Universita degli Studi di Napoli Federico II

DOTTORATO DI RICERCA IN BIOLOGIA

PhD IN BIOLOGY

Cycle - XXXII

Innovative strategies for female fertility preservation: development of a more efficient system for ovarian tissue cryopreservation and in vitro culture.

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INTRODUCTION

Over the last decades, ovarian tissue cryopreservation has been reported as a promising fertility preservation strategy and the only option available for young women with primarily cancer diseases who need immediate anticancer therapies. To date, a pregnancy rate of about 30% has been reported after auto-transplantation of frozen-thawed tissue, corresponding to more than 130 live births [1]. However, the possible risk of reintroducing malignant cells in ovarian cortical grafts remains the major problem associated with this procedure [2]. To overcome this limitation, the only possibility could be represented by a complete in vitro folliculogenesis, in a well-designed culture system.

The possibility of obtaining in vitro mature oocytes in metaphase II starting from the huge reserve of primordial follicles present in the ovary, was born in 1996; when Eppig [3] obtained the birth of mice for the first time after the fertilization of mature oocytes derived from the development of follicles primordial in vitro, and improved in 2003 [4]. Although this promising technology has attracted numerous researchers over the past twenty years, attempts to reproduce these results in superior animals and humans have been largely unsuccessful. Ovarian strip in situ culture has tremendous advantages over isolated primordial follicle culture systems because follicles are maintained within their natural environment. Only recently, mature human oocytes have been developed from primordial follicles through a culture method that involves a multi-stage protocol [5]. Although these results are encouraging, the number and quality of the mature oocytes obtained is still very low.

The causes for this can be traced back to several reasons listed below:

1. The inadequate cryopreservation of the tissue is one reason. It is a known fact that cryopreservation represents a crucial step of fertility preservation. The American Society for Reproductive Medicine still considers it experimental,

despite several pioneers considering ovarian cryopreservation an established technique for preservation of gametes and embryos [6]. Nonetheless, to date we know that almost all live births in cancer patients have been derived from auto-transplantation of cryopreserved ovarian tissue by slow freezing/rapid thawing [7–12] and only two live births have been reported by Suzuki et al. in 2015 [13] following ovarian tissue vitrification in patients with primary ovarian insufficiency.

Ovarian cryopreservation needs to be refined to reduce cryo-damage and the consequent depletion of follicles and stromal cells (SCs) that may impair the full recovery of ovarian function. Some studies suggest that slow freezing may cause extensive DNA fragmentation in primordial follicles and cause injury to stromal cells as well [14–17]. Starting with this knowledge, numerous researchers have tried to improve the efficiency of ovarian tissue cryopreservation.

The research group, with which I worked for my doctorate had already shown that ultra-rapid vitrification of human ovarian tissue with slush nitrogen (SN) improves recovery of healthy follicles in 2016. The group had also demonstrated preservation of granulosa cells (GCs) and stromal cells ultrastructure, DNA integrity of SCs and viability of oocytes, GCs, and SCs over conventional vitrification with liquid nitrogen (LN) [18].

During my PhD I attempted to merge optimal protocols for cryopreservation and culture and assess the functional potential of SN vitrified strips during long term culture [19].

2. The second reason is the insufficient number of secondary follicles that are obtained in the early stages of culture or after the first step of culture, that rarely exceeded 10% [5-20]. This could be due to inadequate culture conditions that limit the number and health of secondary follicles produced during the first step of culture. Despite several studies having investigated the nutritional and endocrine requirements of ovarian tissue to optimize media for cortical strip

culture, only a few studies have investigated the role of oxygen supply for follicular growth.

Therefore, during my second year of PhD, the aim of my project was to study the effects of oxygen on follicular morphology, progression and viability during the culture of the ovarian cortical strips. We made use of oxygen permeable petri dishes to modulate the local oxygen tension in the vicinity of the tissues. To study the effect of media volumes on the performance of the grown strips, we attempted with change in the conditions of ambient oxygen i.e. 5% and 20% oxygen concentration. This was followed by analysis of the influence of the optimal diffusion of oxygen in the in situ culture of the ovarian strip.

3. The third reason is the culture conditions of the current techniques which are still sub-optimal.

   We showed that the in vitro culture of human ovarian cortical strips in gas-Permeable Dishes (PD) enhances follicular health and secondary follicle growth over conventional dishes by improving oxygen availability in situ [4,5]. However the static culture systems adopted so far, lack physiological and biomechanical cues which generates stagnant media layers around the tissue with a consequent deprivation of nutrients and accumulation of toxic/waste metabolites.

   Environmental cues such as oxygen tension and mechanical stimuli may be further enhanced to match the physiological requirements.

   In my last year of PhD, we investigated dynamic culture systems in continuous perfusion bioreactors (PB) that enabled the disruption of solute concentration gradients and application of physiological fluid mechanic stresses on tissue which improved the ovarian cortical tissue culture.

Chapter 1

Title:

"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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Introduction

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 5x2x0.5 mm were manually dissected at 4°C. Fresh and V/W fragments from the same biopsy were further cut in strips of 1x1x0.5 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 mg/mL ascorbic acid at 37°C, in a 5% CO2 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 5x2x0.5 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% CO2, 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 5x2x0.5 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 (Ct) 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 firststrand cDNA by SuperScript III reverse transcriptase. Primers for qRT-PCR validation were designed with Primer 3Plus (http://www.bioinformatics.nl/cgi-bin/primer3 plus/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 Ct values from all the replicates were used to calculate the fold changes relative to the internal control. The comparative DDCt method was used to quantify the level of gene expression. The $2^{-\Delta\Delta}$Ct 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-Sidak 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).

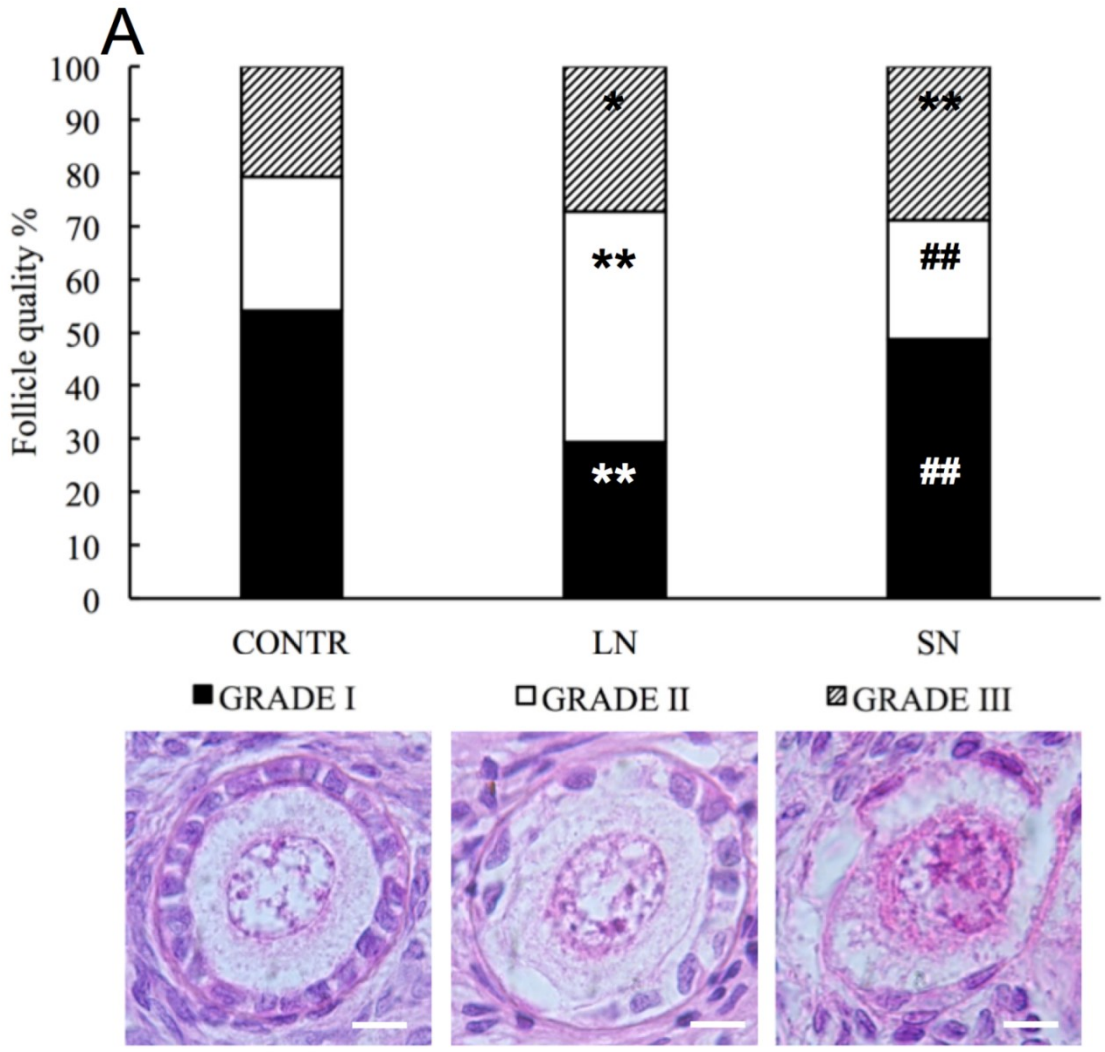
Gene expression in fresh and V/W tissues.

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.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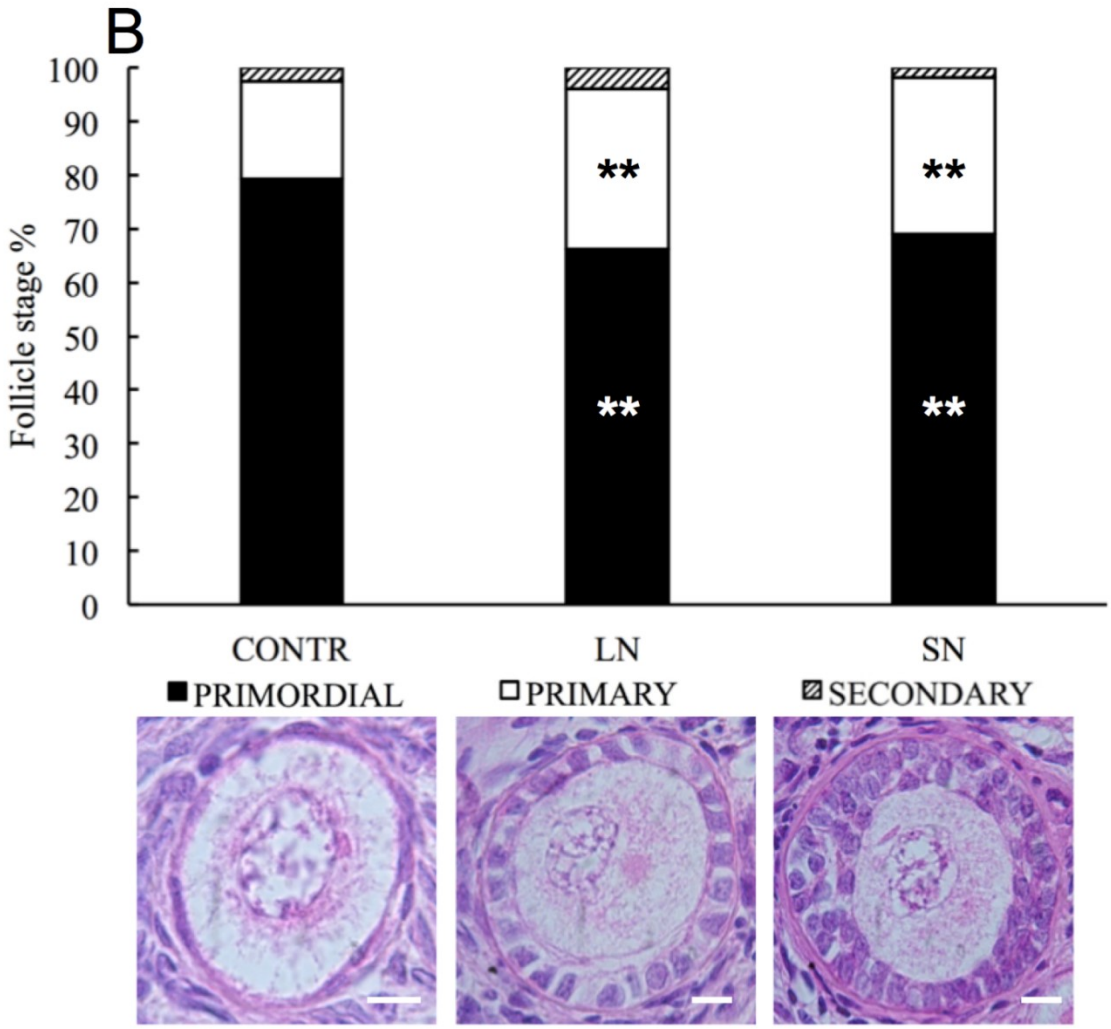


Figure 1. Grading (A) and staging (B) of follicles in fresh (CONTR) and V/W ovarian tissue vitrified by SN or conventional LN. (A) Upper panel: percentage of follicles of varying grade. Lower panel: histology of follicles of varying grade. (B) Upper panel: percentage of follicles of varying stage. Lower 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

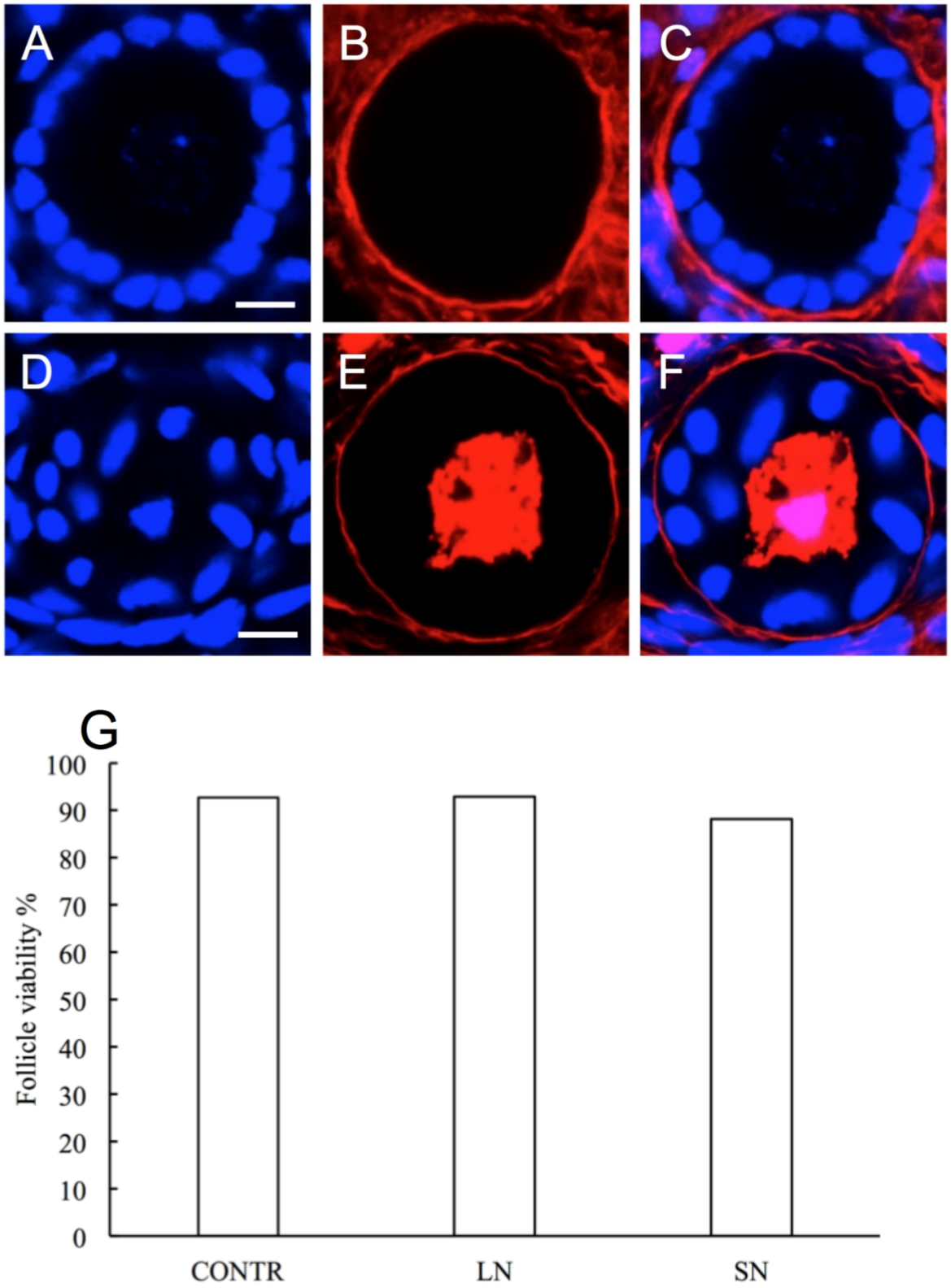


Figure 2. Viability of follicles in fresh and V/W ovarian tissue as obtained by confocal microscopy. Top: representative confocal micrographs of a live (A–C) and a dead (D–F) follicle (red: live–dead far-

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 mm. Bottom (G): percentage of viable follicles in fresh (CONTR) and V/W ovarian tissue vitrified by SN or conventional LN.

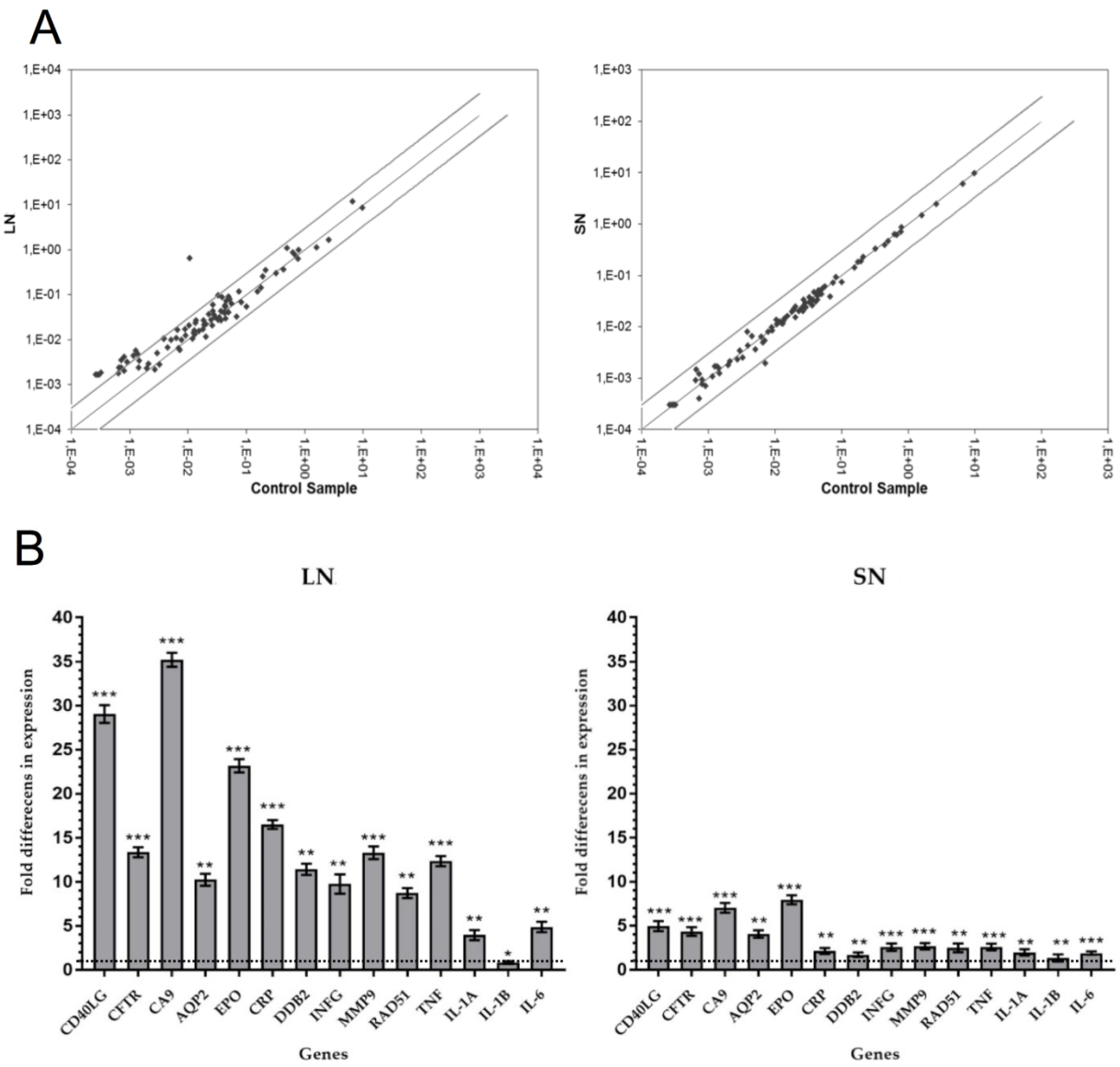
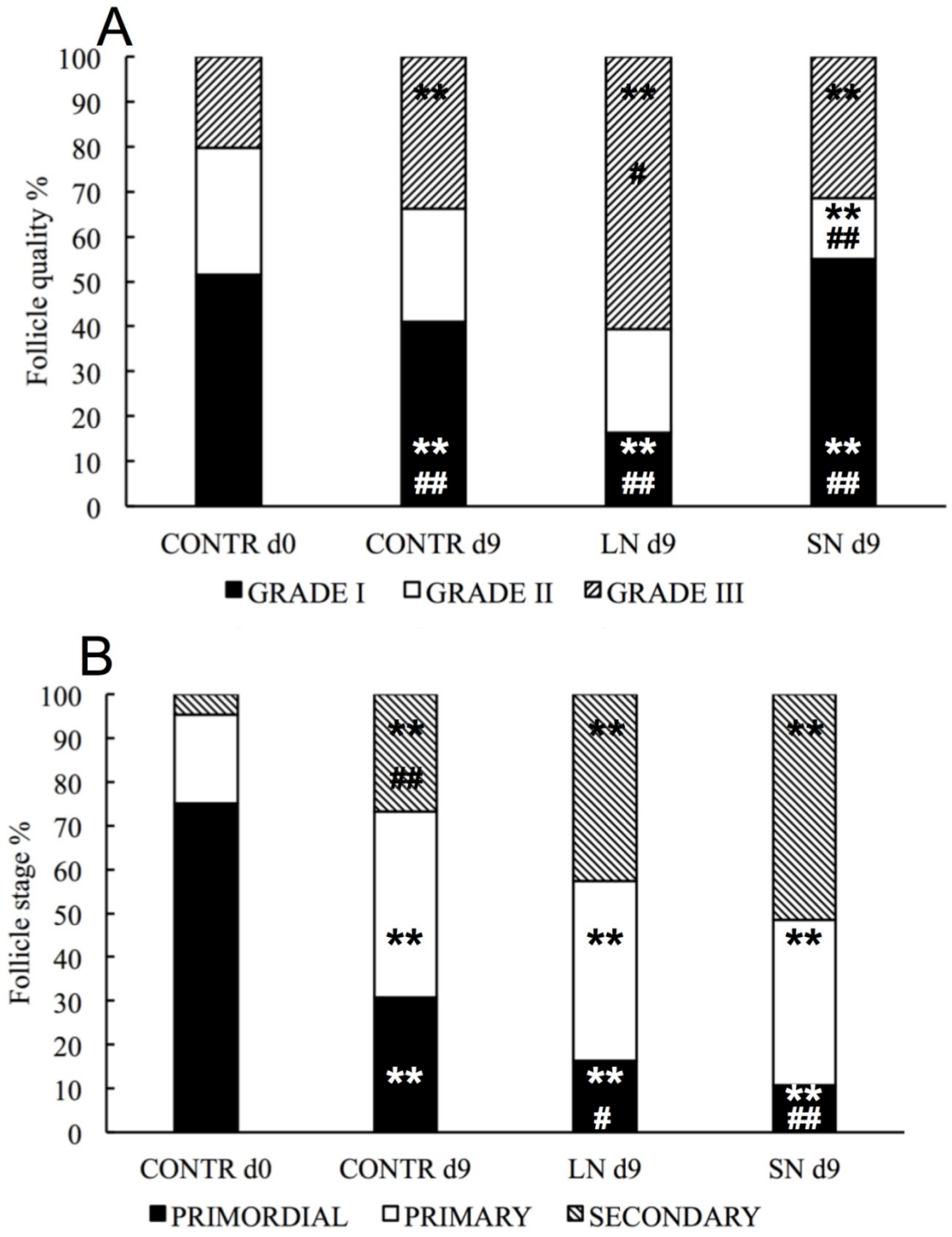


Figure 3. (A) Scatter plots of the PCR array showing the fold expression of the investigated genes involved in stress and toxicity pathways in LN and SN tissue (y-axis) vs. fresh tissue (control sample: x-axis). The lines indicate the zone comprised within a threefold change in gene expression (threshold). (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.

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.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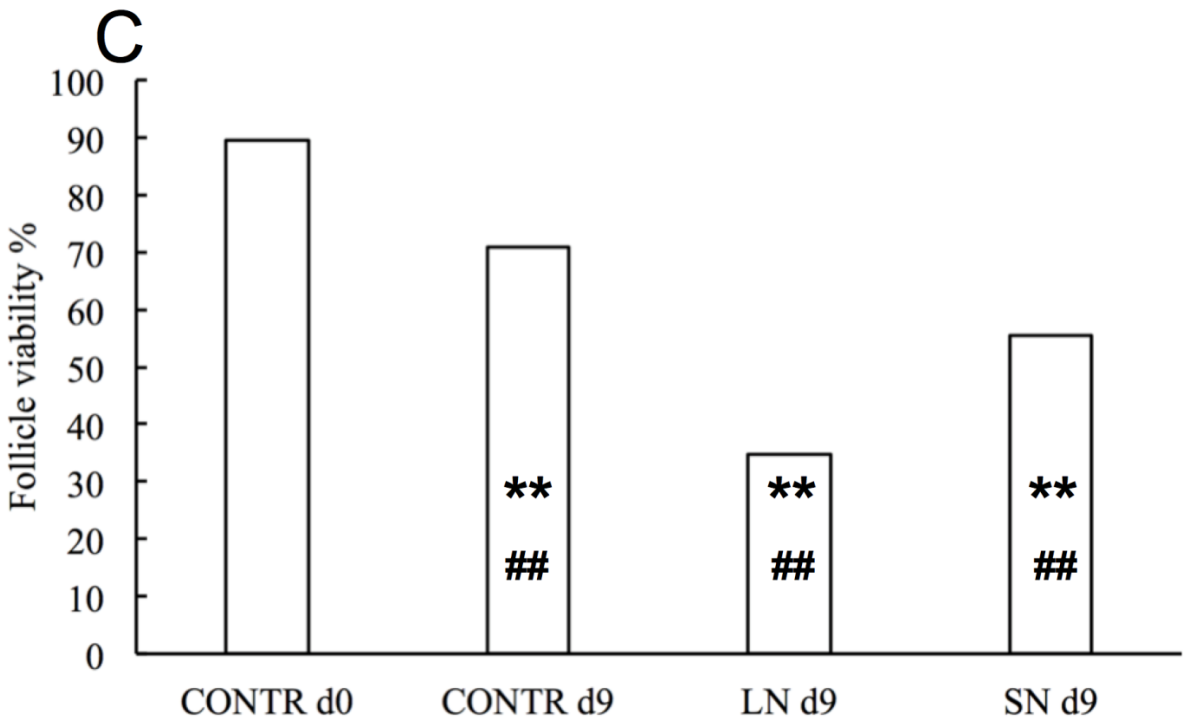

Figure 4. Grading (A), staging (B), and viability (C) of follicles in fresh (CONTR) and V/W ovarian tissue by SN or conventional LN and cultured in vitro for 9 days (d9) as described in Materials and Methods. (A) Percentage of follicles of varying grade. (B) Percentage of follicles of varying stage. (C) Percentage of viable follicles. *, # Statistically significant differences vs. fresh tissue or between V/W tissues, respectively. *, # P<.05; **, ## P<.01. Bar = 10 μm.

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 E2 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 gaspermeable 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 gaspermeable 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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Chapter 2

Title:

"Is oxygen availability a limiting factor for in vitro folliculogenesis?"

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Abstract

Transplantation of ovarian tissue for the preservation of fertility in oncological patients is becoming an accepted clinical practice. However, the risk of re-introducing tumour cells at transplantation has stirred an increased interest for complete in vitro folliculogenesis. This has not yet been achieved in humans possibly for the lack of knowledge on the environmental milieu that orchestrates folliculogenesis in vivo. The main aim of this study was to investigate the effect of oxygen availability on follicle health and growth during in vitro culture of ovarian tissue strips. To this end, a model was developed to predict the dissolved oxygen concentration in tissue under varying culture conditions. Ovarian cortical strips of bovine, adopted as an animal model, and human tissue were cultured in conventional (CD) and gas permeable (PD) dishes under different media column heights and gaseous oxygen tensions for 3, 6 and 9 days. Follicle quality, activation of primordial follicles to the primary stage, and progression to the secondary stage were analysed through histology. Follicle viability was assessed through a live-dead assay at the confocal scanning laser microscope. Findings showed a higher follicle quality and viability after culture of bovine ovarian strips in PD in adequate medium height and oxygen tensions. The best culture conditions found in the bovine were adopted for human ovarian strip culture and promoted a higher follicle quality, viability and progression. Overall, data demonstrated that modulation of oxygen availability in tissue plays a key role in maintaining follicles' health and their ability to survive and progress to the secondary stage during ovarian tissue in vitro culture. Such culture conditions could increase the yield of healthy secondary follicles for subsequent dissection and individual culture to obtain competent oocytes.

Introduction

Advancements in anti-cancer therapy have improved the survival rates of cancer patients [1] and increased the focus on post-treatment quality of life and their future reproductive potential. Anti-cancer regimes, like chemotherapy and ionizing radiations, induce variable degrees of irreversible premature ovarian failure in 100% of patients, threatening their fertility even after complete cancer remission [2,3]. Ovarian tissue cryopreservation is a promising fertility preservation strategy and represents the only option available for pre-pubertal cancer patients due to their ovarian dormancy at this age [4,7]. Thus far, orthotopic ovarian tissue transplantation following cryopreservation has yielded more than 86 pregnancies worldwide [8] suggesting the future potential of this technique. However, transplantation of ovarian tissue following cryopreservation is approached with extreme caution, particularly in the case of blood-borne and highly metastatic malignancies, where the possibility of re-introducing tumour cells back into the patient after cancer remission represents a serious risk [9,10]. Lately, the fact that the follicular basal lamina hinders cancer invasion into the oocyte and follicular cells [11] has triggered widespread interest in the development of culture systems for primordial ovarian follicles that constitute the major share of ovarian reserve in women of all age groups. Several strategies for in vitro follicular growth have been proposed over the years in which follicles were isolated either at the primordial stage or, after a first culture step of ovarian cortical strips, at the secondary stage, and then cultured until full maturation [12,16]. Ovarian strip in situ culture has tremendous advantages over isolated primordial follicle culture systems because follicles are maintained within their natural environment. The ovarian tissue itself is a regulating force, providing cells with an array of highly complex and dynamic bio-mechanical signals [17] which are very difficult to accurately mimic in vitro with an artificial matrix. Success of in vitro follicular culture in the murine model has confirmed the important role played by initiating primordial follicle growth in situ within the ovarian tissue in a two-step culture approach [12].

Unfortunately, the success of current ovarian tissue culture systems in the human is still unsatisfactory being limited by the low efficiency of long-term survival and growth of primordial and primary follicles in situ [18,19]. Over the years, several studies have investigated ovarian tissue nutritional and endocrine requirements with the aim to optimise media for cortical strip culture [20,21]. Nonetheless, the yield of secondary follicles seldom exceeded 10% of the total follicle population obtained post-culture [15,22]. It has been proposed that the inefficient transport of oxygen could be a major hurdle in establishing optimal ovarian tissue cultures because it may hinder oocyte/follicle development and cause tissue necrosis [23]. Only a few studies have investigated the importance of oxygen supply for follicular growth, and results are still controversial [23,27].

The main aim of this study was to investigate the influence of oxygen availability to ovarian cells (e.g. the dissolved oxygen concentrations at the surfaces of the ovarian strip, $C_{O2,TS}$) during in vitro culture on quality, viability and progression of human follicles. To save precious human biological material, adequate culture conditions were identified in preliminary experiments performed in the bovine, and were then used for the culture of human ovarian tissue. To this end, $C_{O2,TS}$ was modulated by using conventional dishes (CD) or gas-permeable dishes (PD), by varying media column height above the strips, and by incubation in air or 5% O_2.

Materials and methods

Chemicals and consumables

Lumox gas-permeable culture dishes and conventional culture dishes 50mm in diameter were from Sarstedt (Nu¨mbrecht, Germany) and Falcon (Sigma-Aldrich, Milan, Italy) respectively. Leibovitz's L-15 medium, α-MEM Glutamax medium (code number 32571), Insulin transferrin selenium (ITS) 100x, Live/dead Fixable far red stain were purchased from Invitrogen (Milan, Italy). Penicillin streptomycin 100x, Amphotericin B 250µg/ml, Bovine serum albumin (BSA), L-Ascorbic acid, L-Glutamine 200mM,

Hoechst 33342, Fructose, α-thioglycerol and Eosin-Y were purchased from Sigma Aldrich (Milan, Italy). Mayers's hematoxylin and paraffin wax were from Carlo Erba (Milan, Italy).

Collection and preparation of ovarian tissue

Bovine ovaries were collected at the Slaughterhouse Straccione (San Marcellino, Caserta, Italy; CEE accreditation number 1403/M) and transported to the laboratory in Leibovitz's L-15, 1% penicillin-streptomycin (Pen-Strep), 1µg/ml Amphotericin-B, at 4˚C, within 2h of slaughter. The use of human 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 biopsies were collected from six women (age = 27.1 ± 6.9 years; range 18–34 years) during laparoscopic surgery for benign gynecologic conditions and transported to the lab as described above.

Bovine ovaries and human ovarian biopsies were transferred to handling medium (Leibovitz's L-15, 2mM glutamine, 3mg/ml BSA, 1% Pen-Strep, 1µg/ml amphotericin B) and cortical slices (~ 0.5mm thick) were manually dissected at room temperature (RT) avoiding areas with visible antral follicles to ensure a predominant primordial follicle population. The cortical slices were further uniformly sliced into 1mm x 1mmx 0.5mm strips using a tissue chopper (Mcilwain, Mickle Laboratory Engineering Company, Ltd, Surrey, UK). The strips were pooled in a 10cm Petri dish, mixed by gentle agitation, washed twice in fresh handling medium, and 10 strips were randomly distributed into each culture dish. Fresh control strips from each ovary were processed for histology and viability assessment as a control.

Strip culture and modulation of oxygen availability

In each experiment, cortical strips from the same ovary were cultured in α-MEM, 3mM glutamine, 0.1% BSA, 1% Pen-Strep, 1% ITS (10 µg/ml Insulin, 5.5 µg/ml Transferrin, 6.7 ng/ml Selenium), 1µg/ml amphotericin-B, 50µg/ml ascorbic acid at 37˚C, 5% CO2 and 95% humidity in air. Half medium was changed every 48h. At the end of culture, 5

strips from each dish were treated for histology and 5 strips for viability assessments. To identify the culture conditions ensuring the most adequate oxygen availability (e.g. dissolved oxygen concentrations at the upper, $C_{O2,TSU}$, and bottom surface, $C_{O2,TSB}$, of the ovarian strip), oxygen supply was modulated by culturing ovarian strips in conventional (CD) or gas permeable dishes (PD) in a volume of medium yielding average column heights from the dish bottom of 1.4mm (HV: high volume, 5ml) or 0.7mm (LV: low volume, 2.5ml) in 5% CO_2 in air or in 5% CO_2, 5% O_2, 90% N_2. The most adequate culture conditions found in the bovine model were applied to human ovarian tissue culture.

Experimental design

In experiment I (n = 3), bovine ovarian strips (BOSs) from the same ovary were cultured as described above in 5ml of medium in permeable vs conventional dishes (conditions hereinafter referred to as PDHV and CDHV, respectively) for 3, 6 and 9 days in 5% CO_2 in air.

Results of experiment I demonstrated a significantly higher follicle quality and viability in PD versus CD both at day 6 (D6) and 9 (D9). Hence, in experiment II (n = 3) BOSs from the same ovary were cultured in 5% CO_2 in air for 6 days in high or low volumes of medium in PD vs CD (conditions hereinafter referred to as PDHV, PDLV, CDHV, CDLV, respectively).

In PD, the strip bottom surface is in direct contact with a gas-permeable polymer which ensures direct oxygen supply and also metabolic CO_2 removal across such surface.

Experiment III (n = 3) was designed to investigate whether the better outcome of BOSs cultured in PDHV in experiment I and II was caused by an adequate oxygen availability or an enhanced removal of metabolic CO_2. To this end, BOSs were cultured in CDHV and PDHV for 6 days under 5% CO_2 in air vs 5% CO_2, 5% O_2, 90% N_2.

Experiment IV (n = 6) was aimed to investigate whether the most adequate culture condition found in bovine experiments could have similar effects on human follicle quality, activation, progression and viability. To this end, human ovarian strips (HOSs)

from the same ovaries were cultured in PDHV vs CDHV under 5% CO_2 in air for 6 and 9 days.

Model of oxygen transport in ovarian tissue

Dissolved oxygen concentration in ovarian cortical tissue during in vitro culture is generally much lower than that in air because of the poor solubility of oxygen in medium, and of the resistance to oxygen diffusion in medium and tissue combined with oxygen consumption by ovarian cells (Fig 1). To account for such effects and gather more realistic information than those derived from the incubator setting, dissolved oxygen concentration anywhere in tissue ($C_{O2,T}$) under varying culture conditions was predicted with a model describing oxygen diffusive transport in medium and tissue at steady state. It was assumed that tissue has uniform properties, that oxygen enters tissue only across the upper surface (i.e. CD) or also from the bottom strip surface (i.e. PD), and that an anoxic zone forms amid the strip thickness. In CD, it is $C_{O2,T} >0$ only in the uppermost strip part (i.e. from $z>\delta_{i,U}$ to $z = \delta_T$), it decreases towards the strip bottom and levels off to zero at a fractional distance $\delta_{i,U}/ \delta_T$ from it (i.e. $C_{O2,T} = 0$ from $z = 0$ to $z = \delta i_{,U}$). In PD, $C_{O2,T}$ at the strip bottom is assumed equal to the dissolved oxygen concentration in medium equilibrating the gaseous oxygen tension ($C_{O2,B}$). As an effect, the dissolved oxygen concentration is $C_{O2,T}>0$ also near the strip bottom but it decreases towards the upper strip surface and levels off to zero at a fractional distance $\delta i,B/\delta_T$ from the bottom (i.e. $C_{O2,T} = 0$ from $z = \delta_{i.B}$ to $z = \delta_{i,U}$). The bias associated to the lack of reliable estimates of ovarian cortex structural, transport and metabolic properties was minimized by lumping such properties in the dimensionless Thiele modulus ϕ and mass Biot number Bi_m, actually determining the dissolved oxygen concentration profile in tissue. The former compares the rate of oxygen metabolic consumption to diffusion in tissue. The latter compares the dissolved oxygen concentration drop in tissue to that in medium to sustain oxygen transport to cells. In CD, an oxygen mass balance about a tissue control volume infinitesimal in z yields the

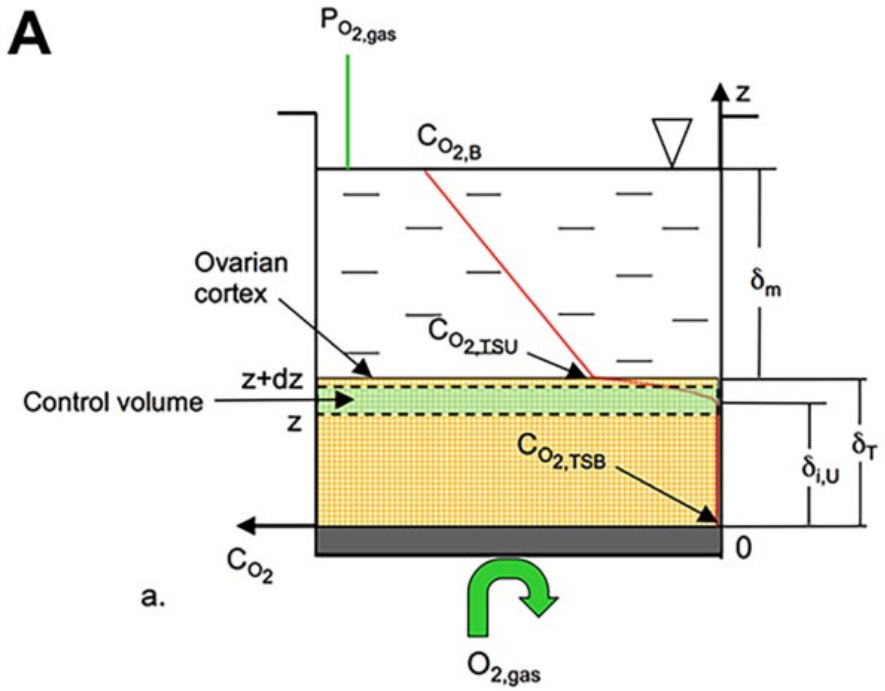
dimensionless dissolved oxygen concentration, $C_{O2,T}$ /$C_{O2,B}$, in the uppermost strip part at a fractional distance z/ δ_T from the bottom as follows (S1 Appendix):

$$\frac{C_{O2,T}(z/\delta_T)}{C_{O2,B}} = \frac{1}{2}\phi^2\left[\left(\frac{\delta_i}{\delta_T}\right)^2 + \left(\frac{z}{\delta_T}\right)^2 - 2\frac{\delta_i}{\delta_T}\frac{z}{\delta_T}\right] \quad (1)$$

with

$$\frac{\delta_i}{\delta_T} = 1 - \left[\frac{1}{\phi}\sqrt{\frac{\phi^2}{Bi_m} + 2} - \frac{1}{Bi_m}\right];\ \ \phi^2 = \frac{G'''}{D_T C_{O2,B}}\delta_T^2;\ \ Bi_m = \frac{D_m \delta_T}{\delta_m D_T} \quad (2)$$

where: G''' is thse volumetric tissue oxygen consumption rate; D_T and D_m are oxygen diffusivity in tissue and medium, respectively; δ_m and δ_T are the medium height above the strip and tissue thickness, respectively.



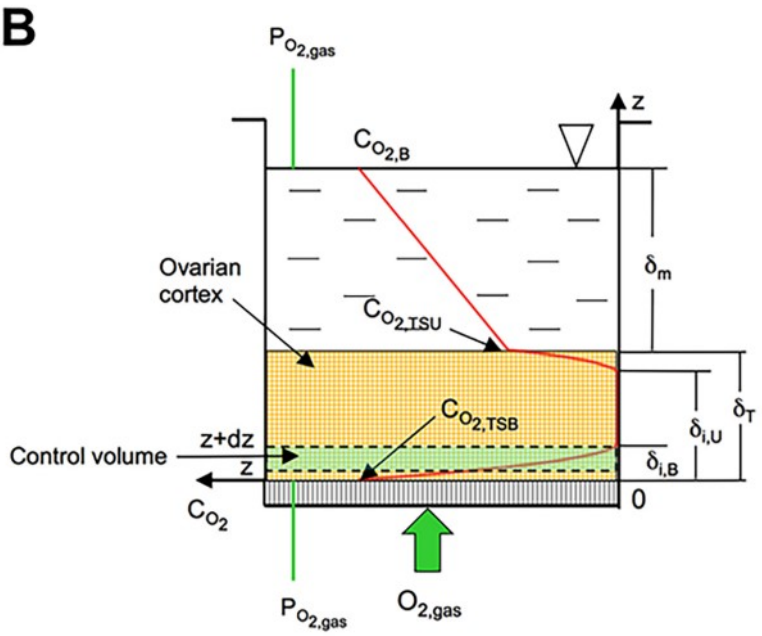


Fig 1. Scheme of ovarian cortex culture in conventional (CD) and gas-permeable (PD) dishes. The continuous red lines show exemplary dissolved oxygen profiles predicted in medium and tissue by the oxygen transport model described in Eqs 1–3 when strips of ovarian cortical tissue are cultured in: A) conventional dishes with gas-impermeable bottom (CD); B) dishes with gas-permeable bottom (PD). The green box identifies the control volume with respect to which the differential mass balance equations were written, as described in S1 Appendix and S2 Appendix for CD and PD, respectively. The meaning of the symbols may be found in text.

In PD, $C_{O2,T}/ C_{O2,B}$ close to the strip upper surface is the same as in Eqs 1 and 2. $C_{O2,T}/ C_{O2,B}$ close to the strip bottom may be gathered with a similar mass balance by imposing that $C_{O2,T}$ equals $C_{O2,B}$ at $z = 0$ and that it levels off to zero at $z = \delta_{i,B}$, to give (S2 Appendix):

$$\frac{C_{O2,T}(z/\delta_T)}{C_{O2,B}} = \left[1 + \frac{1}{2}\phi^2\left(\frac{z}{\delta_T}\right)^2 - \sqrt{2}\,\phi\left(\frac{z}{\delta_T}\right)\right]. \qquad (3)$$

To gather qualitative information on oxygen availability in the ovarian strips for the conditions used for this work, exemplary profiles of the dimensionless dissolved oxygen concentration in tissue, $C_{O2,T}/C_{O2,B}$, were estimated from Eqs 1-3 for representative parameter values, as follows: $G''' = 2x10^{-2}$ mol/(s m^3); $D_m = 3.5x10^{-9}$ m^2/s; $D_T = 2.8x10^{-9}$ m^2/s; $\delta_T = 0.4 \pm 0.5$ mm; $\delta_m = 0.2$ (LV) or 0.9 (HV) mm. $C_{O2,B}$ was set at 0.2 mol/m^3 (i.e. $pCO2_{gas} = 21\%$), unless otherwise noted. These values yield the following dimensionless parameters values: $\phi = 2.5$, $Bi_m = 0.7$ (HV) or 3 (LV). Oxygen availability to ovarian cells was characterized in terms of the dissolved oxygen concentrations at the upper ($C_{O2,TSU}$) and bottom ($C_{O2,TSB}$) strip surface, the average dissolved oxygen concentration in the strip ($C_{O2,T,avg}$), and the percent strip volume operated under anoxic conditions.

Histology

To assess follicular quality, activation and progression, strips were fixed in Bouin's, dehydrated in increasing ethanol concentrations, embedded in paraffin and 5μm serial sections were stained with hematoxylin and eosin. All follicles visualized in serial sections from each strip were graded and staged by two blinded expert observers. Follicles were evaluated only when the germinal vesicle was visible to minimize the chance of re-counting. Follicle quality was graded as previously reported [28]. Briefly: grade 1 follicles were spherical and had homogeneously distributed granulosa cells (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. Follicle stages were scored according to Gougeon's criteria [29], as follows: 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.

Viability assessment

Strips were incubated in Dulbecco's PBS with 1μg/ml Live/Dead Fixable Far Red Stain and 10μg/ml Hoechst 33342, 3 hours at 4˚C under gentle agitation, fixed in 4% paraformaldehyde in PBS 2 hours at RT, washed in fresh PBS and incubated in PBS 10μg/ml Hoechst 33342 at 4˚C overnight [30]. 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 then optically cleared using See DB clearing protocol [31]. Briefly, samples were serially incubated in 5 mL of 20%, 40%, and 60% (wt/vol) fructose, each for 3 hours, 80% and 100% fructose (wt/vol) each for 12 hours, and finally in 115% (wt/vol) fructose for 24 hours with gentle shaking at RT. All fructose solutions were supplemented with 0.5%. α-thioglycerol. To avoid compression, strips were mounted in 115% fructose on a glass slide with 3 spacer coverslips (0.17mm) placed on each side and covered with a coverslip. Analysis was carried out with a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar, Germany) using a 405-nm diode laser for visualizing the nuclear label (Hoechst 33342) and a 633-nm helium neon laser for the live/dead probe. Each strip was traversed using the z-position control and fields to a depth of 300μm from the tissue surface were imaged using a 63x glycerol immersion objective.

Statistical analysis

For each experiment, data is presented as cumulative percentages. Overall, statistical analysis was performed by Fisher's exact test for pairwise comparisons when overall significance was detected.

Results

Model-predicted oxygen availability

As shown in Table 1, when strips were cultured in air, oxygen availability increases with the culture conditions in the following order: CDHV<CDLV<PDHV<PDLV. When strips were cultured at $pCO2_{gas}$ = 5% in CDHV the model predicts that 99% of the strip volume is operated under anoxic conditions. Use of dishes with a gas-permeable bottom in PDHV slightly enhances oxygen availability, yet more than 85% of the strip volume is operated under anoxic conditions.

Experiment I

Histological analysis (n = 1837 follicles: D0, 307; D3 CDHV, 242; D3 PDHV, 265; D6 CDHV, 287; D6 PDHV, 223; D9 CDHV, 275; D9 PDHV, 238) showed that strips cultured in PDHV harboured significantly more grade I follicles both on D6 and 9. By D6 the proportion of grade I follicles in CDHV dropped significantly compared to day 0 (Fig 2A). Both groups (Fig 2B) showed a marked and significant decrease of primordial follicles and a corresponding significant increase of primary follicles at D3 as compared to D0. Interestingly, both groups showed a significantly higher proportion of secondary follicles at D6 and D9 than at D0.

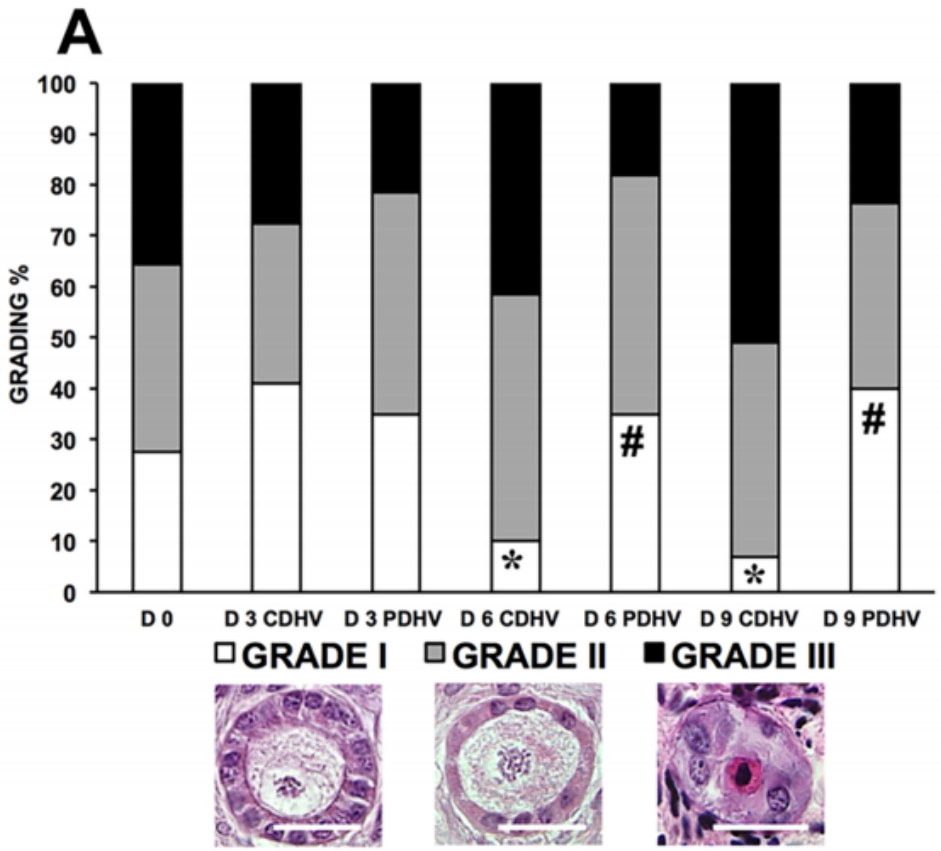
Follicle viability was obtained from a total of 1969 follicles (number of follicles: D0, 254; D3 CDHV, 278; D3 PDHV, 312; D6 CDHV, 323; D6 PDHV, 236; D9 CDHV, 202; D9 PDHV, 364). Fig 3 shows representative confocal micrographs of one viable (A-C) and one dead follicle (D-F). Viability significantly decreased during culture under both conditions. However, viability throughout the culture was about 80% in PDHV and only around 50% in CDHV (Fig 3G).

Taken together, this data (S1 Table) suggests that increased $C_{O2,TS}$ enhances follicular activation and preserves viability.

Table 1. Model-predicted oxygen availability in a tissue strip.

Culture condition	$C_{O2,TSB}/C_{O2,B}$, %	$C_{O2,TSU}/C_{O2,B}$, %	$C_{O2,T,avg}/C_{O2,B}$, %	Anoxic strip volume fraction, %
CDHV	0	3.6	0.053	89.3
PDHV	100	3.6	1.25	32.6
CDLV	0	32.6	0.3	67.7
PDLV	100	32.6	1.55	11.1

Model predictions were obtained for ϕ = 2.5 and Bi_m = 0.7 (HV) and 3 (LV): CO2,TSB−dissolved oxygen concentration at strip bottom surface; C_{O2},TSU−dissolved oxygen concentration at strip upper surface; $C_{O2,T,avg}$−average dissolved oxygen concentration in the strip volume.



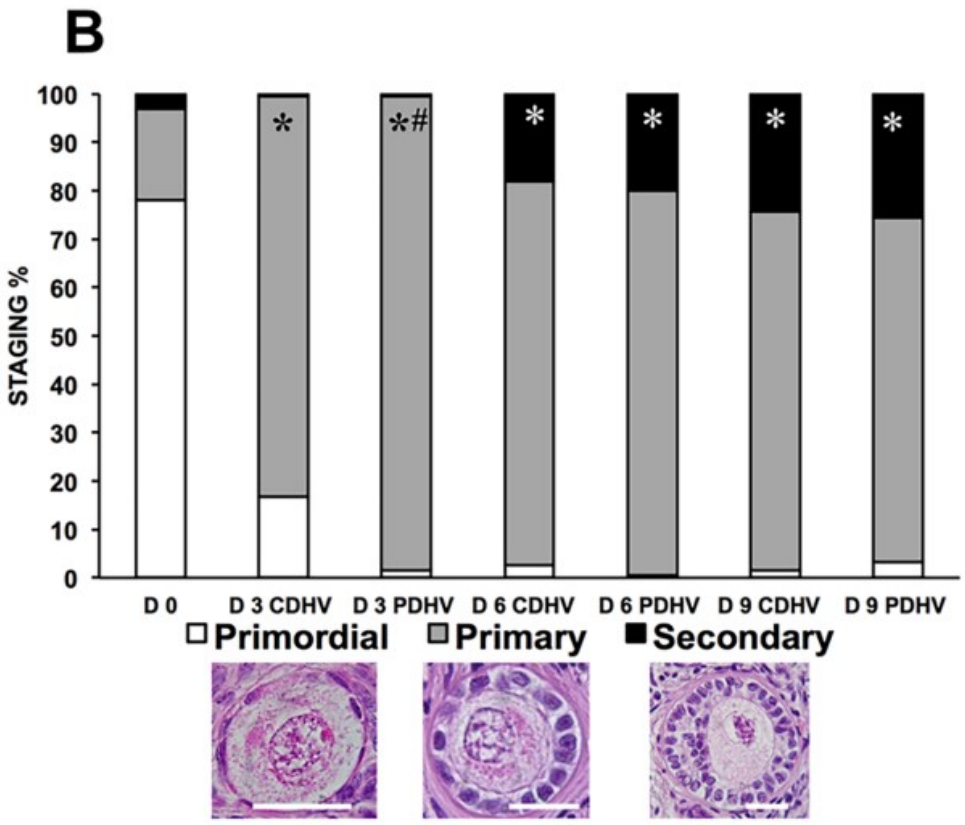


Fig 2. Grading and staging of bovine follicle in strips cultured in air in PDHV vs CDHV. Histological grading (A) and staging (B) of bovine follicles in strips cultured in PDHV and CDHV in air. Bar = 20μm. $P<0.01$ vs D0; $^{\#}$ $P<0.01$ vs corresponding treatments.

Experiment II

The transport model predicts that the combined use of different medium heights and CD or PD yielded progressively increasing oxygen availability in the following order: CDHV<CDLV<PDHV<PDLV (Fig 1 and Table 1).

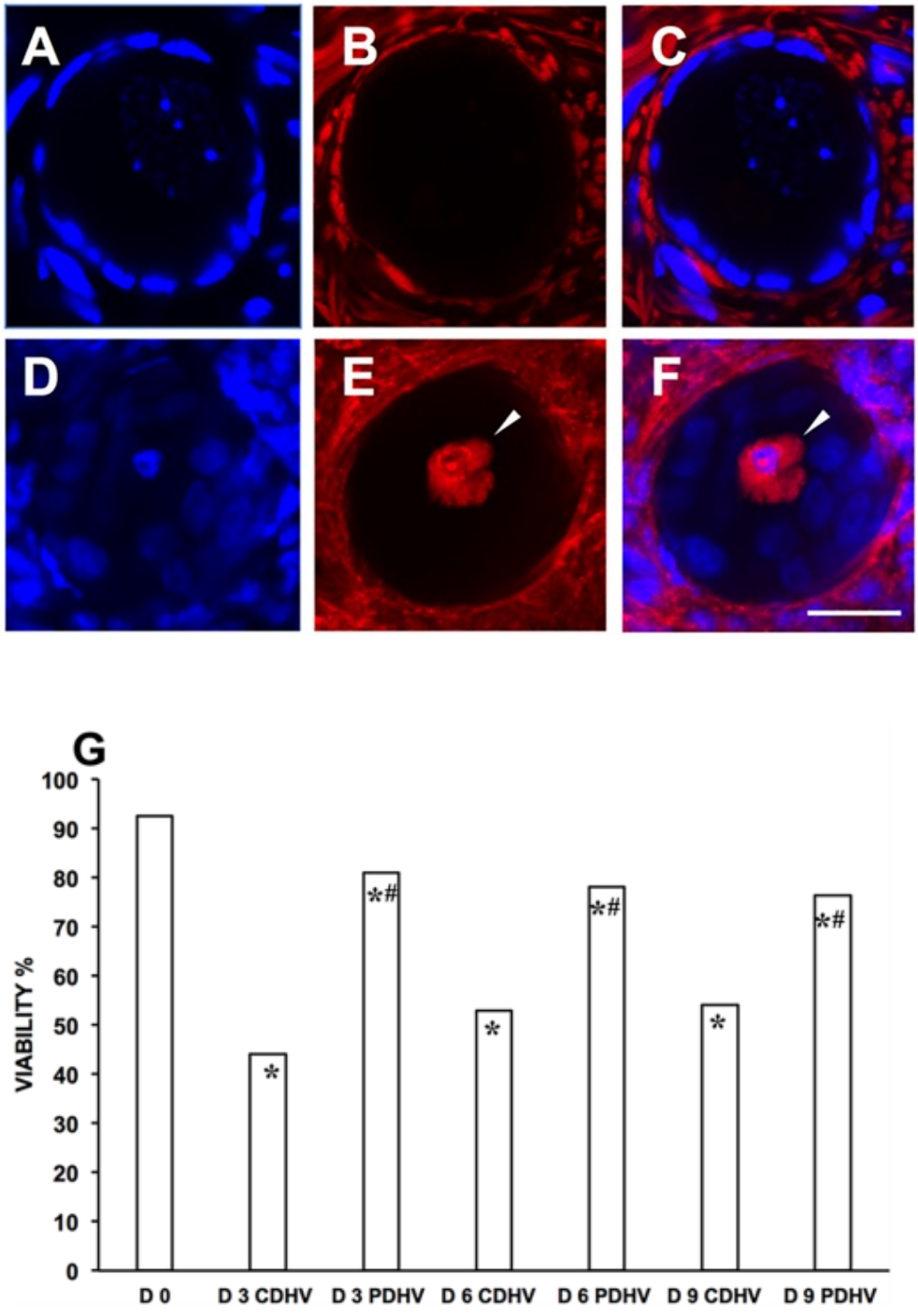
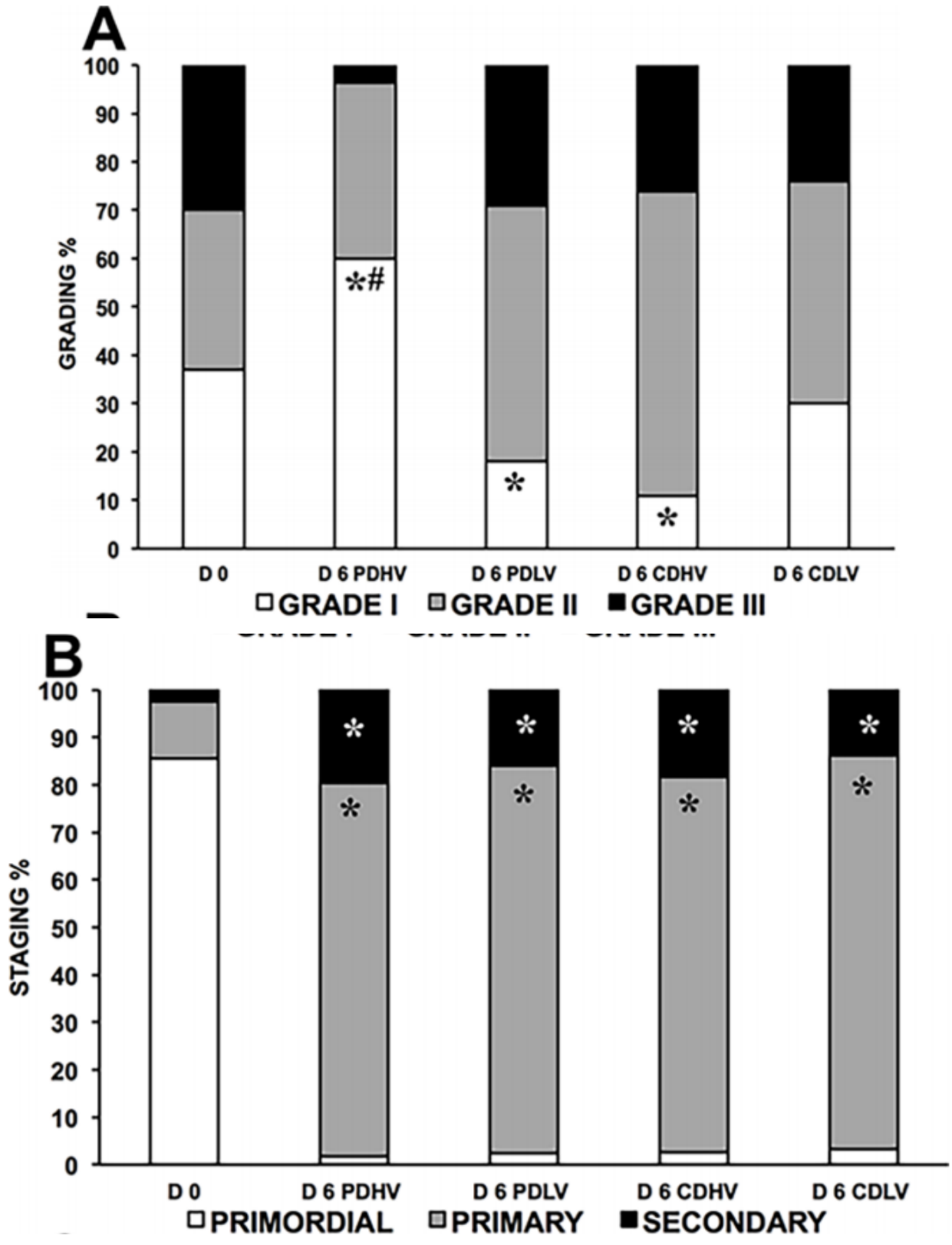


Fig 3. Viability of bovine follicles in strips cultured in air in PDHV vs CDHV. Representative confocal micrographs of a live (A,B,C) and a dead (D,E,F) follicle. (A,D) Hoechst 33342–stained nuclei; (B,E) live–dead far-red probe; (C,F) merge. (G) Follicle viability in fresh and cultured strips. Arrowheads indicate a dead oocyte. Bar = 20μm. $^{\#}P<0.01$ vs corresponding treatments.

Histological analysis (n = 1243 follicles: D0, 273; D6 PDHV, 213; D6 PDLV, 287; D6 CDHV, 199; D6 CDLV, 271) showed that culture in PDHV yielded a significantly higher percentage of grade 1 follicles than both D0 and the other D6 samples (Fig 4A, S2 Table). The higher grading in PDHV compared to the fresh control could be due to the re-absorption of atretic follicles during culture coupled with no further addition of atretic follicles. Grading under the four conditions reflects the model-predicted oxygen availability in tissue. In fact, culture in CDHV and PDLV at the predicted lowest and highest oxygen availability yielded the worst follicle quality. Culture in CDLV outperformed CDHV in terms of grade I follicles possibly showing a positive effect of higher $CO_{2,T}$ caused by reduced media height.

By D6, the majority of the primordial follicles was activated to the primary stage and a significant progression to the secondary stage was observed compared to D0 with no significant differences among groups (Fig 4B, S2 Table).

Viability (n = 971 follicles: D0, 237; D6 PDHV, 173; D6 PDLV, 187; D6 CDHV, 148; D6 CDLV, 226) was 89.5% at D0 and significantly dropped in all groups at D6 (Fig 4C, S2 Table). Viability at D6 in PDHV was significantly better than in PDLV and CDHV, whereas in CDLV it was only slightly lower than in PDHV.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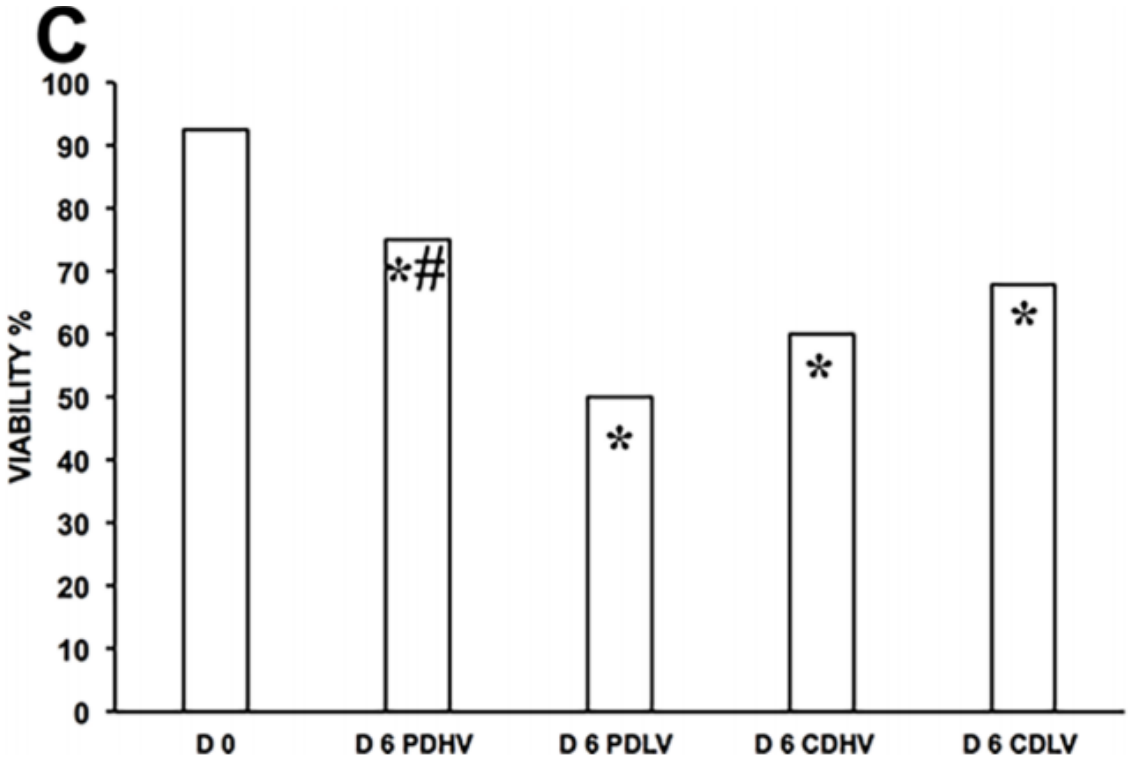
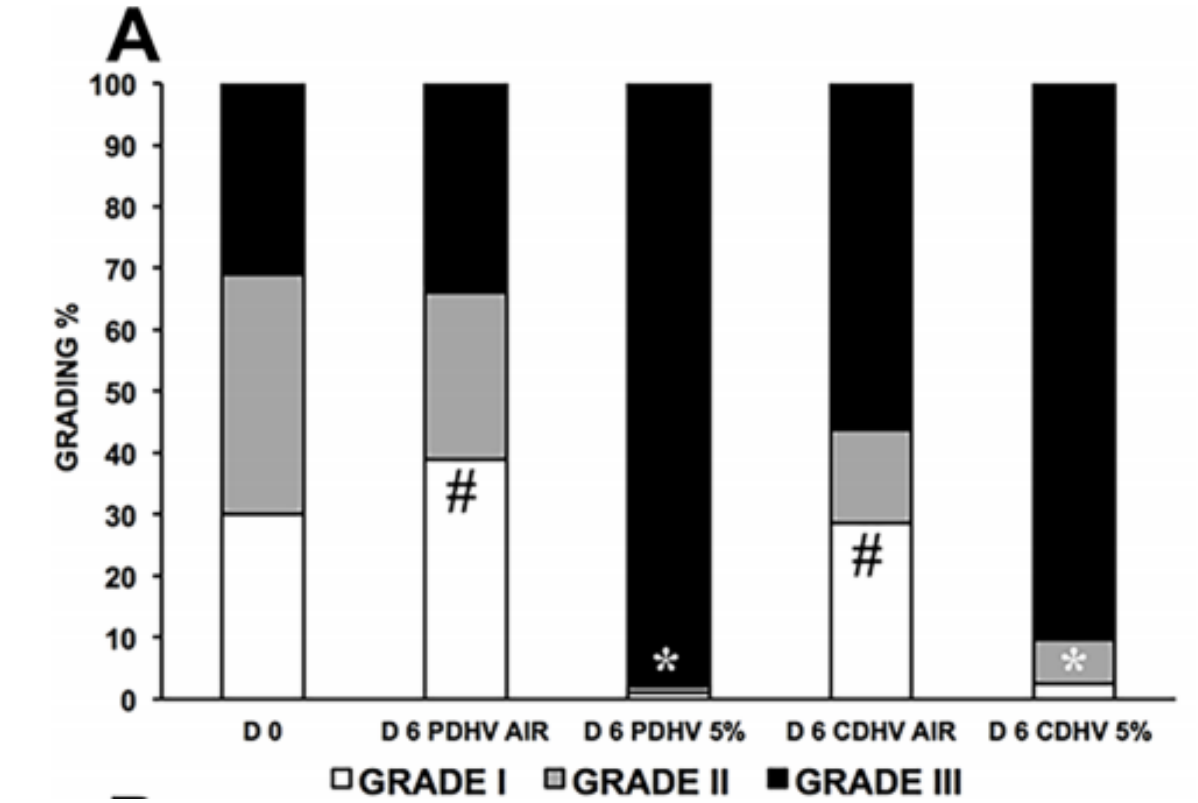


Fig 4. Quality of bovine follicles in strips cultured under various conditions in air. Histological grading (A), staging (B), and viability (C) of bovine follicles in strips cultured in PDHV, PDLV, CDHV and CDLV in air. *P<0.01 vs D0; #P<0.01 vs corresponding treatments.

Experiment III

Histological analysis (n = 978 follicles: D0, 222; D6 PDHV AIR, 187; D6PDHV 5%, 148; D6 CDHV AIR, 265; D6 CDHV 5%, 156) showed that follicle quality was best in PDHV in air. In agreement with the large anoxic strip fraction predicted by the model, culture in 5% O2 markedly and significantly reduced follicle quality in both CDHV and PDHV (Fig 5A, S3 Table). All conditions supported high follicle activation, but culture under 5% O2 impaired progression to the secondary stage (Fig 5B, S3 Table).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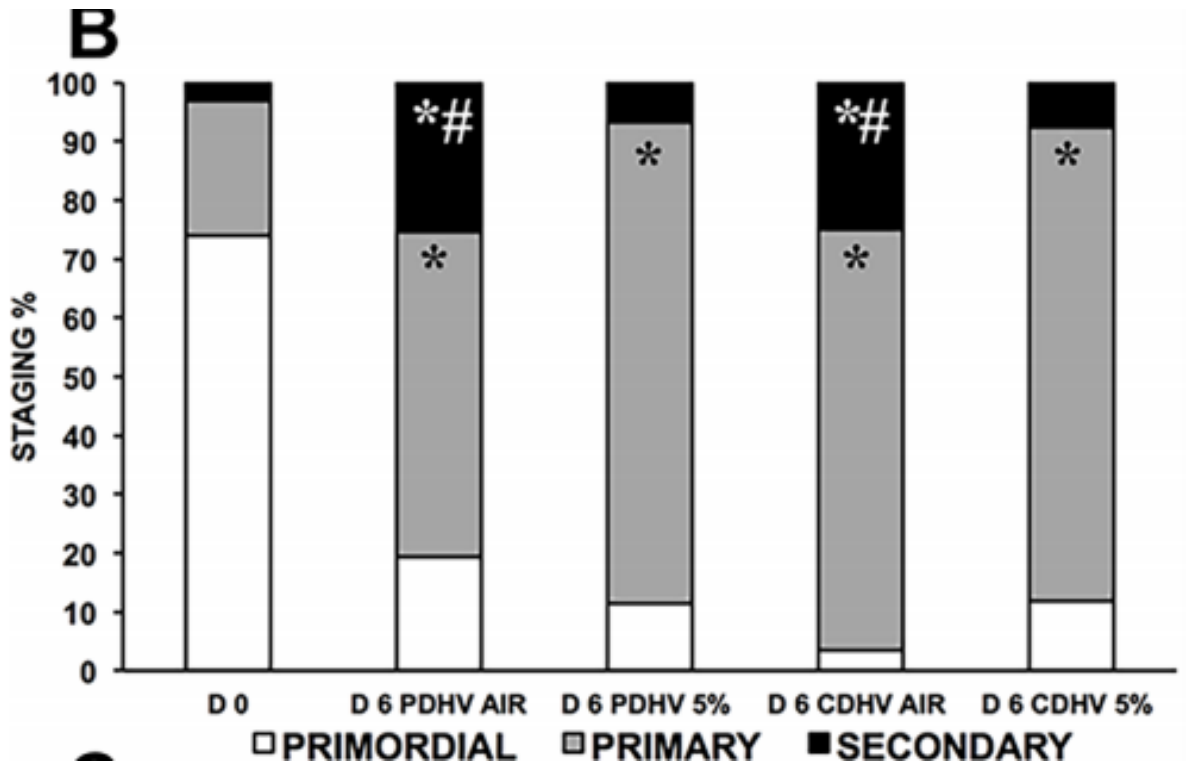
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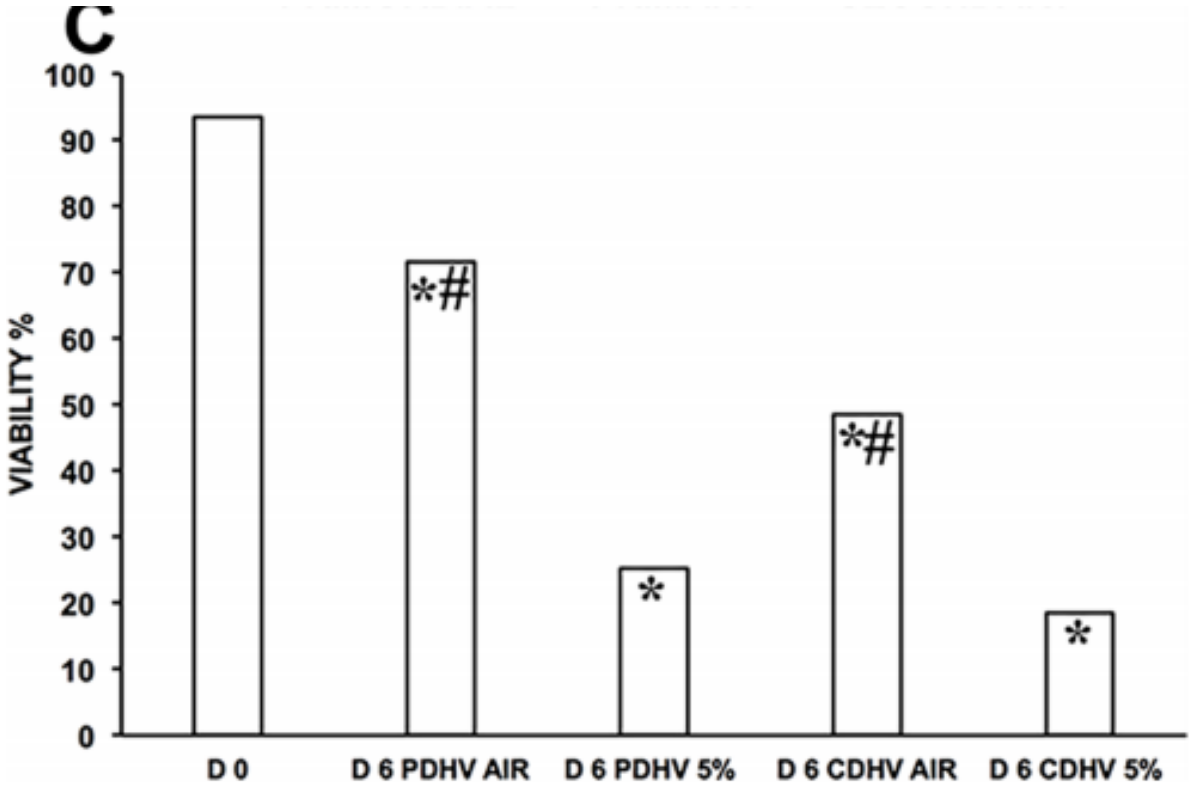
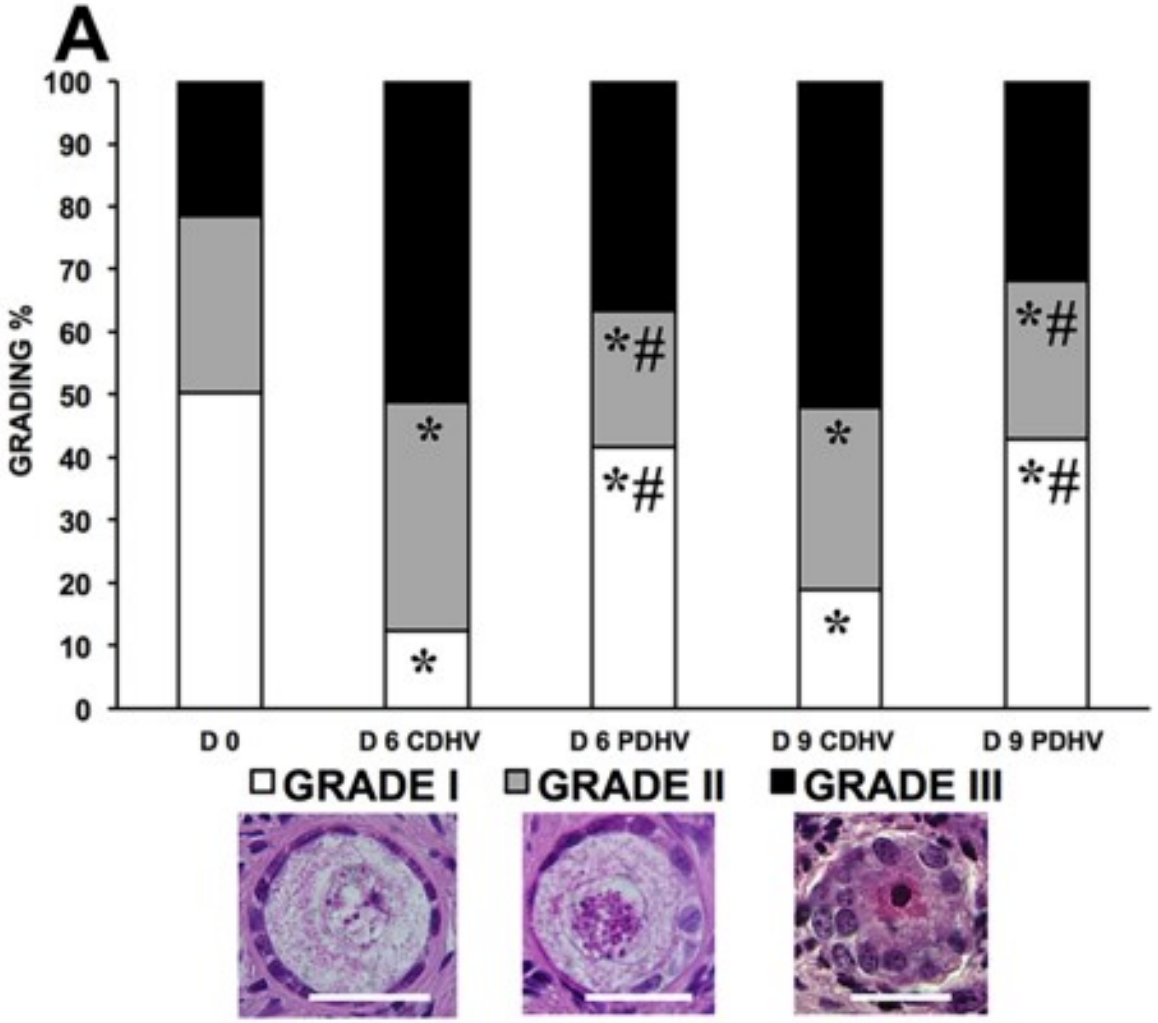


Fig 5. Quality of bovine follicles in strips cultured in air vs. 5%O2. Histological grading (A), staging (B), and viability (C) of bovine follicles in strips cultured in PDHV and CDHV in air versus 5% O2. *P<0.01 vs #P<0.01 vs corresponding treatments.

Viability analysis (n = 587 follicles: D0, 143; D6 PDHV AIR, 96; D6PDHV 5%, 109; D6 CDHV AIR, 121; D6 CDHV 5%, 118) indicates that PDHV in air provides the best culture conditions (Fig 5C, S3 Table).

Experiment IV

Histological analysis of HOSs (n = 1383 follicles: D0, 243; D6 CDHV, 327; D6 PDHV, 302; D9 CDHV, 206; D9 PDHV, 305) (Fig 6A, S4 Table) showed that follicle quality significantly dropped during culture as compared to D0 (Fig 6A). As seen in the bovine, culture in PDHV still provided the best conditions and yielded a greater proportion of grade 1 follicles than in CDHV at both D6 and 9 (Fig 6A). Culture under all conditions supported follicle activation but progression to the secondary stage was significantly higher in PDHV than in CDHV at both D6 and D9 (Fig 6B, S3 Table).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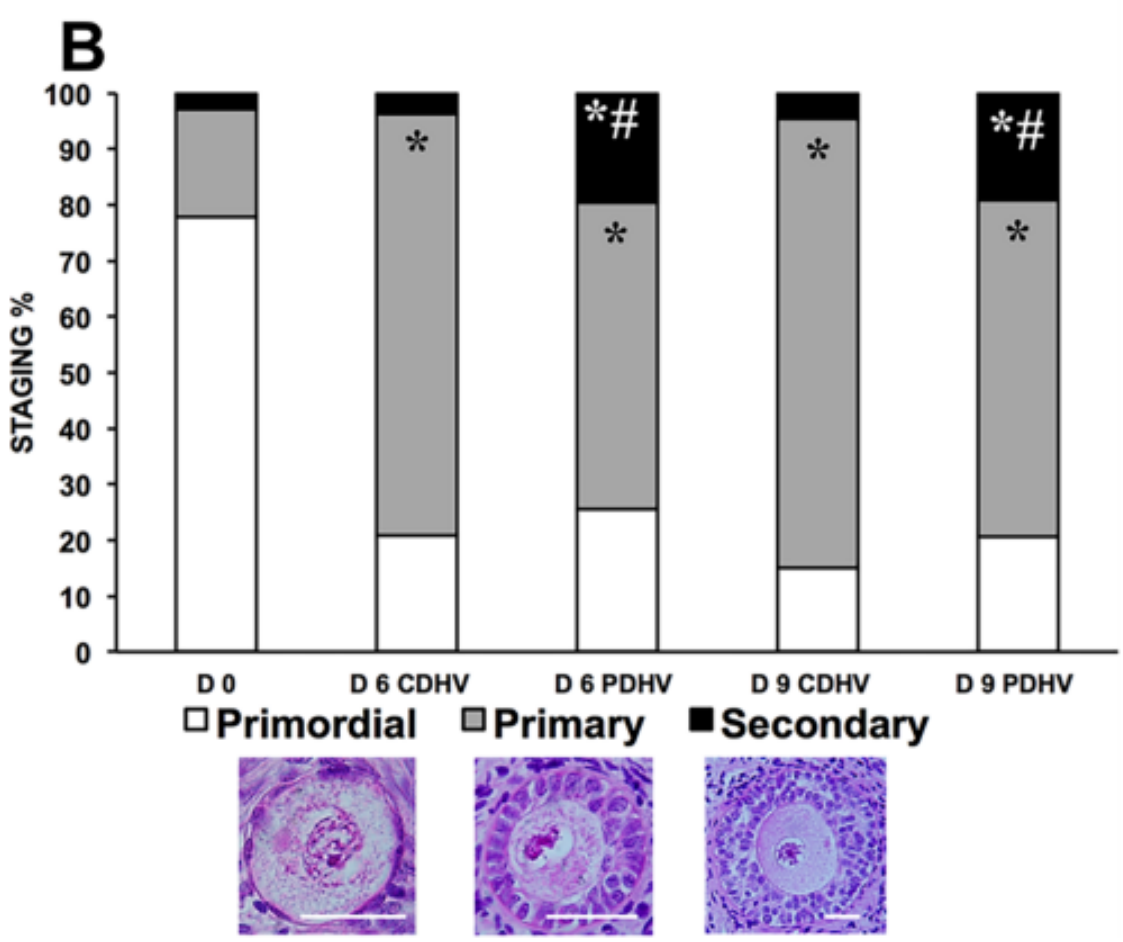

Fig 6. Grading and staging of human follicles in strips cultured in air in PDHV vs CDHV. Histological grading (A) and staging (B) of human follicles in strips cultured in PDHV and CDHV. Bar = 20μm. *P<0.01 vd D0; #P<0.01 vs corresponding treatments.

In agreement with the histology findings, viability (n = 745 follicles: D0, 133; D6 CDHV, 125; D6 PDHV, 231; D9 CDHV, 119; D9 PDHV, 137) decreased during culture as compared to D0 (Fig 7A±7F). Culture in PDHV provided the best conditions yielding a significantly higher viability than in CDHV both at D6 and D9 (Fig 7G, S4 Table).

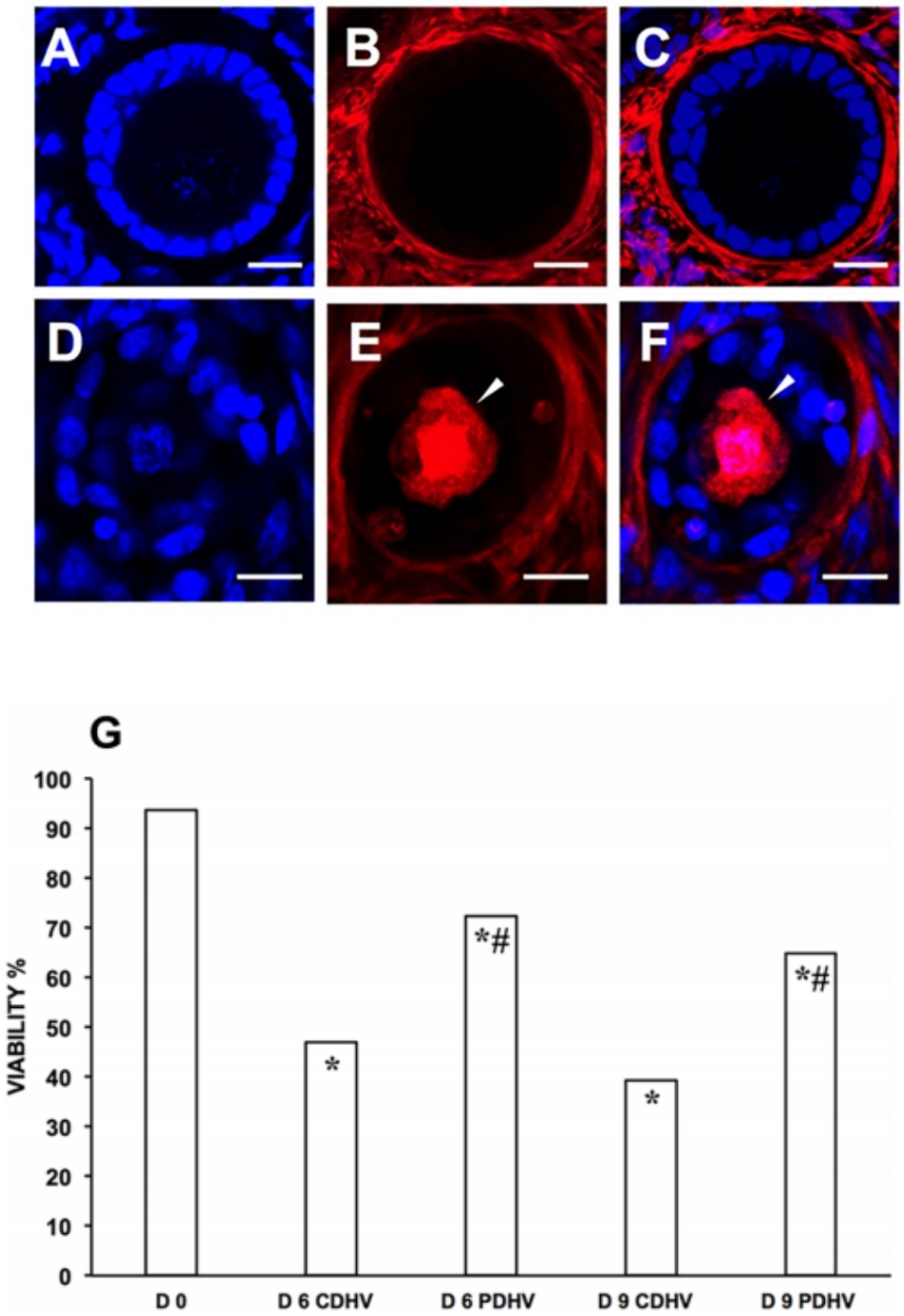

Fig 7. Viability of human follicles in strips cultured in PDHV vs CDHV. Representative confocal micrographs of a live (A,B,C) and a dead (D,E,F) follicle. (A,D) Hoechst 33342–stained nuclei; (B,E) live–dead far-red probe; (C,F) merge. (G) Follicle viability in fresh and cultured strips. Arrowheads indicate a dead oocyte. Bar = 15μm. *P<0.01 vs D0; #P<0.01 vs corresponding treatments.

Discussion

Human folliculogenesis in vivo is a complex process that stretches over a period of more than 290 days [32]. Although follicle growth in vitro is exceptionally accelerated [33], to achieve complete in vitro folliculogenesis it is necessary to develop culture strategies and systems that support long-term maintenance of tissue viability and follicle quality. The two-step culture strategy [12] is among the most promising techniques to realise complete in vitro folliculogenesis. Within this framework, the optimisation of cortical strip culture may play a pivotal role in the preservation of follicle health and in its activation and progression to the secondary stage. When ovarian tissue fragments are cultured in conventional culture dishes, it has been proposed that the poor availability of oxygen may hinder oocyte/follicle development and even cause tissue necrosis [23]. Few studies have investigated the importance of oxygen supply for follicular viability and growth but have not provided yet conclusive evidence [23,27]. In such studies, ovarian strips varying in geometry and thickness have been generally cultured at 100% vs 21% or 21% vs 5% pO2gas for various times. Tissue has also been cultured under uncontrolled heights of media differing for composition and supplements. Albeit simple, the model proposed herein suggests that, in addition to pO2gas, the thickness of the ovarian strip, the metabolic rate at which oxygen is consumed (which changes with the tissue source and the culture medium), and the height of medium above the strip, all affect the resistance to oxygen transport from the gas phase, oxygen concentration in the strip, and ultimately oxygen availability to ovarian cells [34]. This suggests that the findings from the reported studies might be contradictory for the multitude of factors that were varied by different investigators and that could affect the actual oxygen availability to ovarian cells. To the best of our knowledge, this is the first time that the importance of oxygen for cortical ovarian tissue is investigated in culture experiments by systematically varying oxygen availability to ovarian cells. The oxygen transport model was instrumental to seeking those conditions that would permit ovarian cells culture in the strip at varying oxygen availability. Our experiments investigated follicle quality, progression and viability in

BOSs and HOSs cultured at varying oxygen availability by using CD or PD, or by changing medium height or gaseous oxygen tension. The overall results indicate that an adequate oxygen supply is required to maintain follicle quality and viability and to promote follicle progression both in bovine and human tissue. The significantly higher proportion of healthy and viable follicles in BOSs cultured in PDHV vs CDHV suggests that culture in CDHV under the conditions used established very low $CO_{2,T}$`s that compromise follicle health. Culture in PDHV yielded better oxygen availability and preserved follicle quality and viability. Culture under low medium height was used to increase $CO_{2,T}$ in BOSs and mimic the thin medium layer frequently used for strip culture on tissue inserts [34]. Culture in PDHV still offered the best conditions. Culture in CDHV and PDLV, that were predicted to establish the lowest and highest $CO_{2,T}$ respectively, impaired follicle health suggesting that bovine follicles require $CO_{2,T}$ within an optimal range for long-term in vitro culture. Culture in PDs was expected to enhance oxygen supply by providing oxygen also through the tissue bottom (high $CO_{2,TBS}$), but would also enhance removal of metabolic CO_2. When BOSs were cultured in PDHV and CDHV in air vs. 5% O_2, culture under reduced oxygen tension appeared to exert a dominant detrimental effect on follicle quality, viability and progression. This suggests that enhancing CO_2 removal had less relevant effects on follicle health than enhancing the oxygen availability. This is in agreement with the increased growth, estradiol secretion, and decreased lactate production reported when oxygen availability to mouse preantral follicles was enhanced by culturing them at the medium/air interphase in inverted multiwells plates rather than in immersed upright condition [35]. Our findings are in disagreement with those of Jorssen et al. [24] that reported similar rates of follicle survival and progression in BOS cultured in 5% and 20% oxygen. Such a discrepancy is possibly due to the thinner strips used (0.2 vs 0.5mm in the present study) that may have not limited oxygen diffusion. It should be noted that the goodness of the model predictions is limited by the model assumptions and by the fact that model parameters had to be estimated from literature information for similar tissue under similar conditions to those used in this work for the lack of data.

For the sake of the example, DT was assumed equal to that reported for ocular stroma [36] because it was thought that it could better account for the heterogeneous nature of the ovarian cortex. The value of Gº` was extrapolated to 6 days of culture from the values reported in the only paper in which the oxygen consumption rate of ovarian cortical strips has been characterized [37]. The best and worst culture conditions identified for bovine tissue (i.e. PDHV and CDHV) were chosen to culture HOSs from six patients. A large number of follicles was analysed for histology and viability. Also for HOSs, culture in PDHV yielded better follicle quality and viability. Different from BOSs, culture in PDHV yielded also a better follicle progression in HOSs. The different culture outcome in the two species could possibly be due to variations in tissue anatomy, metabolism and resistance to oxygen diffusion, and underlies that, in spite of the fact that bovine tissue is considered a reliable model for human in vitro folliculogenesis [38], it may behave differently from human tissue at least in some respects. Several studies have reported that supplementation of factors like FBS, FSH or activin was not particularly effective in augmenting the growth of primary follicles to the secondary stages [39,42]. It is worth mentioning that in PDHV we obtained one of the highest secondary follicles yield reported in literature by using basal serum-free medium without FSH or activin [43,46]. This suggests that adequate oxygen availability is a key factor to support follicle progression in HOSs culture.

Follicular oxygen requirement is a highly dynamic process in which follicle oxygen consumption increases as follicles progress from the primordial to the primary and secondary stage, outlining the role of both tricarboxylic acid cycle and oxidative phosphorylation in primordial follicle activation and progression [47]. Our results show that all culture conditions promoted follicle activation independent of the predicted oxygen concentration at the strip surfaces or interior, whereas progression to the secondary stage depended more strongly on oxygen availability. This agrees well with the increasingly higher oxygen depletion in tissue caused by the increasing oxygen consumption rates of human follicles as they progress from primordial to primary (2.5-fold) and from primary to the secondary stage (further 3.8-fold) reported by Ishikawa et

al. [47]. In conclusion, culture of ovarian strips using gas-permeable dishes under optimal medium height enabled to maintain follicles health and promote their development. We believe that such an approach holds promise to increase the efficiency of a two-step culture strategy by improving quality and yield of secondary follicles for further isolated culture to achieve complete in vitro folliculogenesis.

Supporting information

S1 Appendix. Supplementary information on the oxygen transport model in conventional dishes. Details on how the concentration profile in ovarian tissue was obtained when ovarian tissue is cultured in conventional dishes. (DOCX)

S2 Appendix. Supplementary information on the oxygen transport imodel in gas-permeable dishes. Details on how the concentration profile in ovarian tissue was obtained when ovarian tissue is cultured in gas-permeable dishes. (DOCX)

S1 Table. Experiment I. Percentages of bovine follicle grading, staging and viability. H = Histology; V = Viability. Number of follicles analysed are indicated in brackets. (DOCX)

S2 Table. Experiment II. Percentages of bovine follicle grading, staging and viability. H = Histology; V = Viability. Number of follicles analysed are indicated in brackets. (DOCX)

S3 Table. Experiment III. Percentages of bovine follicle grading, staging and viability. H = Histology; V = Viability. Number of follicles analysed are indicated in brackets. (DOCX)

S4 Table. Experiment IV. Percentages of human follicle grading, staging and viability. H = Histology; V = Viability. Number of follicles analysed are indicated in brackets. (DOCX)

Acknowledgments

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Chapter 3

Title:

"Dynamic Culture oh human ovarian cortical tissue enhances follicle health, growth and steroids secretion"

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Introduction

The possibility to grow in vitro competent oocytes starting from the huge reserve of primordial follicles in the ovary has always attracted reproductive scientists. In mice, production of mature oocytes from primordial follicles through a two step culture method, subsequent in vitro fertilization and birth of pups has been reported in 1996 and improved in 2003 [3-4]. Nevertheless, translation of these findings in higher animals and humans has not yet been fully achieved due to the differences in the structure and physiology of the ovary. In humans, Telfer et al (2008) reported the activation of primordial follicles and their progression to the secondary stage through a two step culture method in which primordial follicles were first cultured in the tissue to activate and promote their growth to the secondary stage, followed by the culture of isolated secondary follicles. More recently, the same group was able to develop human oocytes from primordial follicles to resumption of meiosis using a four step in vitro culture technique [8]. However, the number of oocytes produced was extremely low and they were clearly abnormal. This could be due to inadequate culture conditions that limit the number and health of secondary follicles produced during the first step of culture. In fact, until now, the progression to secondary stage rarely exceeds 10% of the total population. We demonstrated that an adequate oxygen availability during culture of human ovarian tissue is needed to maintain follicle health and promote the growth of secondary follicles at rates largely exceeding those reported in literature so far [9-10]. As static culture conditions are likely to not properly mimic the in vivo physiology of ovarian tissue, a dynamic culture system enhancing the exchange of nutrients and metabolic products and the removal of waste products, could more efficiently support follicle health and growth. The aim of this paper was to study the effects of static versus dynamic culture of bovine and human ovarian tissue on follicle health and growth. To this end, follicle health and stages were evaluated through histology and steroid secretion in spent media, follicle viability through confocal microscopy after static culture in gas permeable dishes (PD) versus dynamic culture in perfusion bioreactor (PB).

Materials and methods

Chemicals and consumables

Lumox gas-permeable culture dishes 50mm in diameter were from Sarstedt (NuÈmbrecht, Germany). Leibovitz's L-15 medium, α-MEM Glutamax medium (code number 32571), Insulin transferrin selenium (ITS) 100x, Live/dead Fixable far red stain were purchased from Invitrogen (Milan, Italy). Penicillin streptomycin 100x, Amphotericin B 250µg/ml, Bovine serum albumin (BSA), L-Ascorbic acid, L-Glutamine 200mM, Hoechst 33342, Fructose, α-thioglycerol and Eosin-Y were purchased from Sigma Aldrich (Milan, Italy). Mayers's hematoxylin and paraffin wax were from Carlo Erba (Milan, Italy). Estradiol and Progesterone Enzyme Immunoassay kits were from DRG (Germany).

Collection and preparation of ovarian tissue

Bovine ovaries were collected at the time of animals' evisceration from the Slaughterhouse Straccione (San Marcellino, Caserta, Italy; CEE accreditation number 1403/M) and transported within 2h to the lab in Leibovitz's L-15, 1% penicillin-streptomycin (Pen-Strep), 1µg/ml Amphotericin-B, at 4°C.

After obtaining written informed consent, human ovarian biopsies were collected from five women (age = 24.8 ± 6.4 years) during laparoscopic surgery for benign gynaecologic conditions and transported to the lab as above. Approval of human tissue use was signed by the Ethics Committee of Regione Campania (ASL NA1 Centro, Naples, Italy; reference number 57 CE 2±2017).

Cortical slices (~ 0.5mm thick) were manually dissected from bovine ovaries and human ovarian biopsies in handling medium (Leibovitz's L-15, 2mM glutamine, 3mg/ml BSA, 1% Pen-Strep, 1µg/ml amphotericin B) at room temperature (RT), avoiding areas with visible antral follicles. Cortical slices were homogeneously sliced into 1mm x 1mmx 0.5mm strips through a tissue chopper (Mcilwain, Mickle Laboratory Engineering Company, Ltd, Surrey, UK). Strips were then washed twice in fresh handling medium, and 10 strips were randomly distributed into PD or PB for

culture. Fresh control strips from each sample were processed for histology and viability assessment as a control.

Culture of ovarian tissue in gas-permeable dishes and perfusion bioreactor

In each experiment, strips from the same ovary were cultured in PD or in PB in α-MEM, 3mM glutamine, 0.1% BSA, 1% Pen-Strep, 1% ITS (10 µg/ml Insulin, 5.5 µg/ml Transferrin, 6.7 ng/ml Selenium), 1µg/ml amphotericin-B, 50µg/ml ascorbic acid at 37°C, 5% CO2 and 95% humidity in air for 6 days. Half medium was changed every 48h. At the end of culture, 5 strips from each dish were treated for histology and 5 strips for viability assessments.

Histology

For histological analysis, cortical strips were fixed in Bouin's solution, dehydrated in increasing ethanol concentrations, embedded in paraffin and 5µm serial sections were stained with hematoxylin and eosin. Grading and staging of follicles was evaluated by two blinded expert observers: only follicles in which the germinal vesicle was well visible were classified. Follicle quality was graded as previously described [16]. Briefly: grade 1 follicles were spherical with homogeneously distributed granulosa cells (GCs) and oocyte presenting a homogenous cytoplasm and slightly granular nucleus, with well visible spherical condensed chromatin; grade 2 follicles had GCs non-uniformly distributed around the spherical oocyte; grade 3 follicles with pyknotic GCs and distorted and/or vacuolized oocyte. Follicle staging was scored according to Gougeon's criteria [17], as follows: 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.

Viability assessment

Strips were incubated, under shaking, for 3 hours at 4°C, in Dulbecco's PBS with 1µg/ml Live/Dead Fixable Far Red Stain and 10µg/ml Hoechst 33342, fixed in 4% paraformaldehyde in PBS for 2 hours at RT, washed in fresh PBS and incubated at 4°C

overnight in PBS plus 10µg/ml Hoechst 33342 [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 then optically cleared using See DB clearing protocol [19]. Briefly, samples were serially incubated in 5 mL of 20%, 40%, and 60% (wt/vol) fructose, each for 6 hours, 80%, 100% and 115% fructose (wt/vol) each for 12 hours, with gentle agitation at RT. All fructose solutions were supplemented with 0.5%. α-thioglycerol. To avoid compression, strips were mounted in 115% fructose on a glass slide with 3 spacer coverslips (0.17mm) placed on each side and covered with a coverslip. Analysis was carried out with a Leica TCS SP5 confocal scanning laser microscope (Leica Microsystems, Wetzlar, Germany) using a 405-nm diode laser for visualizing the nuclear label (Hoechst 33342) and a 633-nm helium neon laser for the live/dead probe. Each strip was traversed using the z-position control and fields to a depth of 300µm from the tissue surface were imaged using a 63x glycerol immersion objective.

Steroid secretion

Spent media (500µl) were collected at the end of the culture period (day 6) and stored at −80°C for subsequent 17 beta-estradiol (E_2) and progesterone (P_4) quantification using ELISA Kits (Salimetrics, DBA Italia, Milan, Italy) according to the manufacturer's instructions. Absorbance was calculated at 450 nm. The analytical sensitivity of the assay was 0.1 pg/mL (assay range, 1–32 pg/mL) for E_2 and 0.045 ng/mL (assay range, 0–40 ng/mL) for P_4. Steroid levels were expressed as E_2 and P_4 secreted by ten 1x1x0.5mm ovarian cortical strips cultured in PD and PB.

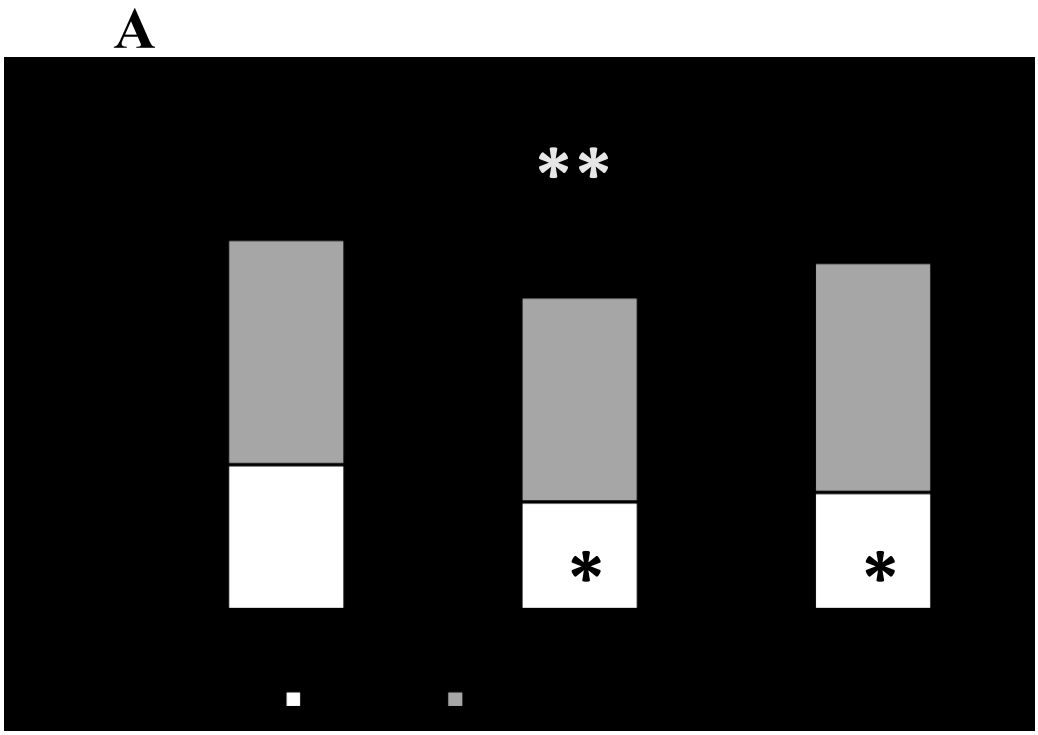
Statistical analysis

For each experiment, data is presented as cumulative percentages. Overall, statistical analysis was performed by Fisher's exact test for pairwise comparisons when overall significance was detected.

Results

Bovine cortical strip culture

Histological analysis (n = 1064 follicles: day 0, 535; day 6 PD, 229; day 6 PB, 300) showed that compared to day 0, strips cultured on PD and PB had a slight and significant reduction of grade I follicles. However, a marked and significant increase of grade III follicles was observed in PD compared to day 0 and PB (Fig 1A). At day 6, both groups showed a marked and significant decrease of primordial follicles and a concomitant significant increase of primary and secondary follicles, compared to day 0 (Fig 1B). Interestingly, the percentage of secondary follicles in PB was about thrice than in PD. Follicle viability was not affected by culture (n = 619 follicles: day 0, 254; day 6 PD, 132; day 6 PB, 233) (Fig 2). Overall, data demonstrate that although follicle viability is maintained under both culture conditions, follicular progression to the secondary stage is markedly enhanced by culture in PB.



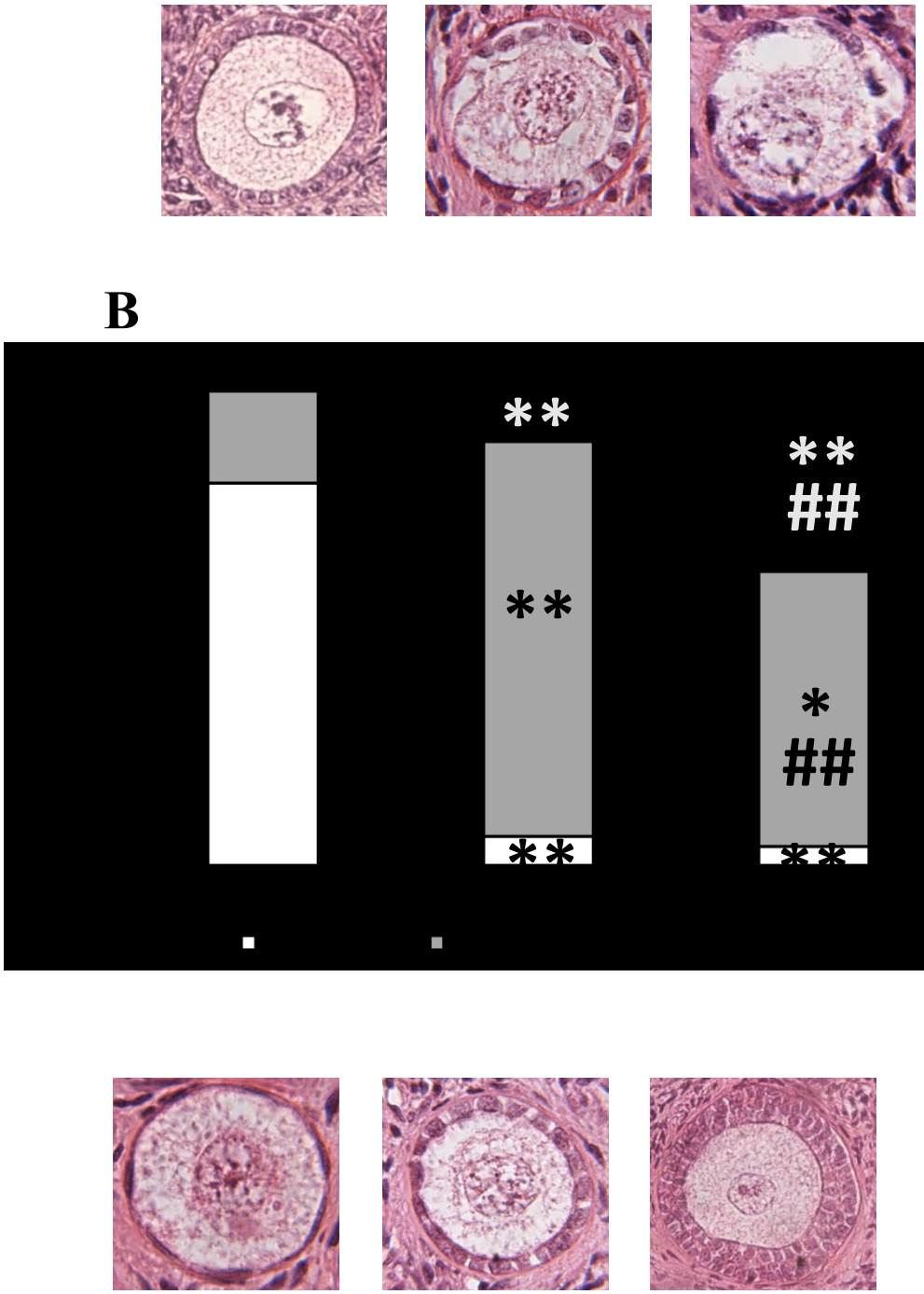


Figure 1. Grading and staging of bovine follicle in strips cultured for 6days in PD and PB. Histological grading (A) and staging (B) of bovine follicles in strips cultured in PD and PB. * $p<0.05$ and ** $p<0.01$ vs. DAY 0 | ## $p<0.01$ vs. DAY 6 PD

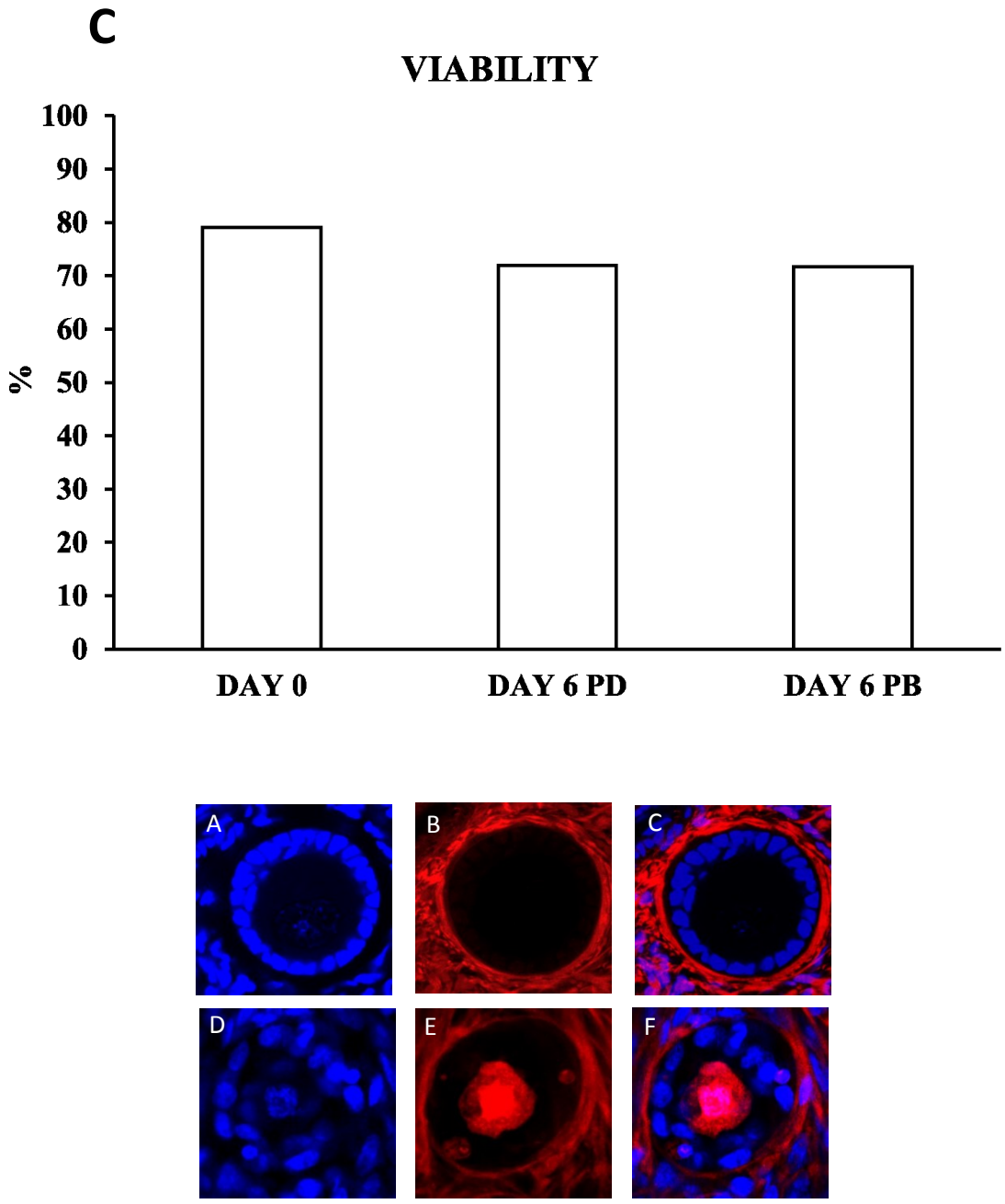
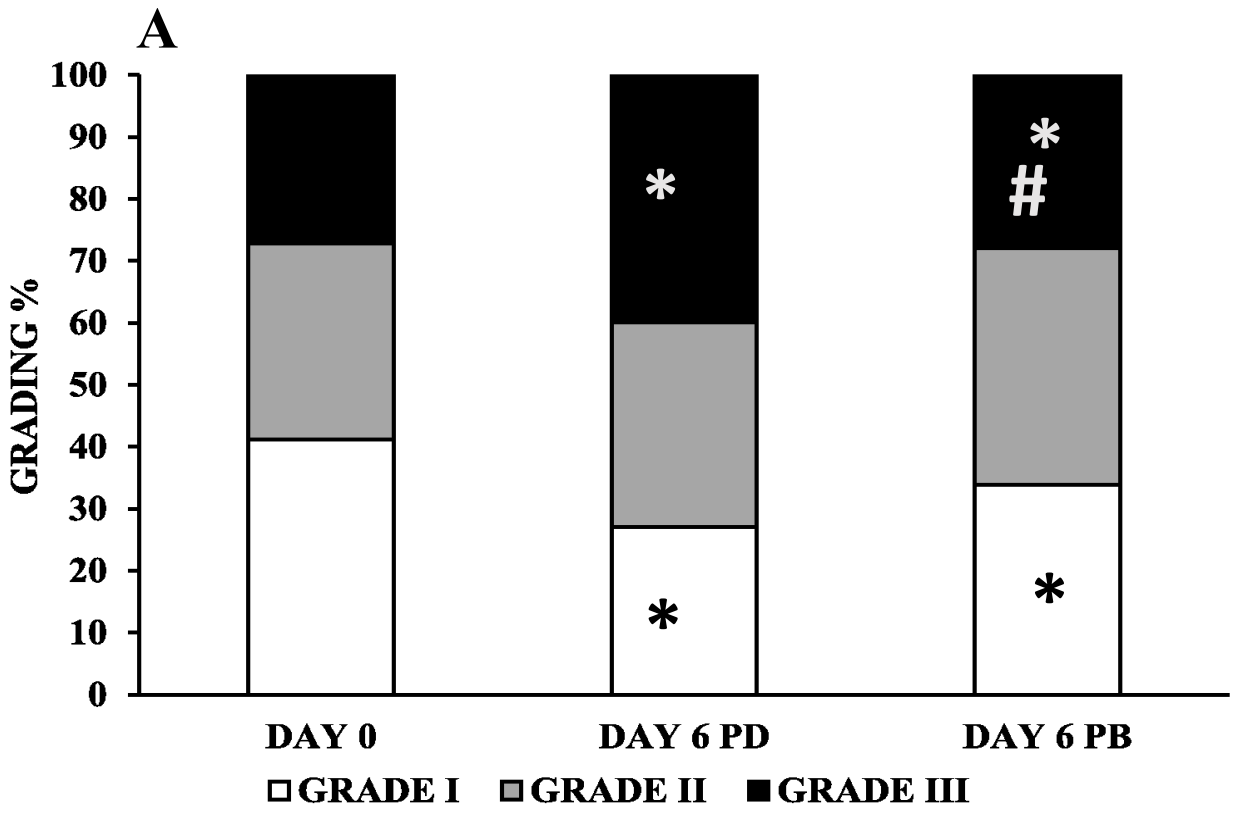


Figure 2. Viability of bovine follicles in strips cultured in PD vs PB. Representative confocal micrographs of a live (A,B,C) and a dead (D,E,F) follicle. (A,D) Hoechst 33342–stained nuclei; (B,E) live–dead far-red probe; (C,F) merge. (C) Follicle viability in fresh and cultured strips.

Human cortical strip culture

To evaluate whether the PB culture could provide significant improvements in follicle health and growth in the human, cortical strips from five consenting patients were cultured and analysed as described in the materials and methods section. Histological analysis (n = 1696 follicles: day 0, 1052; day 6 PD, 343; day 6 PB, 301) showed that culture in PD led to a significant decrease of grade I follicles and a corresponding increase of grade III follicles compared to both fresh and PB cultured tissue. Culture in PB yielded a slight reduction of grade I and an increase of grade II follicles compared to fresh tissue (Fig 3A). Analysis of follicle stages at day 6 showed a significant decrease of primordial follicles and a concomitant increase of primary and secondary follicles in cultured samples. Interestingly, the percentage of resting follicles in PB cultured tissues was significantly lower and the percentage of secondary follicles 1.5 folds higher than in PD cultured tissue (Fig. 3B). Compared to fresh tissue, a slight and similar reduction of follicle viability occurred under both culture conditions (Fig. 4) (n = 1176 follicles: day 0, 412; day 6 PD, 399; day 6 PB, 365).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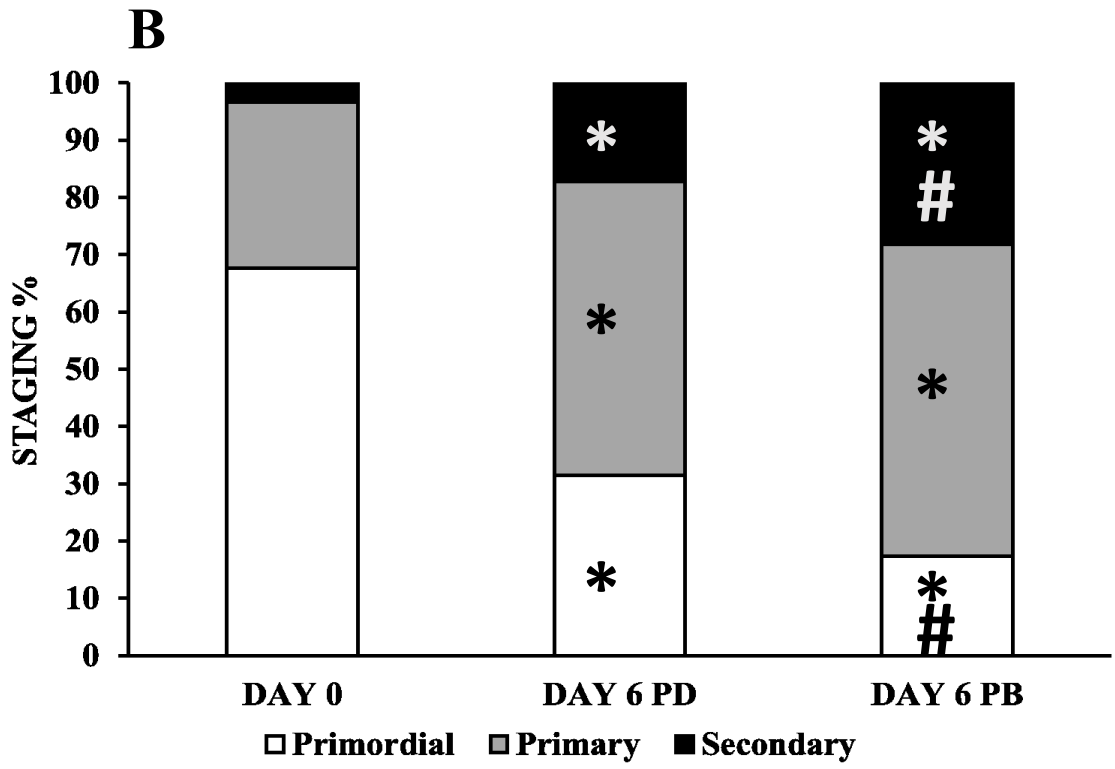
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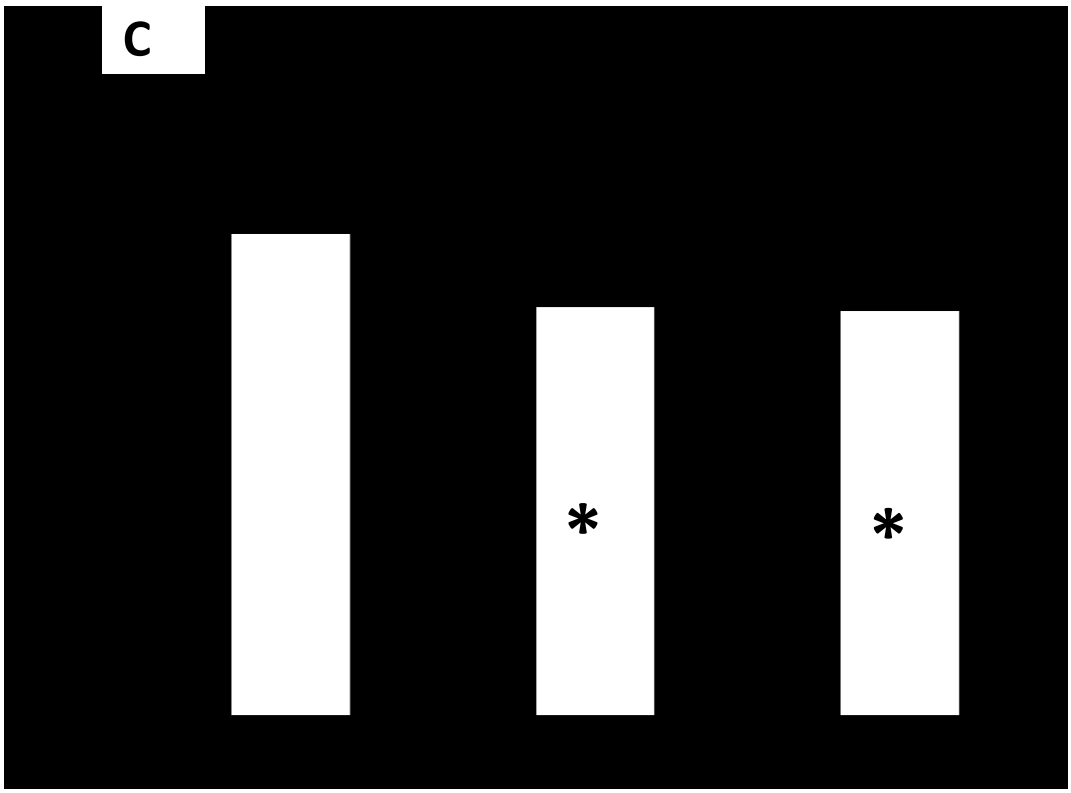

Figure 3. Grading, staging and viability of human follicles in strips cultured in air in PD vs PB. Histological grading (A) staging (B) and viability (C) of human follicles in strips cultured in PD and PB. * $p<0.05$ vs. DAY 0. # $p<0.01$ vs. DAY 6 PD

Steroid secretion

Steroid secretion in spent media was assessed to evaluate growth and physiology of follicles under the two culture conditions herein tested. To this end, conditioned media were collected from PD and PB cultures at day 6 of each experiment and stored at -80°C. At thawing, aliquots from each experiment were pooled for analysis. In both bovine and human ovarian strip culture, E2 levels were markedly higher in the PB than in PD condition whereas P4 levels were remarkably higher in PD than in PB (Table I). As E2 concentration is directly proportional to the number and health of granulosa cells, and consequently to the different stages of follicle growth (32) whereas a high concentration of P4 is considered an indicator of a premature luteinization (33), E2/P4

ratio was calculated (Table I). Ratio values were about 50 and 25 times higher in PB versus the PD culture condition in bovine and human respectively.

A

BOVINE	Day 6 Pool PD	Day 6 Pool PB
Estradiol (pg)	**0,7**	**4,6**
Progesterone (ng)	**20,8**	**2,8**
Ratio E2/P4	**0,00003**	**0,0016**

B

HUMAN	Day 6 Pool PD	Day 6 Pool PB
Estradiol (pg)	**2,5**	**4,2**
Progesterone (ng)	**16,75**	**1,1**
Ratio E2/P4	**0,00015**	**0,0038**

Discussion

Successful in vitro follicle growth combined to ovarian tissue cryopreservation, represents a promising alternative method for fertility preservation. Moreover, it could provide an in vitro model to better understand the complicate mechanisms that regulate the process of folliculogenesis in vivo and for drug testing as well (20). The most recent achievements in this field were reported by McLaughlin et al. (2018), who successfully obtained mature metaphase II human oocytes starting from primordial follicles through a multi-step culture strategy (8). However, only a very limited number of mature oocytes were obtained and their morphological features were not optimal. Therefore, further optimization of culture conditions is surely required, and functional characterization of these oocytes is needed. One of the main factors affecting the low efficiency of the multistep culture strategy may be represented by the low number and quality of secondary follicles grown during the first step of in situ culture. Although numerous efforts have been done to implement the culture conditions of human ovarian cortical strips, currently follicle progression to the secondary stage is limited around 10% (9-10). We recently demonstrated that modulation of oxygen availability in ovarian cortical tissue represents a key factor in maintaining follicles' health and viability and in the promotion of growth to the secondary stage during in situ tissue culture (11). In particular, static culture of human ovarian strips in gas permeable dishes under an optimal medium column height increased the yield of healthy secondary follicles about 2-fold compared to literature data (9-11). These encouraging results provide proof of concept that the physical microenvironment in which follicles are cultured may be crucial for growth activation and progression. In this regard, a dynamic culture system providing a continuous flow of culture medium that guarantees a constant exchange of nutrients and metabolic products could better mimic physiological processes involved in the follicle activation and growth (13-15). In the present paper we investigated the efficiency of a prototype dynamic perfusion bioreactor versus static culture in gas permeable dishes in supporting the early stages of folliculogenesis in the

bovine and human through evaluation of follicle quality, progression, viability and E2 and P4 levels in conditioned media.

A series of preliminary experiments carried out on bovine ovarian cortical tissue allowed to set up the optimal bioreactor operating conditions (unpublished data) that were herein applied for culture of bovine and human ovarian cortical tissue. Overall, data demonstrated that culture in PB improved follicle quality, progression to the secondary stage, E2 secretion and E2/P4 ratio whereas a high and similar follicle viability was found under the two culture conditions.

In the last years, several papers studied the ability of bioreactors to support organ culture and model systems for lungs, livers, and kidneys of large animals have been successfully developed showing highly beneficial effects on cell physiology, viability and differentiation (23–27). Dynamic bioreactors provide a number of advantages over static culture such as the continuous and adjustable flow of culture medium into and around tissue permitting an adequate nutrition supply for proliferation, and the precise monitoring of pH, temperature, flow rate, mechanical forces, and biochemical gradients, creating an adequate environment for in vitro cell/tissue growth.

Application of bioreactor for ovarian tissue culture has been poorly investigated but some encouraging results of using a dynamic system for early folliculogenesis were reported. Different experimental approaches were applied: agitation of medium during the culture period of cortical strips, use of microdiaphragm pump for fluidic circulation of the medium around the cultured tissue, and a novel in vitro culture system with a peristaltic pump that provides a continuous flow of culture medium (12-14). More recently a custom bioreactor was developed for the culture of bovine ovaries to investigate the effects of doxorubicin on follicle apoptosis (15). Although such studies were carried out on ovaries from different species and under various operating conditions, beneficial effects were demonstrated on follicle viability, growth and steroid secretion.

Our data agree with those reported until now, confirming that dynamic culture could better mimic the environment in which follicles grow in vivo. In addition, it is

considerable that ovarian strips cultured in PB in only basal serum-free medium harboured at day 6 the highest secondary follicles yield reported in literature. However, the viability, as observed through confocal analysis decreased in both groups compared to day 0. The use of the serum-free medium, without supplementation with FSH and/or growth factors, could explain this data. It is well established the beneficial role of FSH supplementation in culture medium on preservation of viability and promoting of development and antrum formation of isolated preantral follicles (28-31), but the impact of these factors in culture of ovarian strips is still controversial. Therefore, further experiments will be aimed to investigate the effect of supplementation of FSH as well as other hormones and growth factors on follicle viability during in situ culture.

Determination of steroid hormones levels in conditioned media is another important aspect, in order to better understand the efficacy of follicle development. It is well known that production of E2 is closely related to follicles' maturity, particularly E2 concentration is directly proportional to the number and health of granulosa cells, and consequently to the different stages of follicles (32). Our results demonstrated that E2 levels are enhanced at the end of culture period in all culture conditions, both in human and in bovine. Interestingly, culture in PB promotes an increased production of E2, compared to correspondent culture in PD. This is in according with Liebenthron et al. (13) that firstly investigate the positive impact of dynamic culture conditions on follicle development in human cortical biopsies.

Instead, it is still under investigation the correlation between production of P4 and follicle development, as a massive increase in P4 production is associated to the late gonadotropin-dependent antral follicle development. It looks likely that a high concentration of P4 in early folliculogenesis could indicate a premature luteinization, caused by oxidative stress (33). It has been reported that static culture could lead to accumulation of toxic factors in culture media, responsible of this process (13). In support of these findings, our results show that P4 levels are significantly increased after culture in PD, compared to PB, both in human and in bovine. Therefore, ratio E2/P4 is higher in PB, demonstrating again that dynamic culture better supports early

follicle development. Differences between our revealed concentration of steroid hormones and the reported values in literature could be attributed to the different experimental parameters, such as number and size of cortical fragments, volume and any exchange of culture media, heterogeneity of ovarian tissue and thus number of follicles analysed.

In conclusion, use of dynamic perfusion bioreactor for culture of ovarian cortical strips significantly promotes follicle progression to secondary stage, maintaining their health and functionality in terms of E2/P4 production. Taken together, this data suggest that such an approach could open new interesting and promising prospectives in order to increase the efficacy of multi-step culture strategy. This could lead to an implementation of both quality and percentage of secondary follicles at the end of first step of culture, and then the possibility to further culture of isolated follicle for a complete in vitro folliculogenesis.

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DISCUSSION

The common thread of research work developed during my Ph.D. has been the enhancement of the cryopreservation and culture condition of the cortical ovarian tissue finalized to fertility preservation and in vitro folliculogenesis. My first purpose has been to evaluate a cryopreservation protocol that could increase the responsiveness of follicles at warming. The data obtained by Talevi et al. (18) had clearly demonstrated that at warming the damages caused by LN cryopreservation of ovarian tissue can be significantly reduced using a protocol that avoids the Leidenfrost effect caused by the LN. However, less was known about the ability of the SN cryopreserved tissue to fully recover the follicle competence to grow and progress after warming and in vitro culture.

To this end we have analyzed the gene expression, the DNA damage and the follicle progression of V/W tissue in long-term in vitro culture to provide more detailed information on the health of the ovarian strips.

Data showed that after 9 days of in vitro culture, the quality, progression and viability of the follicles was better in SN than in LN V/W tissue confirming what already reported in the earlier study (18). Interestingly, in this study, human ovarian tissue subjected to SN vitrification and warming yielded a progression to the secondary follicle stage almost twice compared to 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. More detailed studies should be undertaken to explain such a marked difference between V/W and fresh tissue. Currently we can only speculate that vitrification positively modifies the ovarian microenvironment that drives follicle growth. SN vitrification improves human ovarian tissue preservation and could provide 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.

Another aspect treated in my Ph.D work has been the study of the culture conditions that could allow a complete in vitro folliculogenesis to avoid the risk of reintroducing malignant cells during autotransplantation [21, 22]. So far, numerous attempts have been made to investigate the metabolic and hormonal requirements of human ovarian tissue in order to optimize the in vitro culture media [23, 24, 25]. Unfortunately, up to now, very few results have been obtained. Only recently, Telfer et al. [5] have demonstrated the possibility of developing in vitro metaphase II oocytes from the primordial follicle stage of fresh human ovarian tissue in a multi-step process. This encouraging result have demonstrated that a complete in vitro folliculogenesis can be achieved , even though the low number and quality of the secondary follicles obtained still limits their clinical application. Indeed, the progression towards the secondary follicles rarely exceeds 10% of the total follicle population at the end of the in vitro culture [5, 26].

I have devoted my second year of Ph.D. to investigate the ovarian culture conditions and the role played by oxygen in the follicular growth. To this end, we have studied in Bovine and Human Ovarian Strips the quality, progression, and viability of the follicles cultured under different oxygen availability. Overall, results suggest that an adequate supply of oxygen is required to maintain follicle quality and viability and to promote follicle progression in both bovine and human tissue. Using Permeable Dishes we obtained one of the highest secondary follicle yields reported in the literature using a basal serum-free medium without FSH or activin [28-31]. This indicates that adequate oxygen availability is a key factor in supporting follicle progression in the culture of ovarian strips. These encouraging results provide evidence that the microenvironment in which the follicles grow is essential. Even though the factors that regulate the initial activation of the follicles are not yet fully understood, we know that the primordial follicles are located within the ovarian cortex (32) which is poorly irrorated by hematic vessels (33), and their concentration decreases in the medulla. Therefore, there is a slow and continuous diffusion that guides the exchange of nutrients and metabolic products.

In the last period of my Ph.D., to better mimic the physiological processes involved in early follicle activation, we decided to develop a dynamic culture system to enhance the in vitro folliculogenesis. The effectiveness of the dynamic perfusion system has been compared with the best culture condition obtained in the previous experiments performed with in vitro static Permeable Dish culture. In the last years, the dynamic culture approach has generated the development of bioreactors for several organ cultures and even though some systems for the culture of ovarian tissue have been reported [34-36] the efficiency of these systems is still questionable. In the perfusion bioreactor (PB) that we have developed (the data related to the perfusion bioreactor are not present in this thesis being the bioreactor under patent) we have cultured Bovine and Human cortical strips analysing the follicles quality, progression, viability and the levels of hormons present in the conditioned media collected at the end of the culture. Data showed that the culture in PB improved follicular progression to the secondary stage and increased quality and ratio between estradiol and progesterone levels.

Our data enrich those reported in the literature until now, confirming that the dynamic culture could better mimic the environment in which the follicles grow in vivo, providing a continuous flow of the medium, which grants a constant exchange of nutrients. Again, it is noteworthy that ovarian strips grown in PB in serum-free basal media housed (on day six) the highest yield of secondary follicles reported in the literature.

In conclusion, in the present thesis, we have developed a more efficient system for the cryopreservation of ovarian tissue based on the use of super-cooled nitrogen, and an innovative system for the dynamic in vitro culture of primordial follicles present inside ovarian tissue strips. Such an approach could open new interesting and promising perspectives in order to increase the efficacy of in vitro folliculogenesis.

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