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Ultrastructure of follicles after vitrification of mouse ovarian tissue

Transplantation of cryopreserved ovaries has been used to enhance the reproductive efficiency of women with cancer, in whom chemotherapy or radiotherapy may have caused hypogonadism (1, 2). Many attempts have been made to improve cryopreservation conditions by using simple and efficient vitrification procedures (3). The earliest method, introduced in 1963 (4) has the advantage of preventing intracellular ice formation because the cryoprotectant solution is very concentrated.

Recently, Sugimoto et al. (5) and Kagabu and Umezu (6) showed that after vitrification of ovarian tissue, the morphology of follicles remains normal at the light microscopic level. We also showed that the integrity of follicles was well preserved after vitrification of mouse ovaries by using ethylene glycol as the cryoprotectant (7).

Transmission electron microscopy is a good technique for evaluating the subcellular organelles and structural changes during freezing and thawing. We investigated the ultrastructural changes of follicles in mouse ovarian tissue that was vitrified by using ethylene glycol as the cryoprotective agent.

Fifteen ovaries from 8- to 10-week-old NMRI mice were collected after cervical dislocation. Intact ovaries were vitrified by using a modified Pedro protocol (8). The vitrification solution was Roswell Park Memorial Institute (RPMI 1640) medium containing 30% (w/v) Ficoll 70 (average molecular weight, 70,000), 0.5 M of sucrose, 10.7% (w/v) acetamide, and 40% (v/v) ethylene glycol, supplemented with 4 mg/mL of bovine serum albumin. The ovarian tissues were transferred to the cryoprotectant solution at room temperature and held for 5 minutes. Each ovary was individually loaded into a cryovial containing vitrification solution and placed directly into liquid nitrogen. The vitrified samples were warmed rapidly at room temperature, then placed in a 25°C water bath for 20 seconds. The content of each cryovial was expelled into 1 mL of 1 M sucrose in RPMI for 5 minutes and washed in RPMI medium for at least 15 minutes.

Ovaries were fixed in 2.5% glutaraldehyde in 0.1 M of phosphate buffer (pH, 7.4) for 1 hour at 4°C. They were then washed in the same buffer for 10 minutes and post-fixed in 1% osmium tetroxide for 1 hour. After dehydration in an increasing ethanol concentration followed by acetone, the tissues were embedded in Epon 812 resin (TABB Laboratories Ltd., Berkshire, United Kingdom). Semi-thin sections were stained with 1% toluidine blue. Ultra-thin sections were stained with alcoholic uranyl acetate (for 7 minutes) and aqueous lead citrate (13 minutes) and were then examined by using Zeiss transmission electron microscopy.

After vitrification, the fine structure of oocytes and follicular cells was the same as that in nonfrozen control oocytes. The integrity of cell organelles was well preserved; however some mitochondria were swollen, and their cristae had partially disappeared. One or two cuboidal cells surrounded the oocyte in the primary follicles (Fig. 1). These cells were separated by a narrow perivitelline space. The dens amorphous materials filled the perivitelline space to form zona pellucida. Oocyte microvilli and follicular cell processes projected into the perivitelline space. The oocytes had clear cytoplasm with a centrally located germinal vesicle. The nucleus was spherical and, in vitrified oocytes, had vesiculated euchromatin.

In vitrified tissue, some mitochondria were swollen and their cristae had disappeared. Free ribosomes were abundant and scattered between organelles. The follicular cells had a round or ovoidal nucleus with a moderately dense appearance on electron microscopy, peripheral heterochromatin, and one nucleolus. The follicular cells had characteristics of protein synthesis and contained fat droplets with moderate density on electron microscopy.

The follicular cells of large follicles consisted of more than one or two layers, and the antral spaces were present between granulosa cells. The perivitelline space was wider than that of small follicles. Many cross-sections and longitudinal sections of cytokeratin intermediate filament were observed in the oocytes of large follicles. Cortical granules were filled with dense materials and seen throughout the oocyte cytoplasm.

Our ultrastructural studies showed that after thawing of ovaries, no noticeable changes occurred in the organelles of oocytes and follicular cells. The integrity of subcellular structure was well preserved. Our results confirm those of Oktay et al. (9), who showed that ovarian tissue has no signs of ultrastructural sign of damage or necrosis after isolation and cryopreservation.

In contrast, Nisolle et al. (10) showed that some frozen-thawed follicles seemed to become degenerated and

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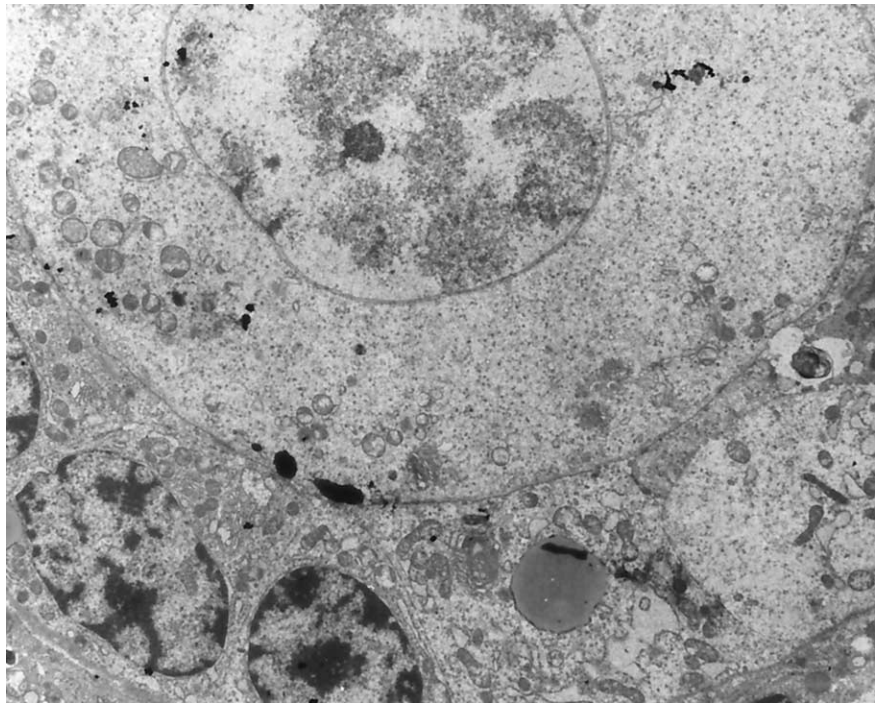
FIGURE 1

Electron micrograph of vitrified-thawed mouse ovarian tissue. One primary follicle is shown. In the cytoplasm of follicular cells, some mitochondria, RER, and large lipid droplets are seen. The oocyte nucleus had vesiculated euchromatin. Magnification, ×3000.



Salehnia. Ultrastructure of vitrified ovary. Fertil Steril 2002.

extensively vacuolated, but other follicles had well-preserved structure. Gook et al. (11) found that oocyte abnormalities significantly increase when slow cooling of human ovaries is performed.

We found that follicular cells and prophase I oocytes of small follicles had characteristics of protein-synthesizing cells: They had a large proportion of polysomes and rough endoplasmic reticulum (RER) compared with mature follicles. The characteristic of follicles in vitrified and nonvitrified ovaries was similar to that found by Sathananthan et al. (12). Cytokeratin intermediate filaments were also observed in the mature oocyte but not in the small follicles; thus, these filaments may be an indicator of maturation in oocytes.

Overall, we found that vitrification using ethylene glycol as cryoprotectant produced little change in the fine structure of follicles during cooling and warming. This method may be an efficient alternative for cryopreservation of mammalian ovarian tissue and can be used to improve fertility among patients with cancer.

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