal study.
Setting: Academic research environment.
Animal(s): Adult ewes.
Intervention(s): Eighty-one ovaries were randomly assigned to fresh control, conventional freezing (CF), and directional freezing (DF) group. Ovaries of CF and DF groups were perfused via the ovarian artery with Leibovitz L-15 medium, 10% fetal bovine serum, and 1.5 M dimethyl sulfoxide for 5 minutes. Each ovary was inserted into a glass test tube containing 10 mL of the same solution and cooled to −100°C or −70°C, respectively. Ovaries were stored in liquid nitrogen for a minimum of 2 weeks.
Main Outcome Measure(s): Structural integrity of cortical and medulla regions, vascular integrity, follicle in vitro development, cell proliferation, and DNA damage and repair.
Result(s): All examined parameters indicate that the structure of DF ovaries remains largely intact and comparable to fresh controls, whereas significant damages were observed in CF ovaries.
Conclusion(s): Directional freezing allows good preservation of whole ovaries, with most of the parameters taken into consideration almost identical to those recorded in fresh control samples. This encourages a reconsideration of the possible use of whole-ovary cryopreservation as a viable alternative to cortical fragments. (Fertil Steril® 2013;100:1122–31. ©2013 by American Society for Reproductive Medicine.)
Key Words: Whole ovary, cryopreservation, directional freezing, sheep

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Current cancer treatments improve the long-term survival rates of young women with malignancies. However, many of these therapies have lasting effects on fertility because they cause severe injuries to the ovarian reserve, which may lead to consequent premature ovarian failure, with a negative impact on life quality of young cancer survivors (1, 2).

Cryopreservation of ovarian tissue has been suggested as an alternative to oocyte and embryo freezing for preserving fertility of these patients, and two options are available: cryopreservation of ovarian cortical fragments and cryopreservation of the whole ovary. The first technique is currently the most widely used, owing to the higher efficiency of the cryopreservation methods and to the less-invasive nature of the retransplantation procedures (3–8). On the other hand, the relatively small size of grafted cortical fragments and their avascular nature may limit their functional longevity upon transplantation (9). However, recent follow-up studies of ovarian fragment transplantation suggest that this may not be the case because, in some cases,

Received February 22, 2013; revised June 1, 2013; accepted June 3, 2013; published online July 3, 2013.
S.M. has nothing to disclose. M.H. has nothing to disclose. G.P. has nothing to disclose. J.R.V.S. has nothing to disclose. T.A.L.B. has nothing to disclose. A.A. has nothing to disclose. F.G. has nothing to disclose.
Supported by AIRC IG 10376, Carraresi Foundation, and by Legge 7 R.A.S.; J.R.V.S. is the recipient of a grant from the CAPES Foundation, Ministry of Education of Brazil.
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Fertility and Sterility® Vol. 100, No. 4, October 2013 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2013.06.003

both fresh and cryopreserved tissue may remain functional for up to 7 years (10, 11).

Whole ovary cryopreservation with vascular reanastomosis, in theory, represents an appealing method to reduce ischemic time and prolong graft longevity (12). In fact, successful cryopreservation and transplantation of whole ovaries have been reported in different experimental animal species (mice, rat, rabbit, dog, and sheep) (13–17). However, the method suffers from a number of drawbacks. In particular, orthotopic transplantation has proved technically very challenging and cannot prevent ischemic damage (18, 19). In addition, the possibility exists to reintroduce malignant cells, even if this risk is also present when ovarian fragments are transplanted (20).

Nevertheless, it is important to improve the efficiency of cryopreserving whole organs, because in large mammals and humans this has been more problematic than in small animals, owing to heat transfer in such large organs (21) caused by the large thermal gradient existing between the surface and the interior of the system (22). At present there are two main methods for cryopreserving whole ovary: conventional freezing (CF) and directional freezing (DF). Conventional freezing operates on the principle of heat transfer by convection (23, 24). Directional freezing is an alternative approach to preserve the large organs, based on the physical concept of DF, which enables precise adjustment of the temperature gradients to achieve an accurate cooling rate through the entire tissue (17, 25, 26). This method has solved the problems associated with heat release by maintaining a uniform cooling rate through the entire organ. Although whole ovary cryopreservation with DF followed by successful retransplantation has been performed once in sheep (17), data regarding this new method are limited. In particular a direct comparison with CF has never been performed. The aim of this study was to compare the efficiency of CF with DF of whole ovaries. On the basis of our results (27) and those in the literature (17, 28), the sheep ovary was chosen as a model because of its similarity to the human ovary.

MATERIALS AND METHODS

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich.

Ovaries Collection

Sheep female reproductive tracts were collected at the local slaughterhouse and transported to the laboratory in cold (0–4°C) 0.9% saline solution. A total of 81 ovaries were randomly assigned to a fresh control group (CTR, n = 27), CF group (n = 27), or DF group (n = 27). Upon arrival to the laboratory, ovaries of the CF and DF groups were perfused via the ovarian artery with Ringer's solution and 10 UI/L heparin for 10 minutes, followed by perfusion with cryoprotectant solution containing Leibovitz L-15 medium (Life Technologies), 10% fetal bovine serum (vol/vol; Life Technologies), and 1.5 M dimethyl sulfoxide for 5 minutes (29). Each ovary was inserted into a 16-mm-diameter glass test tube (Manara) containing 10 mL of the same solution.

Conventional Freezing

Conventional freezing was performed in a Kryo 560M apparatus (Planer) with the following program: [1] from 4° to −40°C using a cooling rate of 0.5°C/min (seeding was induced at −7°C), [2] from −40° to −100°C at 5°C/min, and [3] immersion in liquid nitrogen (29).

Directional Freezing

Directional freezing was performed with a Multi-Thermal-Gradient (IMT). Briefly, the technology operates on the physical concept of DF and is based on a series of four heat-conductive blocks arranged in a line. Different temperatures are set along the blocks, thereby imposing a temperature gradient. Freezing tubes were pushed along the thermal gradient (+4° to −70°C), and the speed was set at 0.01 mm/s, resulting in a cooling rate of 0.3°C/min, after which samples were plunged into liquid nitrogen (17, 25).

Ovarian Thawing

Ovaries frozen with CF and DF were thawed by plunging the test tubes into a 68°C water bath for 20 seconds and then into a 37°C water bath for 2 minutes. The cryoprotectant solution was rinsed out of the ovaries by perfusion via the ovarian artery with 10 mL *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid–Tyrode's albumin lactate pyruvate medium (HEPES-TALP medium), supplemented with 0.5 mol/L sucrose and 10 IU/mL heparin at 38°C, followed by another 10 mL rinse with no sucrose (17, 25).

Morphologic Analysis

Nine ovaries from each group were allocated to morphologic and immunochemical analysis. To improve fixation, ovaries were cut in half, embedded in paraffin, and stained with hematoxylin and eosin. Samples were observed under an Eclipse E600 microscope (Nikon), and pictures were acquired with Nis Elements Software (version 4.0). Follicles were classified as primordial (1 layer of flattened granulosa cells around the oocyte), intermediate (1 layer of flattened to cuboidal granulosa cells around the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). Follicles were further classified as normal, when a spherical oocyte with a non-pyknotic nucleus was surrounded by granulosa cells organized in discrete layers; or degenerated, when a misshapen oocyte with or without vacuolation with a pyknotic nucleus was surrounded by disorganized granulosa cells detached from the basement membrane (30). Stromal cell density was quantified by counting nuclei with ImageJ software (National Institutes of Health) as described below.

Immunohistochemistry

Ovarian cortical fragments were fixed with 10% formaldehyde, embedded in paraffin, and sectioned before immunostaining. Slides were then boiled for 5 minutes in antigen-unmasking solutions (Vector Laboratories) and then blocked with a