Slush nitrogen vitrification of human ovarian tissue does not alter gene expression and improves follicle health and progression in long-term in vitro culture

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Objective: To study whether slush nitrogen (SN) vs. liquid nitrogen (LN) vitrification affects human ovarian tissue gene expression and preserves follicle health during extended in vitro culture.
Design: Randomized experimental study.
Setting: University research laboratory.
Patient(s): Ovarian biopsies collected by laparoscopic surgery from patients with benign gynaecologic conditions.
Intervention(s): None.
Main Outcome Measure(s): Ovarian strips were vitrified with LN or SN, warmed, and analyzed before or after culture for 9 days (d9) in gas-permeable dishes. Expression of genes involved in stress and toxicity pathways was analyzed in fresh and warmed strips by polymerase chain reaction (PCR) array and quantitative real-time–PCR. Fresh and vitrified/warmed strips were analyzed for follicle quality, progression, and viability before or after culture.
Result(s): The SN vitrification preserved follicle quality better than LN (% grade 1 follicles: fresh control, 54.2; LN, 29.3; SN, 48.8). Quantitative reverse transcription–PCR demonstrated a noticeable up-regulation of 13 genes in LN samples (range, 10–35) and a markedly lower up-regulation of only 5 genes (range, 3.6–7.8) in SN samples. Long-term in vitro culture evidenced worse follicle quality and viability in LN samples than in both fresh and SN samples (% grade 1 follicle: fresh d0, 51.5; fresh d9, 41; LN d9, 16.4; SN d9, 55) and a highly significant reduction of primordial follicles and a concomitant increase of primary and secondary follicles in all samples. Follicle growth to the secondary stage was significantly higher in vitrified tissue than in fresh tissue, being better in SN than in LN vitrified tissue.
Conclusion(s): Follicle quality, gene expression, viability, and progression are better preserved after SN vitrification. (Fertil Steril® 2018;110:1356–66. ©2018 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Follicle, gene expression, histology, ovarian cryopreservation, viability

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Ovarian tissue cryopreservation is an effective option to preserve the fertility of cancer patients undergoing chemo- and radiotherapy. Endocrine function has been restored in approximately 64% of cryopreserved ovarian tissue auto-transplantation cases (1), and more than 86 babies have been born to date (2–4). Although the first birth was reported in 2004 (5), and despite the fact that several pioneers consider ovarian cryopreservation an established procedure (6), the American Society for Reproductive Medicine still considers it experimental (7).

Vitrification has gained wide acceptance as the preferred cryopreservation method for gametes and embryos. Nonetheless, all live births after autotransplantation in cancer patients have derived from ovarian tissue cryopreservation by slow freezing/rapid thawing (1, 8–12). To the best of our knowledge, the only two live births following ovarian tissue vitrification have been reported by Suzuki et al. (2015) (13) in patients with primary ovarian insufficiency. Although data are still controversial, some studies suggest that slow freezing may cause extensive DNA fragmentation in primordial follicles and injury to stromal cells (9,14–17). This has started the quest for new and more efficient cryopreservation procedures. Their development and validation are hampered by the small number of clinical cases and the long interval between tissue cryopreservation and autotransplantation. To overcome such limitations, basic research studies should be undertaken that consider physiologic endpoints of tissue health and the potential to restore the endocrine and reproductive function of cryopreserved tissue.

Recently we demonstrated that the ultrarapid vitrification of human ovarian tissue with slush nitrogen (SN) improves recovery of healthy follicles, preservation of granulosa cells (GCs) and stromal cells (SCs) ultrastructure, DNA integrity of SCs, and viability of oocytes, GCs, and SCs over conventional vitrification with liquid nitrogen (LN) (18). In this study we investigated whether SN vitrification affects human ovarian tissue competence to support folliculogenesis after warming. Possible cryoinjuries were investigated by performing on fresh, SN-, and LN-vitrified tissue molecular analysis of genes known to be involved in stress and toxicity pathways, by polymerase chain reaction (PCR) array and quantitative real-time (qRT)-PCR. In fact, some studies evidence cryoprotectant toxicity and that cryopreservation may cause oxidative and osmotic stress and ultimately DNA damage (19–22). The efficacy of SN vs. LN vitrification in preserving the reproductive function was assessed in long-term in vitro culture experiments of vitrified/warmed (V/W) tissue under conditions previously shown to foster follicle activation and progression in bovine and human ovarian strips (23). Follicle health, viability, activation, and progression were characterized with histology and live–dead assay in fresh and V/W tissue before and after in vitro culture.

MATERIALS AND METHODS

In this study we used materials and methods very similar to those of prior studies published by our group (18, 23) and occasionally reproduced them verbatim in the following.

Chemicals and Consumables

Gas-permeable 50-mm Lumox culture dishes were from Sarstedt. Leibovitz's L-15 medium, α-minimum essential medium (MEM) Glutamax medium (code number 32571), insulin transferrin selenium 100×, and Live/Dead fixable far red stain were from Invitrogen. Penicillin streptomycin 100×, amphotericin B 250 μg/mL, bovine serum albumin (BSA), human serum albumin, L-ascorbic acid, L-glutamine 200 mM, dimethylsulfoxide, ethylene glycol, polyvinylpyrrolidone, sucrose, Hoechst 33342, fructose, α-thioglycerol, and eosin-Y were from Sigma Aldrich. Mayers's hematoxylin and paraffin wax was from Carlo Erba. The NucleoSpin RNA kit was from Macherey Nagel. RT2 First Strand kit, RT2 ProfilerTM PCR Array Stress and Toxicity pathways, RT2 SYBR Green qPCR Mastermix, and SYBR green PCR kit were from Qiagen. SuperScript III reverse transcriptase was from Invitrogen.

Experimental Design

Experiment I was aimed to evaluate the effects of vitrification and warming on ovarian tissue health and gene expression. Tissue from four patients (aged 26–35 years) was vitrified and then warmed as described below. Fresh and V/W tissue was analyzed for follicle quality and staging by histology, for viability by live/dead assay, and for gene expression analysis by PCR array and qRT-PCR.

Experiment II aimed to study the capacity of V/W tissue to preserve reproductive function. To this end, cortical strips of fresh and V/W tissue from four patients (aged 20–26 years) were cultured for 9 days (d9). At the end of culture, follicle quality, staging, and viability were evaluated as described below.

In each experiment the same amount of tissue from any given patient was allocated to controls and treatment groups.

Collection, Preparation, and Culture of Ovarian Tissue

The use of human ovarian tissue was approved by the Ethics Committee of Regione Campania (ASL NA1 Centro, Naples, Italy; reference number 57 CE 2-2017). After obtaining written informed consent, ovarian tissue was harvested with laparoscopic surgery from eight women (age 20–39 years) with benign gynecologic conditions, was transported within 2 hours to the laboratory in Leibovitz's L-15, penicillin–streptomycin 1% (Pen-Strep), and amphotericin-B 1 μg/mL, at 4°C, and was transferred to handling medium (Leibovitz's L-15 with 2 mM glutamine, 3 mg/mL BSA, 1% Pen-Strep, and 1 μg/mL amphotericin B). Fragments of cortical tissue of approximately 5 × 2 × 0.5 mm were manually dissected at 4°C. Fresh and V/W fragments from the same biopsy were further cut in strips of 1 × 1 × 0.5 mm with a tissue chopper (Mcilwain, Mickle Laboratory Engineering). Then the strips were put in a 10-cm Petri dish, gently mixed, and washed twice in fresh handling medium. In experiment II, 10 strips of either fresh or V/W tissue were randomly positioned in a gas-permeable dish (23) and cultured for 9 days in α-MEM supplemented with 3 mM glutamine, 0.1% BSA,

1% Pen-Strep, 1% insulin transferrin selenium (10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 ng/mL selenium), 1 μg/mL amphotericin-B, and 50 μg/mL ascorbic acid at 37°C, in a 5% CO_2 and 95% humidity air gaseous atmosphere. Half medium volume was exchanged every 48 hours.

Fresh strips from each ovary were used as controls and processed for histology and viability assessment as described below.

Vitrification and Warming

Fragments of cortical tissue of approximately 5 × 2 × 0.5 mm were vitrified and warmed according to the method suggested by Amorim et al. (24), as described elsewhere (18). Tissue was immersed in vitrification and warming solutions at room temperature and 37°C, respectively. The vitrification solution (VS) consisted of basal medium (BM: minimum essential medium with 20 mg/mL human serum albumin) supplemented with 10% dimethylsulfoxide, 26% ethylene glycol, 2.5% polyvinylpyrrolidone, and 1 M sucrose. The fragments were sequentially equilibrated in 25% VS (5 minutes) and 50% VS (5 minutes), transferred to 100% VS (1 minute), plunged in LN or SN, and stored in Nunc Cryotubes. Slush nitrogen was prepared by maintaining 750 mL of LN in a polystyrene container in a vacuum chamber (Vacutherm, Thermo Scientific Heraeus) at a negative pressure of 65–70 mBar for 15 minutes and used as previously reported (18). The fragments were dropped in SN within 5 minutes after returning the SN to atmospheric pressure.

The warming solutions (WS) consisted of BM with sucrose concentrations ranging from 0.25 to 1 M. Tissue was sequentially immersed in WS1 (1 M sucrose) for 15 seconds, in WS2 (0.5 M sucrose), WS3 (0.25 M sucrose), and BM for 5 minutes each, transferred to fresh BM, and incubated for 30 minutes at 37°C, in 5% CO_2, 95% humidity air.

Histology

To evaluate follicle quality, activation, and progression, the cortical strips were processed for histology as reported elsewhere (18). Follicles were graded and staged on serial sections from each strip by two blinded expert observers. Follicles were scored when the germinal vesicle was present in the section to avoid recounting. Follicle quality was scored as previously reported (14), and follicle stages were classified according to Gougeon (25): primordial follicles featuring a layer of flat GCs, primary follicles featuring one complete layer of cuboidal GCs, secondary follicles featuring two or more layers of cuboidal GCs.

Viability Assessment

The cortical strips were treated with 1 μg/mL Live/Dead fixable far red stain and 10 μg/mL Hoechst 33342 in Dulbecco's PBS for 3 hours at 4°C under gentle shaking. Then they were fixed in 4% paraformaldehyde in PBS for 2 hours at room temperature, rinsed in fresh PBS, and treated with 10 μg/mL Hoechst 33342 in PBS at 4°C overnight (18). The live/dead probe is resistant to fixation, reacts with free amines both in the cell interior and on the cell surface, and is excluded by cells with intact membranes. Strips were eventually optically cleared according to the SeeDB clearing protocol (26) and mounted and imaged with a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems), as previously reported (18, 23).

Tissue Preparation and Messenger RNA Extraction

The total messenger RNA (mRNA) was extracted from fresh, LN, and SN tissue of each patient. To this aim, one cortical fragment of 5 × 2 × 0.5 mm was homogenized in lysis buffer, and total mRNA was extracted using the NucleoSpin RNA kit, according to the manufacturer's instructions. The RNA samples were stored at −20°C until use. The concentration and purity of the RNA samples was determined with the NanoDrop ND1000 (Thermo Scientific). The RNA integrity was checked by electrophoresis on 2.0% agarose gel.

Custom RT-PCR Arrays

Single-strand complementary DNA (cDNA) was synthesized from 1 μg of high-quality mRNA extracted from fresh and V/W cortical fragments of each patient with the RT2 First Strand kit. For the analysis the RT2 Profiler PCR Array Stress and Toxicity pathways were used, containing primers and controls for reverse transcription and PCR reactions for 84 tested and 5 housekeeping genes involved in oxidative stress, hypoxia, osmotic stress, cell death, inflammatory response, DNA damage signaling, heat shock, and unfolded protein response. The entire volume of each cDNA sample was used for the preparation of the reaction mixture. Each well in a 96-well plate was filled with 25 μL of reaction mixture based on the RT2 SYBR Green qPCR Mastermix. The thermal cycling protocol suggested by the plate manufacturer for IQ5 was used throughout. All plates had positive PCR and reverse transcription controls. Contamination with mouse genomic DNA was assessed as suggested by the manufacturer and evidenced presence of genomic DNA in an acceptable range. Values of the cycle threshold (C_t) obtained in quantification were used for estimating the fold changes in mRNA abundance according to the $2^{-\Delta\Delta}$Ct method. Beta2-microglobulin was selected as the best housekeeping gene. Changes in the mRNA level of the investigated genes were estimated in all groups in relation to the tissue control group, with mRNA abundance set arbitrarily at 1.

Quantitative RT-PCR

The RNAs extracted from fresh and V/W cortical fragments of the four patients were pooled in three groups (fresh, LN, SN). Two micrograms of such RNA was used to synthesize first-strand cDNA by SuperScript III reverse transcriptase.

Primers for qRT-PCR validation were designed with Primer 3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) on sequences found in Genbank (Supplemental Table 1). The RT-PCR reactions were performed with the SYBR green PCR kit and the gene-specific primers in 96-well optical reaction plates in a reaction volume

of 20 μL. The PCR cycling conditions were as follows: 10 minutes at 95°C to activate the HotStart DNA polymerase, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Reactions were performed in an iCycler iQ system (Bio-Rad, Milan, Italy). For transcript quantification, samples were normalized to the expression level of endogenous reference genes (Beta2-microglobulin and hypoxanthine phosphoribosyltransferase 1) to take into account possible differences in cDNA quantity and quality. The average C_t values from all the replicates were used to calculate the fold changes relative to the internal control. The comparative $^{\Delta\Delta}C_t$ method was used to quantify the level of gene expression. The $2^{-\Delta\Delta}C_t$ method was used to calculate the relative fold change in gene expression.

Statistical Analysis

Data are generally presented in terms of cumulative percentage. Data were analyzed by pairwise comparisons using Fisher's exact test when overall differences were statistically significant. Statistical significance of relative fold changes in gene expression was assessed with the Holm-Šidák *t* test for multiple comparisons in GraphPad Prism 6.

RESULTS

Experiment I

Follicle histology and viability in fresh and V/W tissues. The effect of vitrification on follicle quality and progression was assessed by histology in 656 fresh and in 910 vitrified/warmed strips (LN = 410; SN = 500) (Fig. 1). As compared with fresh tissues, a significant reduction of grade 1 ($P<.01$) and a concomitant increase of grade 2 follicles ($P<.01$) was observed in LN but not in SN tissue (Fig. 1A). In all V/W samples, the number of primordial follicles significantly decreased, and that of primary follicles significantly increased with respect to fresh tissue (Fig. 1B).

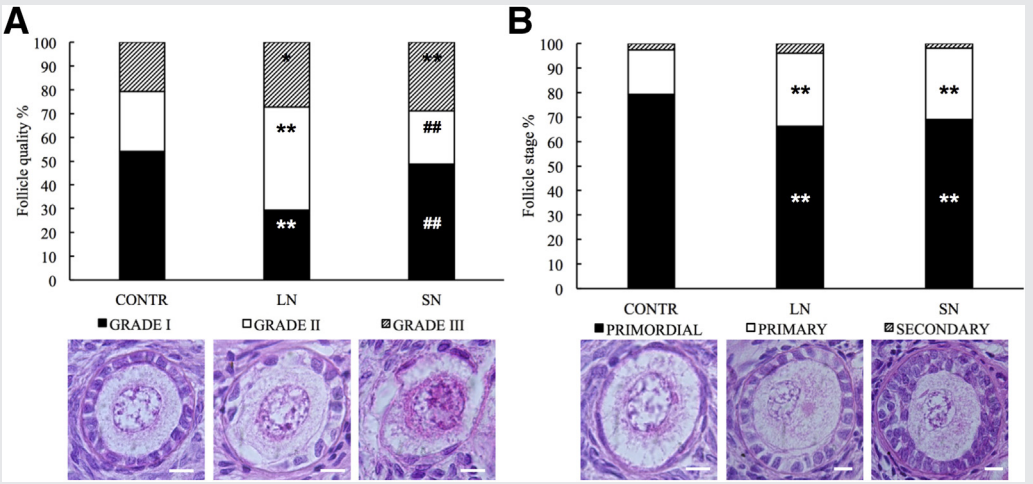
Follicle viability was assessed in 2,188 follicles: 628 in fresh tissue, 594 in LN, and 966 in SN V/W tissue. The analysis demonstrated that the vitrification and warming treatment did not affect follicle viability (Fig. 2G, Supplemental Table 2).

Gene expression in fresh and V/W tissues. We evaluated the expression of 84 genes known to be involved in stress and toxicity pathways, such as oxidative and osmotic stress, inflammatory response, cell death, and DNA damage signaling. Gene expression in V/W tissue was considered significantly altered at a threshold threefold value with respect to fresh tissue. In LN tissue, 13 of the 84 investigated genes (i.e., *DDB2*, *CA9*, *IL6*, *IL1A*, *IFNG*, *CD40LG*, *MMP9*, *RAD51*, *TNF*, *EPO*, *AQP2*, *CFTR*, *CRP*) were up-regulated beyond the threshold value. In SN tissue, all genes were expressed to within the threshold value, the only exception being the *IL1B* gene, whose number of transcripts decreased (Fig. 3A, Supplemental Table 3).

qRT-PCR validation. The qRT-PCR analysis of fresh and V/W tissues confirmed the differential gene expression detected by microarray analysis. The analysis validated the up-regulation of the 13 genes that were found altered after LN vitrification (i.e., *DDB2*, *CA9*, etc.). Interestingly, in LN tissue the genes of the interleukin inflammatory pathway (i.e., *IL-1A*, *IL-1B*, and *IL6*) were expressed to a smaller extent than the other investigated genes.

The analysis of SN tissue evidenced the up-regulation of only 5 of the 13 genes that were altered in LN tissue (i.e., *AQP2*, *CFTR*, *CD40LG*, *CA9*, and *EPO*). Their fold values ranged from 3.6 to 7.8 and were markedly lower than in LN tissue (Fig. 3B). In the latter, genes up-regulation ranged from 10.0- to 35-fold.

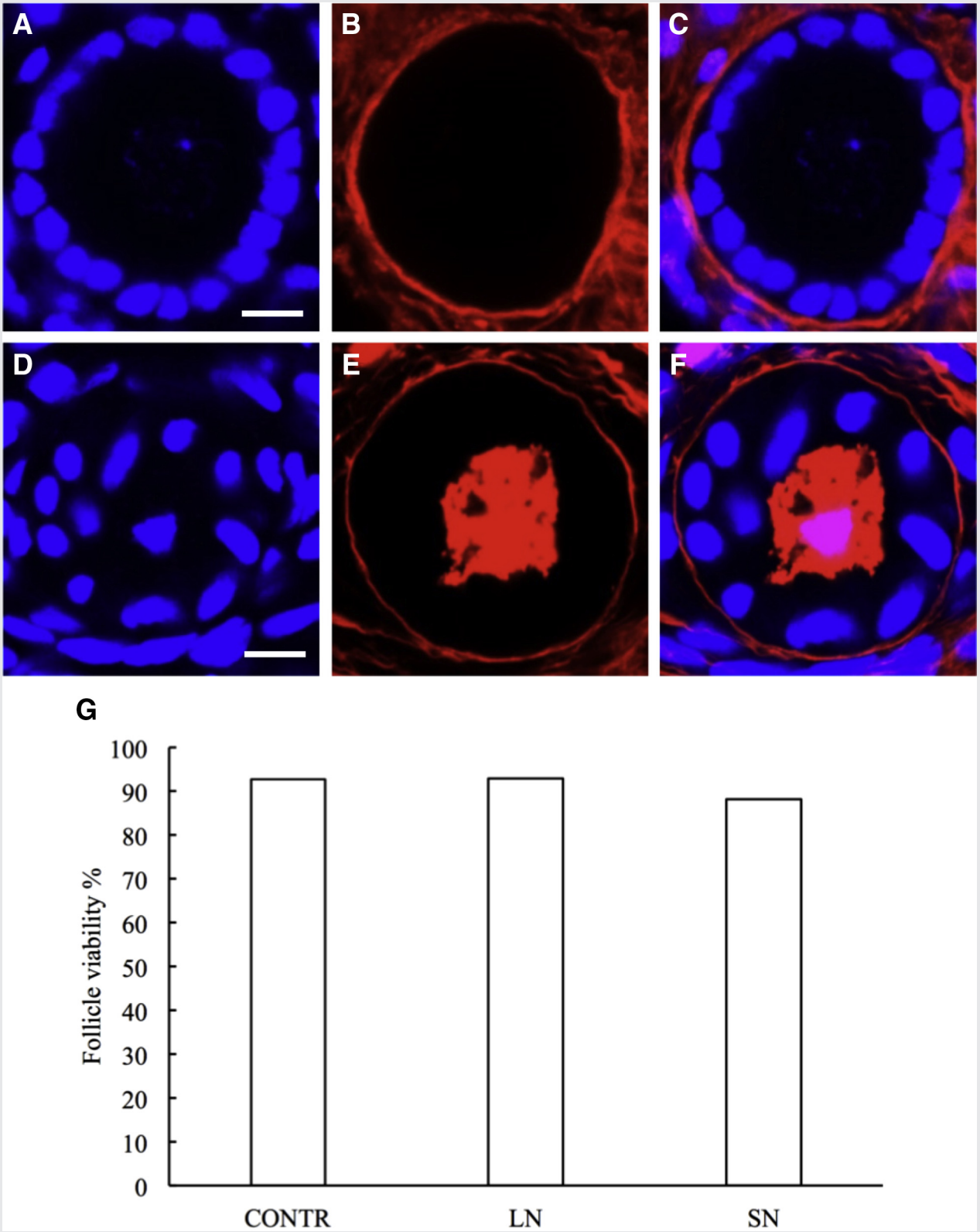
FIGURE 1



Grading (**A**) and staging (**B**) of follicles in fresh (CONTR) and V/W ovarian tissue vitrified by SN or conventional LN. (**A**) Upper panel: percentage of follicles of varying grade. Lower panel: histology of follicles of varying grade. (**B**) Upper panel: percentage of follicles of varying stage. Lower panel: histology of follicles of varying stage. *,#Statistically significant differences vs. fresh tissue or between V/W tissues, respectively. *,#$P<.05$; **,##$P<.01$. Bar = 10 μm.

Barbato. Vitrified ovarian tissue gene expression. Fertil Steril 2018.

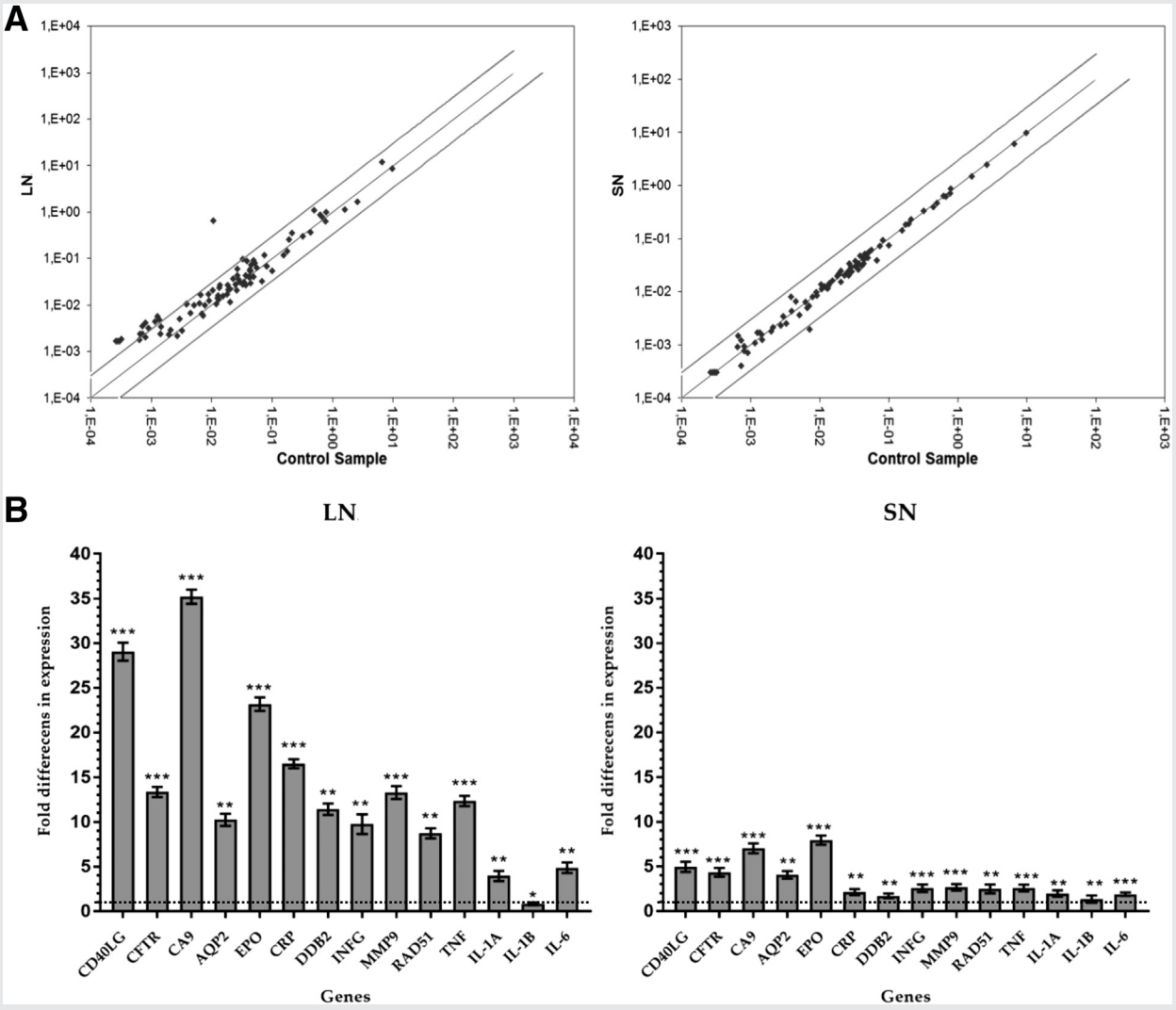
FIGURE 2



Viability of follicles in fresh and V/W ovarian tissue as obtained by confocal microscopy. Top: representative confocal micrographs of a live (**A–C**) and a dead (**D–F**) follicle (red: live–dead far-red probe; blue: Hoechst 33342–stained nuclei). (**A, D**) Hoechst 33342; (**B, E**) live–dead far-red; (**C, F**) merge. GV = germinal vesicle; BL = basal lamina. Bar = 25 μm. Bottom (**G**): percentage of viable follicles in fresh (CONTR) and V/W ovarian tissue vitrified by SN or conventional LN.

Barbato. Vitrified ovarian tissue gene expression. Fertil Steril 2018.

FIGURE 3



(**A**) Scatter plots of the PCR array showing the fold expression of the investigated genes involved in stress and toxicity pathways in LN and SN tissue (*y*-axis) vs. fresh tissue (control sample: *x*-axis). The lines indicate the zone comprised within a threefold change in gene expression (threshold). (**B**) Quantitative RT-PCR validation of PCR array for the 14 genes whose expression profiles were found altered in LN and SN as compared with fresh tissue. Data are reported as means ± SD. *$P<.05$; **$P<.01$; ***$P<.001$.

Barbato. Vitrified ovarian tissue gene expression. Fertil Steril 2018.

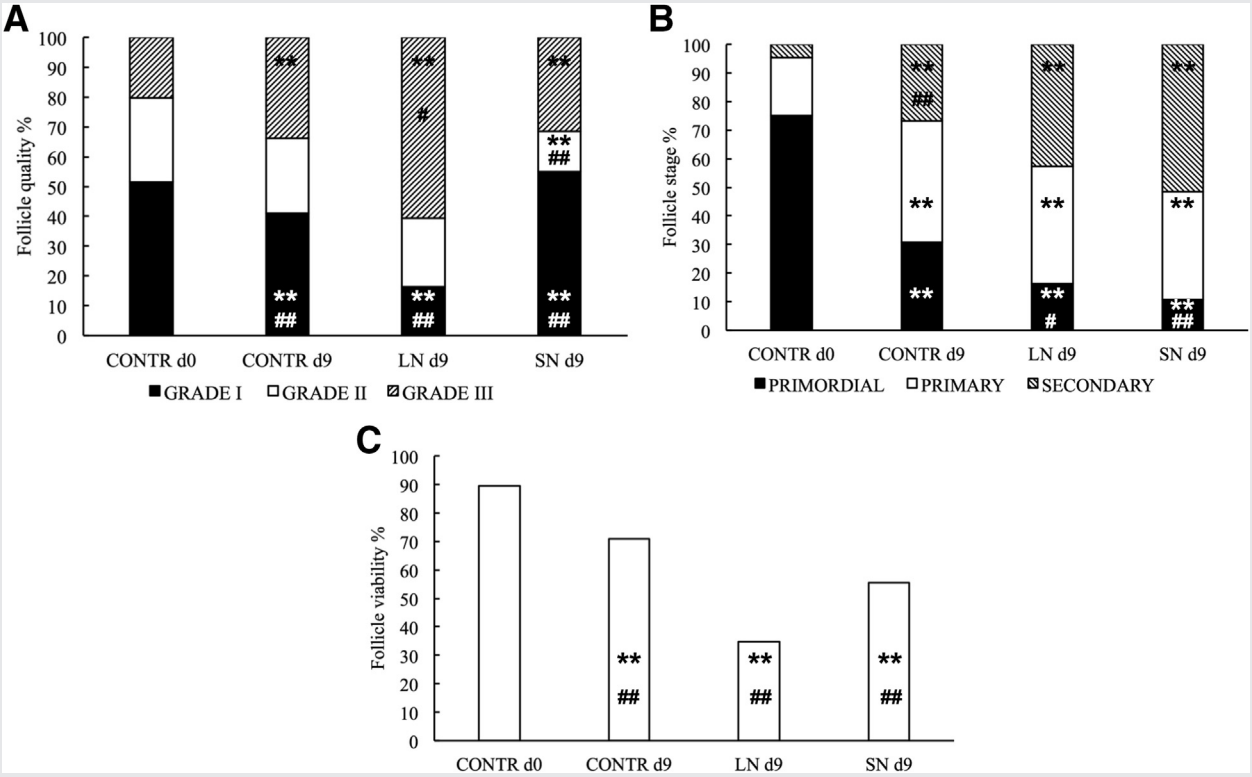
Experiment II

Long-term culture of fresh and V/W tissues: follicle histology and viability. To evaluate the effects of vitrification and warming on follicle quality and progression in long-term in vitro culture, the histology of follicles in fresh tissue at d0 (n = 617) was compared with follicles in fresh (n = 522) and V/W tissues after 9 days of culture (d9) (n = 122 in LN, n = 280 in SN) (Fig. 4A and 4B, Supplemental Table 2). Culture of fresh tissue for 9 days yielded a significant reduction of grade 1 follicles and an increase of grade 3 follicles as compared with d0 (Fig. 4A). A significant reduction of grade 1 follicles and a corresponding increase of grade 3 follicles were observed after 9 days of culture in LN tissue as compared with both fresh (d0 and d9) and SN tissue (Fig. 4B). At day 9, the percentage of grade 1 follicles in SN tissue was significantly higher than in fresh tissue.

Analysis of follicle stages at day 9 showed that long-term in vitro culture yielded a significant decrease of primordial follicles and a concomitant increase of primary and secondary follicles in all samples. Interestingly, at day 9 the percentage of secondary follicles in V/W tissues was significantly higher than in fresh tissue (Fig. 4B). The percentage of follicles progressing to the secondary stage was higher in SN than in LN tissue, although differences were not statistically significant.

Follicle viability (n = 1,976) significantly decreased in all samples after 9 days of culture (Fig. 4C). Follicle viability was significantly higher in SN than in LN tissue, although it was generally lower than in fresh tissue.

FIGURE 4



Grading (**A**), staging (**B**), and viability (**C**) of follicles in fresh (CONTR) and V/W ovarian tissue by SN or conventional LN and cultured in vitro for 9 days (d9) as described in Materials and Methods. (**A**) Percentage of follicles of varying grade. (**B**) Percentage of follicles of varying stage. (**C**) Percentage of viable follicles. $^{*,\#}$Statistically significant differences vs. fresh tissue or between V/W tissues, respectively. $^{*,\#}P<.05$; $^{**,\#\#}P<.01$. Bar = 10 μm.

Barbato. Vitrified ovarian tissue gene expression. Fertil Steril 2018.

DISCUSSION

Recently vitrification has emerged as an alternative to slow freezing for ovarian tissue cryopreservation (6–12,15,18) but its application and spread in clinical practice requires further basic research studies. Slush nitrogen vitrification has recently been considered as a new procedure to increase the cooling rate of tissue because it avoids the physical phenomenon termed "Leidenfrost effect." This refers to the quick development of nitrogen gas bubbles around a tissue, at a higher temperature than the nitrogen boiling point, that thermally shield tissue from the direct contact with the liquid nitrogen and cause a decrease of tissue cooling rate (27). Recently we reported that vitrification of human ovarian tissue with SN preserves the health of follicles and stromal cells after 24 hours of culture after warming better than with LN (18).

The aim of this study was to understand whether cryopreservation affects gene expression and follicle health and development in long-term culture. Human ovarian cortical tissue was vitrified with SN or LN and was analyzed for gene expression, follicle quality, survival, and growth immediately after warming and after 9 days of in vitro culture in gas-permeable dishes. Both vitrification procedures preserved the viability of follicles immediately after warming. However, in LN V/W tissue follicle quality was worse, and several genes involved in stress and toxicity pathways were overexpressed to a greater extent than in SN tissue.

Confirming that obtained after 24 hours of in vitro culture (18), the potential of SN to limit cryoinjury became more evident after 9 days of in vitro culture after warming. In fact, follicle survival, quality, and growth in SN tissue was markedly better than in LN tissue. This suggests that the evaluation of a cryopreservation procedure by characterizing follicle survival immediately after warming may not reflect the follicle developmental potential.

To provide a broader spectrum of information on the effects of cryopreservation, in this study we analyzed the expression of 84 genes known to be involved in stress and toxicity pathways, such as osmotic and oxidative stress, DNA damage, inflammatory response, and apoptosis. In LN V/W tissue, 13 of the 84 genes analyzed by qRT-PCR markedly exceeded the set threshold value. Among the altered genes, the increased overexpression of *DDB2* and *RAD51*, the markers recognizing early DNA damage, evidenced the extent of DNA damage caused by LN vitrification. In fact, in LN vitrified mouse follicles, *RAD51* expression has been correlated with an increased expression of *gamma-H2AX*. *Gamma-H2AX* is a biomarker of DNA double-strand breaks (28) established within a few minutes of DNA damage that has a critical role in the recruitment of repair factors to

nuclear foci. Interestingly, the levels of *DDB2* and *RAD51* were only slightly altered after SN vitrification suggesting that nuclear DNA in oocytes and somatic cells is well preserved by this cryopreservation procedure.

The transcript numbers for proteins involved in the inflammatory response, such as the interleukins, INFG, TNF, CD40LG, CFTR, and CRP, were also higher in LN than SN V/W tissue. The up-regulated expression of the genes for interleukins in LN V/W tissue is particularly interesting. In fact, CRP interacts with DNA and histones and may scavenge nuclear material released from damaged cells. Its level greatly increases during the acute phase response to tissue injury, and it is induced by interleukin-1 and interleukin-6 (29). In the ovary, interleukin-1 and interleukin-6 regulate the physiologic apoptosis and the follicular atresia (30). Their altered expression was reported in patients with polycystic ovary syndrome or ovarian cancers (31, 32).

Aquaporins (AQPs) allow a rapid, osmotically driven passage of water across the plasma membrane and play an important role in the transport of water and cryoprotectants (33). In cryopreserved mouse oocytes, hyperosmosis caused by the cryoprotectants has been reported to up-regulate the protein levels of AQP7 (34). This suggest that the overexpression of *AQP2* to a larger extent in LN than in SN V/W ovarian tissue could represent an attempt of tissue to withstand the osmotic stress induced by the cryoprotectants.

The markers of hypoxia carbonic anhydrase IX, matrix metalloproteinase 9 (MMP9), and erythropoietin (EPO) were among the most up-regulated genes in LN V/W ovarian tissue. Carbonic anhydrase IX is a hypoxia-induced enzyme with a key role in the protection against hypoxia and acidosis (35). Matrix metalloproteinase 9 is a gelatinase expressed by several cell types able to digest a broad spectrum of extracellular matrix molecules (36). Increased circulating levels of the matrix metalloproteinases MMP2 and MMP9 are involved in polycystic ovary syndrome through their effects on ovarian tissue remodelling (37). Erythropoietin is a hematopoietic cytokine with antioxidant, antiapoptotic, and anti-inflammatory properties that promotes cell survival and angiogenesis. Injection of erythropoietin in mice autografted with ovarian tissue transplants has been reported to reduce the oxidative stress and apoptosis associated with ischemia/reperfusion injury, and to increase follicle survival (38). The marked up-regulation of the genes expressing these three proteins in LN V/W tissue might represent an attempt of tissue to survive the injuries caused by the cryopreservation procedure.

Taken altogether, these data suggest that the use of SN for the vitrification of human ovarian tissue significantly reduces the alteration of gene expression caused by conventional LN vitrification, and the DNA damage associated with it.

The capacity of V/W tissue to support follicular development is a fundamental endpoint in the evaluation of a cryopreservation procedure, but its assessment still is a matter of debate. Several reports indicate that LN vitrification of human ovarian tissue does not affect follicle, GCs, and SCs morphology, viability, and E_2 synthesis (8–12, 18, 39, 40). However, this outcome is possibly to be blamed on the heterogeneity of the cryopreservation protocols and on the varying endpoints used for evaluating tissue health. Assessing the capacity of V/W tissue to support folliculogenesis in long-term in vitro culture might provide more appropriate information on ovarian tissue health. In a recently published study on the influence of oxygen availability in fresh human ovarian tissue strips cultured in gas-permeable dishes, we demonstrated that oxygen plays a key role in the maintenance of follicles health, survival, and capacity to progress to the secondary stage (23). In this study, we cultured SN or LN V/W tissue in the same gas-permeable dishes and operating conditions as in the earlier study (23) to ensure suitable oxygen availability in tissue. After 9 days of in vitro culture, tissue analysis showed that follicle progression, quality, and viability were better in SN than in LN V/W tissue. This confirms the superior efficiency of SN vitrification previously demonstrated after 24 hours of in vitro culture after warming (18).

The possibility to perform a complete folliculogenesis in vitro after human ovarian tissue vitrification and warming is a valuable and promising strategy to avoid the risk of reintroducing malignant cells after autotransplantation (41, 42). To date, great research effort has been made to investigate the metabolic and hormonal requirements of human ovarian tissue as a means to optimize the media for its in vitro culture (23, 43, 44). Recently, McLaughlin et al. (45) have demonstrated the possibility developing in vitro few metaphase II oocytes from the primordial follicle stage of fresh human ovarian tissue, thus raising new hopes for the achievement of complete human folliculogenesis in vitro. However, the insufficient number and quality of secondary follicles that have been obtained from fresh tissue with conventional culture strategies still limits its clinical application. In fact, the progression to the secondary follicles seldom exceeds 10% of the whole follicle population at the end of in vitro culture (45, 46). Recently, we reported that culturing fresh human ovarian tissue in gas-permeable dishes increases the yield of secondary follicles up to approximately 20% (23). In this study the in vitro culture, under the same conditions as in the earlier study (23), of human ovarian tissue that had been vitrified and then warmed yielded a progression to the secondary follicle stage almost twice as high as that of fresh tissue. The culture of tissue vitrified by SN consistently yielded better results than tissue vitrified by LN in terms of secondary follicles rate, quality, and viability. Deeper studies should be undertaken to explain such a marked difference between V/W and fresh tissue. We can only speculate that vitrification positively modifies the ovarian microenvironment that drives follicle growth.

In conclusion, SN vitrification improves human ovarian tissue preservation and provides a more efficient strategy for cancer patient fertility preservation than slow freezing. Slush nitrogen vitrification coupled with long-term culture after warming under conditions that ensure optimal oxygen availability in tissue could allow for the recovery of a more competent tissue capable of generating a higher number of metaphase II oocytes that can be used for programs of assisted reproduction.

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La vitrificación de tejido ovárico humano mediante nieve carbónica no altera la expresión génica y mejora la calidad y la progresión de los folículos en cultivo *in vitro* a largo plazo

Objetivo: Estudiar si la vitrificación mediante nieve carbónica (NC) vs. nitrógeno líquido (NL) afecta la expresión génica del tejido ovárico humano y preserva la calidad de los folículos durante el cultivo *in vitro* prolongado.

Diseño: Estudio experimental aleatorizado.

Lugar: Laboratorio de investigación universitaria.

Paciente(s): Biopsias de ovario obtenidas mediante cirugía laparoscópica de pacientes con afecciones ginecológicas benignas.

Intervenciones: Ninguna.

Medidas de los resultados principales: Las tiras de ovario fueron vitrificadas con NL o NC, descongeladas y analizadas antes o después del cultivo durante 9 días (D9) en placas permeables a gases. Se analizó la expresión de los genes implicados en las rutas de estrés y toxicidad mediante Q3 Array reacción en cadena de la polimerasa (PCR) y PCR cuantitativa a tiempo real en las tiras de ovario frescas y descongeladas. Se analizó la calidad de los folículos, así como la progresión y viabilidad de las tiras frescas y vitrificadas/descongeladas antes o después del cultivo.

Resultado(s): La vitrificación mediante NC conservó la calidad de los folículos mejor que la vitrificación mediante NL (% de folículos grado 1: fresco control 54,2; NL 29,3; NC 48,8). PCR cuantitativa mostró una notable regulación al alza de 13 genes en las muestras NL (rango: 10-35) y una regulación al alza notablemente menor de solo 5 genes (rango: 3,6-7,8) en las muestras NC. El cultivo *in vitro* a largo plazo mostró una peor calidad y viabilidad de los folículos en las muestras vitrificadas mediante NL que en las muestras frescas y vitrificadas mediante NC (% de folículos grado 1: fresco D0 51,5; fresco D9 41; NL D9 16,4; NL D9 55) y una significativa reducción de los folículos primordiales, así como un aumento concomitante de los folículos primarios y secundarios en todas las muestras. El crecimiento del folículo en estadio secundario fue significativamente mayor en el tejido vitrificado que en el tejido fresco, siendo mejor en NC que en tejido vitrificado mediante NL.

Conclusión(es): La calidad, expresión génica, viabilidad y progresión de los folículos, se conserva mejor después de la vitrificación mediante NC.

Palabras clave: Folículos, expresión génica, histología, criopreservación ovárica, viabilidad