Successful slush nitrogen vitrification of human ovarian tissue

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Objective: To study whether slush nitrogen vitrification improves the preservation of human ovarian tissue.
Design: Control vs. treatment study.
Setting: University research laboratory.
Patient(s): Ovarian biopsies collected from nine women (aged 14–35 years) during laparoscopic surgery for benign gynecologic conditions.
Intervention(s): None.
Main Outcome Measure(s): Ovarian cortical strips of 2 × 5 × 1 mm were vitrified with liquid or slush nitrogen. Fresh and vitrified cortical strips were analyzed for cryodamage and viability under light, confocal, and transmission electron microscopy.
Result(s): Compared with liquid nitrogen, vitrification with slush nitrogen preserves [1] follicle quality (grade 1 follicles: fresh control, 50%; liquid nitrogen, 27%; slush nitrogen, 48%); [2] granulosa cell ultrastructure (intact cells: fresh control, 92%; liquid nitrogen, 45%; slush nitrogen, 73%), stromal cell ultrastructure (intact cells: fresh control, 59.8%; liquid nitrogen, 24%; slush nitrogen, 48.7%), and DNA integrity (TUNEL-positive cells: fresh control, 0.5%; liquid nitrogen, 2.3%; slush nitrogen, 0.4%); and [3] oocyte, granulosa, and stromal cell viability (oocyte: fresh control, 90%; liquid nitrogen, 63%; slush nitrogen, 87%; granulosa cells: fresh control, 93%; liquid nitrogen, 53%; slush nitrogen, 81%; stromal cells: fresh control, 63%; liquid nitrogen, 30%; slush nitrogen, 52%).
Conclusion(s): The histology, ultrastructure, and viability of follicles and stromal cells are better preserved after vitrification with slush nitrogen compared with liquid nitrogen. (Fertil Steril® 2016;105:1523–31. ©2016 by American Society for Reproductive Medicine.)
Key Words: DNA fragmentation, follicle, histology, ovarian cryopreservation, ultrastructure

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Chemo- and radiotherapy administered to female cancer patients can lead to premature ovarian failure and subsequent loss of fertility. Ovarian tissue (OT) cryopreservation has emerged as a promising option to safeguard the fertility of prepubertal girls or patients who need immediate chemotherapy. The possibility of restoring both endocrine and reproductive function of the gonads represents the main advantage of autotransplantation of frozen/thawed OT. Although autotrasplantation of ovarian cortical fragments cryopreserved through slow cooling has resulted in approximately 35–40 live births worldwide to date (1–3), this procedure is still considered experimental, and further research is warranted to avoid cryodamage (4, 5). Slow cooling has been suggested to negatively affect the ovarian stroma (6–12), granulosa cells (GCs) (13), and the development of the thecal layer during in vitro culture (11). Although vitrification has gained a wider acceptance than slow freezing in the cryopreservation of oocytes and embryos in human assisted reproduction, only recently has it been applied for clinical OT cryopreservation, relying on a limited number of biological comparative studies (9, 10,14–16), and two live births have been reported to date (17, 18). Compared with conventional slow cooling, vitrification avoids the development of ice crystals, promoting the formation of an amorphous glassy solid state, through the use of higher cooling rates and cryoprotectant (CPA) concentrations (19). However, the efficacy of vitrification is still controversial

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(9, 16, 20), and this is probably due to several variables, such as tissue size, choice and concentrations of CPAs, and nature of devices used to vitrify the tissue in open or closed systems that in turn affect the cooling rates (21). A further fundamental factor that can result in poor heat transfer and limits the cooling rate is represented by the Leidenfrost effect (22). Indeed, as soon as an object is plunged into liquid nitrogen (LN), it enters into the so-called film boiling regime caused by the large temperature difference between the object and LN (23). Heat flow from the object to LN, causes the latter to boil in the immediate proximity of the object, generating a pocket of nitrogen vapours that acts as an "insulator" and further delays heat transfer. Recently, slush nitrogen (SN) has been proposed as a new strategy that increases the cooling rate, avoids the Leidenfrost effect, and allows the use of lower concentrations or time of exposure to toxic CPAs. Fresh and SN-vitrified mouse oocytes were reported to have the same ability to develop to the blastocyst stage and produce healthy offspring, and SN has been shown to improve the clinical outcome of human oocyte vitrification (23–27). However, the efficacy of SN vitrification on OT cryopreservation has not been evaluated to date.

The main aims of the present study were to compare the efficiency of SN vs. LN vitrification of human OT in an open system, studying the quality and viability of follicles and stromal cells (SCs) through histology, ultrastructure, TUNEL, and live–dead assay.

MATERIALS AND METHODS

Collection and Processing of OT

The use of human tissue in this study was approved by the Ethics Committee of the Azienda Ospedaliera San Giuseppe Moscati (Avellino, Italy; reference number CE 6-2009, approval granted June 5, 2009). After obtaining written informed consent, ovarian biopsies were collected from nine women (aged 14–35 years) during laparoscopic surgery for benign gynecologic conditions. The tissue was transported to the laboratory within 2 hours in minimum essential medium (Sigma Aldrich), supplemented with 5% human serum albumin (Octapharma) at 4°C. The cortical OT was manually dissected from medullar tissue and divided using a scalpel into cortical strips of approximately 2 × 5 mm and 1 mm thick. For each patient, fresh tissue was directly fixed or vitrified and cultured after warming as detailed below. Fresh tissue and vitrified/warmed cortical strips were fixed for light and transmission electron microscopy (TEM). Fresh, LN-vitrified, and SN-vitrified cortical strips from two younger patients were also treated for the assessment of viability as detailed below.

Vitrification and Warming

Vitrification and warming were performed with minimum essential medium (Sigma Aldrich) supplemented with 20 mg/mL human serum albumin as basal medium (BM). Exposure to VS and WS was performed at room temperature (RT) and at 37°C, respectively (14). The vitrification solution (VS) consisted of BM supplemented with 10% dimethyl sulfoxide (Sigma Aldrich), 26% ethylene glycol (Sigma Aldrich), 2.5% polyvinylpyrrolidone (Sigma Aldrich), and 1 M sucrose (Sigma Aldrich). Cortical strips were equilibrated in 25% VS (5 minutes), 50% VS (5 minutes), and transferred into 100% VS (1 minute). The strips were then blotted on aseptic gauze to remove the remaining VS, dropped into LN or SN, and then stored in Nunc Cryotubes (Sigma Aldrich). The SN was obtained, exposing a polystyrene container holding 750 mL of LN to a negative pressure of 65–70 mBar for 15 minutes in a vacuum chamber (Vacutherm, Thermo Scientific Heraeus). Strips were dropped into SN within 5 minutes after return to normal atmospheric pressure.

For warming, strips were transferred in warming solutions (WS) consisting of BM supplemented with different concentrations of sucrose. Briefly, the strips were immersed in WS1 (1 M) for 15 seconds, then in WS2 (0.5 M), WS3 (0.25 M), and BM for 5 minutes each. Warmed strips were cultured 24 hours in BM supplemented with insulin, transferrin, and selenium 1× (41400-045, Gibco) at 37°C, 5% CO^2 in air and then fixed for histology, TEM, or assessment of viability as detailed below.

Histology

Samples were fixed in Bouin's solution, dehydrated through sequential passages in increasing concentrations of ethanol, embedded in paraffin wax, serially sectioned at 5 μm, stained with haematoxylin/eosin, and observed. The quality of primordial/primary follicles was graded as previously reported (12) by an expert blinded operator. Briefly, grade 1 follicles were spherical and had homogeneously distributed GCs and an oocyte with homogenous cytoplasm and slightly granular nucleus, in the center of which condensed chromatin in the form of a dense spherical structure is detected; grade 2 follicles had GCs pulled away from the edge of the follicle but still a spherical oocyte; and grade 3 follicles had GCs with pyknotic nuclei and misshapen oocyte with or without vacuolation. The number and stage of follicles was determined in all sections by one blind operator following each follicle through serial sections to avoid double counting. Follicle density was expressed as the number of follicles per square millimeter of the ovarian cortex. Follicle stages were scored according to Gougeon's criteria (28): primordial with a single layer of flat GCs, primary with a complete single layer of cuboidal GCs, secondary with two or more complete layers of cuboidal GCs.

Transmission Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde (SIC) in 0.1 M sodium cacodylate (Sigma Aldrich) at pH 7.3 for 1.5 hours at RT, washed 3 × 10 minutes in the same buffer, postfixed in 1% osmium tetroxide (SIC) in 0.1 M sodium cacodylate at pH 7.3 on ice, washed 3 × 10 minutes in the same buffer, treated with 0.1% tannic acid in buffer for 10 minutes, and dehydrated in increasing concentrations of ethanol (Sigma Aldrich) on ice. All samples were treated 2 × 5 minutes with propylene oxide (Fluka), infiltrated with propylene oxide/agar 100 (1:1; Agar Scientific) overnight, and individually embedded in fresh resin. Thick (0.5–1 μm) and thin

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