RESEARCH ARTICLE

Is oxygen availability a limiting factor for *in vitro* folliculogenesis?
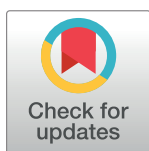
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Abstract

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 (Nümbrecht, 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% CO_2 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):

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

with

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

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. In PD, $C_{O2,T}/C_{O2,B}$ close to the strip upper surface is the same as
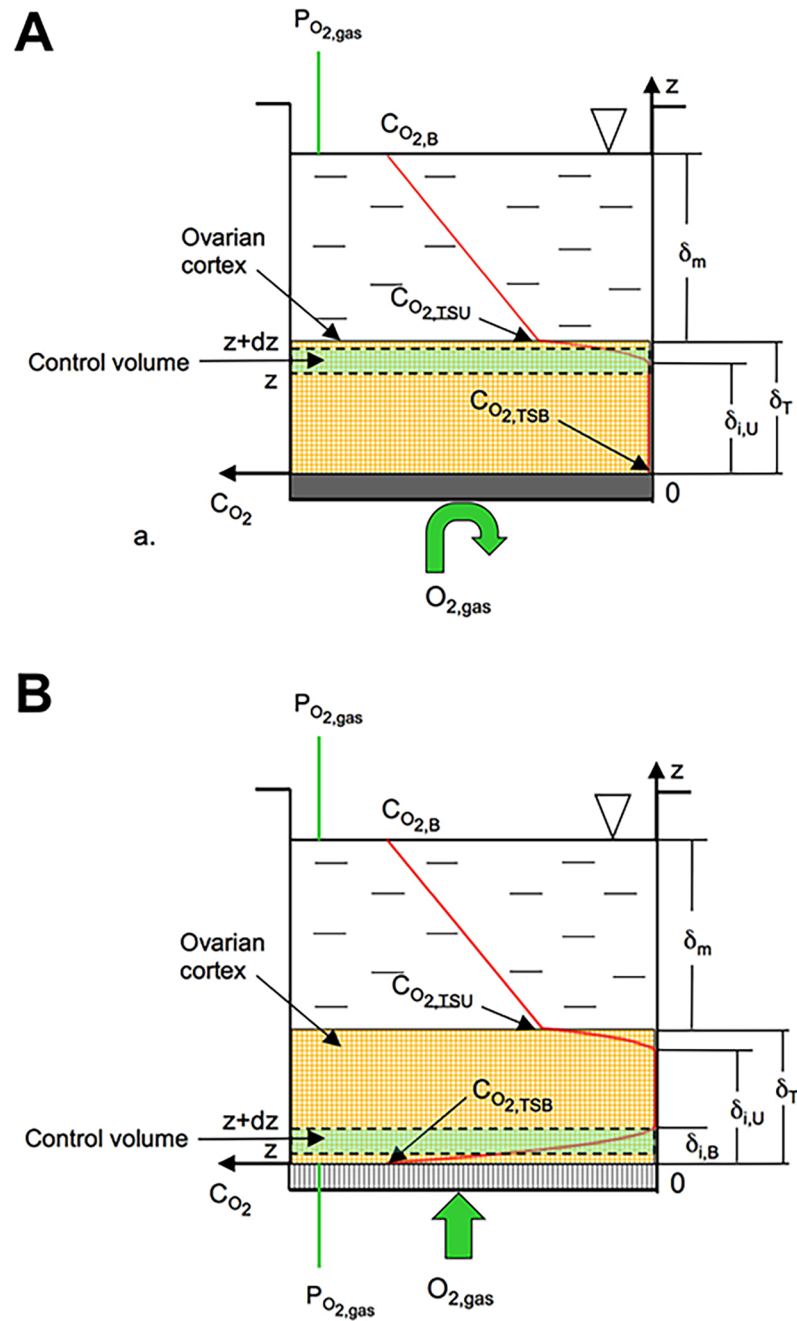
Fig 1. Scheme of ovarian cortex culture in conventional (CD) and gas-permeable (PD) dishes. The continuous red lines show exemplary dissolved oxygen profiles predicted in medium and tissue by the oxygen transport model described in Eqs 1–3 when strips of ovarian cortical tissue are cultured in: A) conventional 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.

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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):

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

(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: $G''' = 2 \times 10^{-2}$ mol/(s m^3); $D_m = 3.5 \times 10^{-9}$ m^2/s; $D_T = 2.8 \times 10^{-9}$ m^2/s; $\delta_T = 0.4$–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): $C_{O2,TSB}$–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.

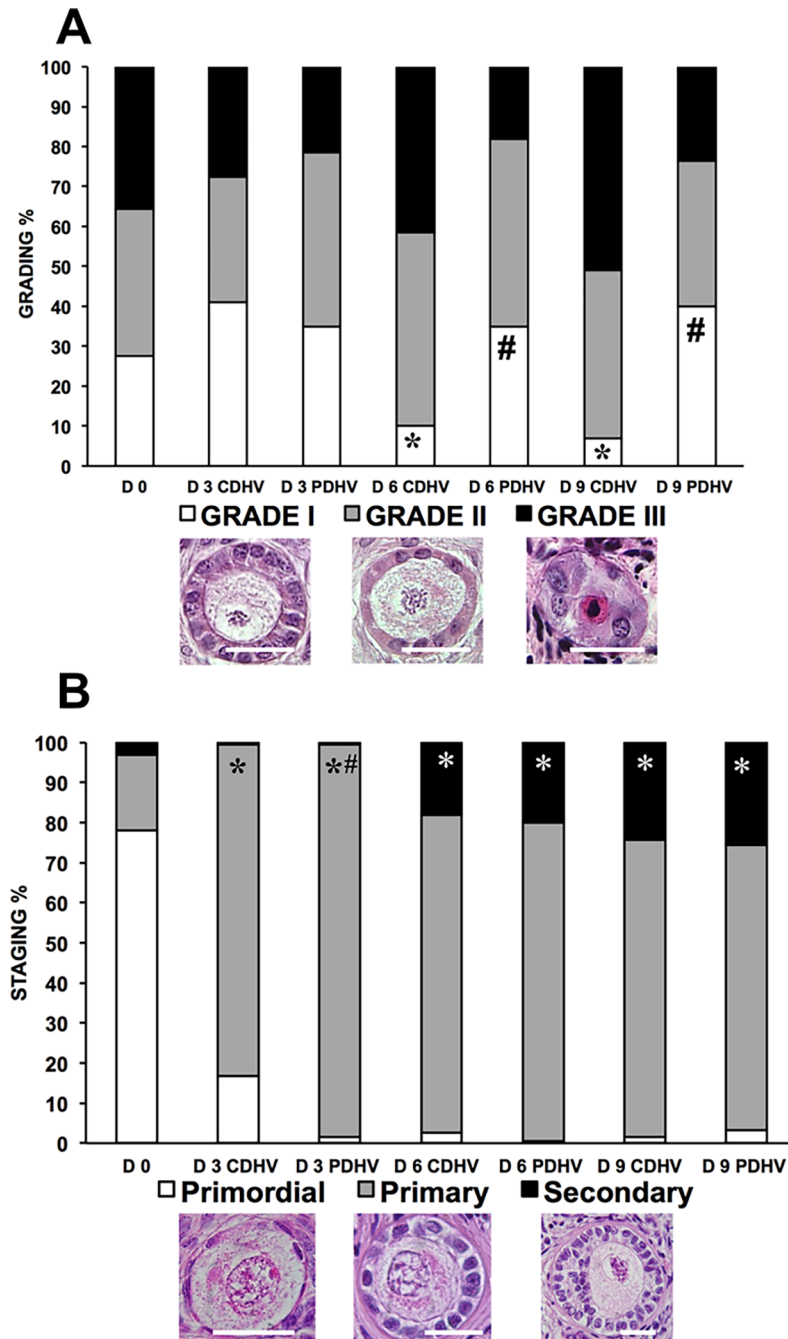https://doi.org/10.1371/journal.pone.0192501.t001

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.

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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). Histological analysis (n = 1243 follicles: D0, 273; D6 PDHV, 213; D6 PDLV, 287; D6 CDHV, 199; D6 CDLV, 271) showed that culture
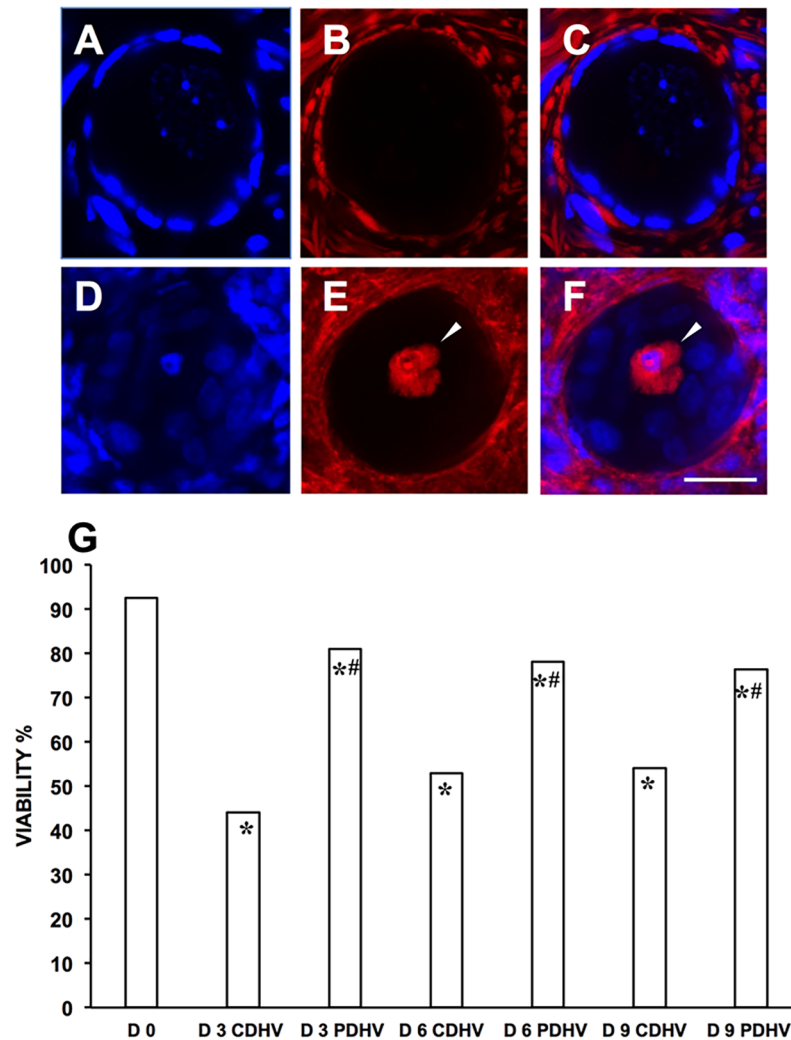
Fig 3. Viability of bovine follicles in strips cultured in air in PDHV vs CDHV. Representative confocal micrographs of a live (A,B,C) and a dead (D,E,F) follicle. (A,D) Hoechst 33342–stained nuclei; (B,E) live–dead far-red probe; (C,F) merge. (G) Follicle viability in fresh and cultured strips. Arrowheads indicate a dead oocyte. Bar = 20μm. $^*P<0.01$ vs d0; $^#P<0.01$ vs corresponding treatments.

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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 $C_{O2,T}$ 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).
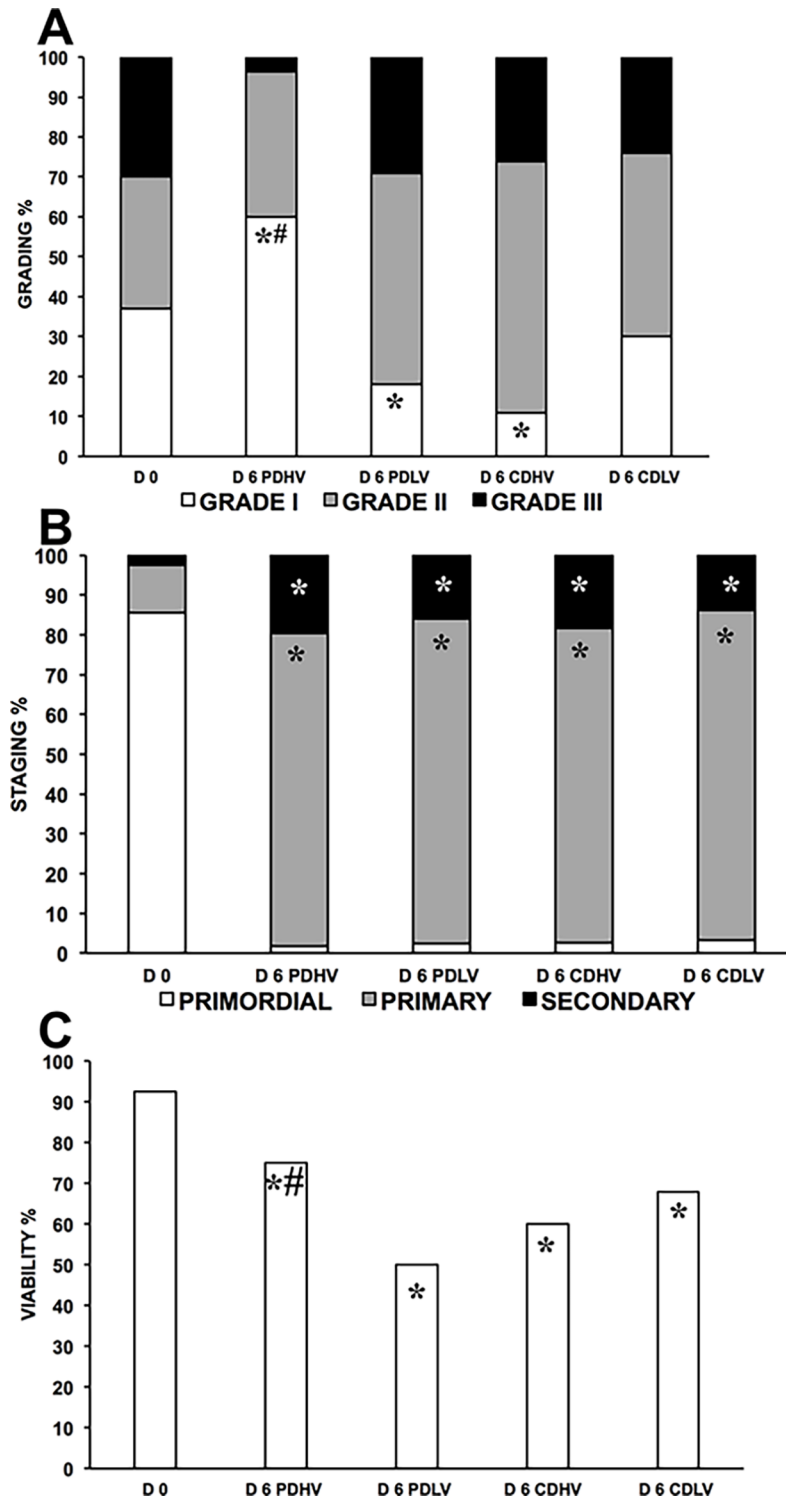
Fig 4. Quality of bovine follicles in strips cultured under various conditions in air. Histological grading (A), staging (B), and viability (C) of bovine follicles in strips cultured in PDHV, PDLV, CDHV and CDLV in air. *P<0.01 vs D0; #P<0.01 vs corresponding treatments.

Viability at D6 in PDHV was significantly better than in PDLV and CDHV, whereas in CDLV it was only slightly lower than in PDHV.

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% O_2 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% O_2 impaired progression to the secondary stage (Fig 5B, S3 Table). 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). 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).

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% $pO2_{gas}$ 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 $pO2_{gas}$, 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
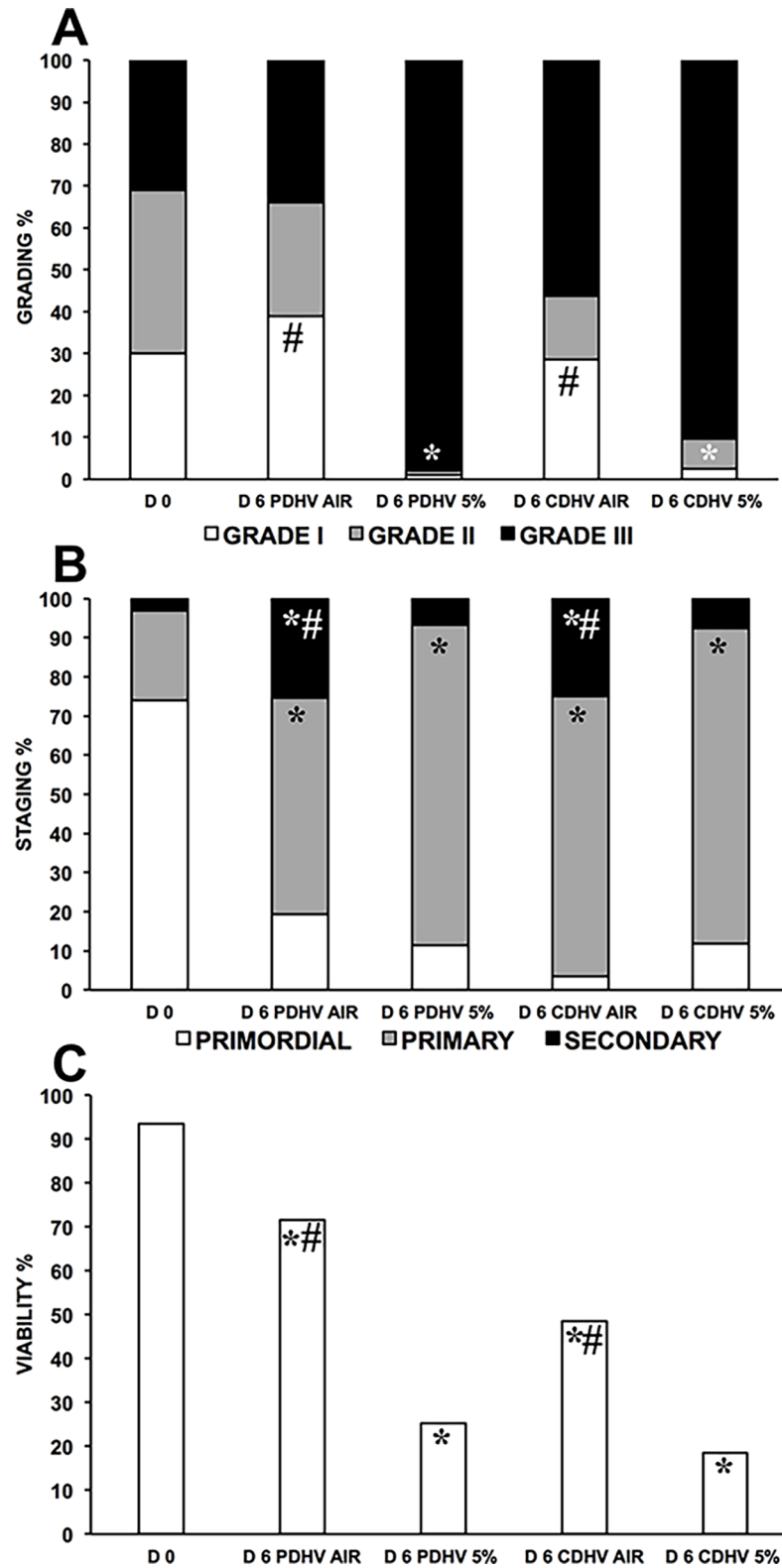
Fig 5. Quality of bovine follicles in strips cultured in air vs. 5%O2. Histological grading (A), staging (B), and viability (C) of bovine follicles in strips cultured in PDHV and CDHV in air versus 5% O2. *P<0.01 vs D0; $^\#$P<0.01 vs corresponding treatments.

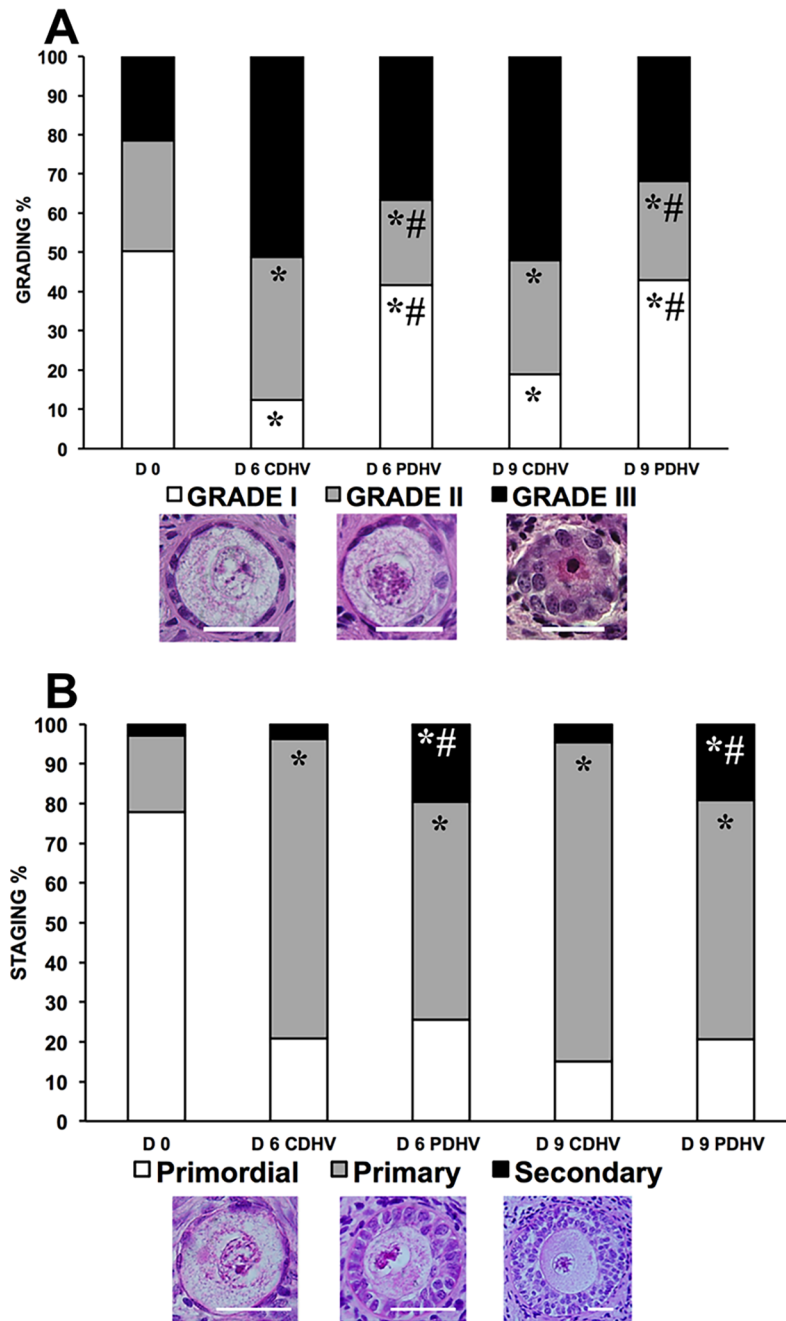https://doi.org/10.1371/journal.pone.0192501.g005

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 vs D0; $^{#}$P<0.01 vs corresponding treatments.

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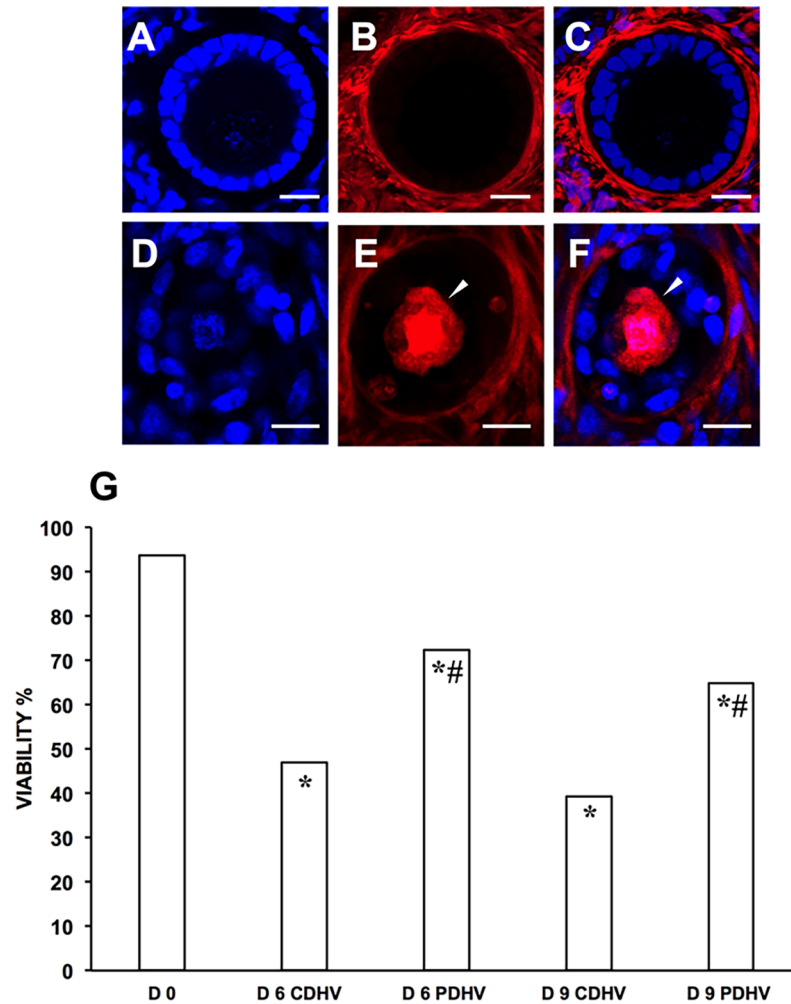
Fig 7. Viability of human follicles in strips cultured in PDHV vs CDHV. Representative confocal micrographs of a live (A,B,C) and a dead (D,E,F) follicle. (A,D) Hoechst 33342–stained nuclei; (B,E) live–dead far-red probe; (C,F) merge. (G) Follicle viability in fresh and cultured strips. Arrowheads indicate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

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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 $C_{O2,T}$'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 $C_{O2,T}$ 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 $C_{O2,T}$ respectively, impaired follicle health suggesting that bovine follicles require $C_{O2,T}$ 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 $C_{O2,TBS}$), 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, D_T 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 $G^{\sim}$ 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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References

1. Jemal A, Ward EM, Johnson CJ, Cronin KA, Ma J, Ryerson B, et al. Annual Report to the Nation on the Status of Cancer, 1975–2014, Featuring Survival. J Natl Cancer Inst. 2017;109.

2. Donnez J, Dolmans MM, Pellicer A, Diaz-Garcia C, Sanchez Serrano M, Schmidt KT, et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. Fertil Steril. 2013; 99:1503–1513. https://doi.org/10.1016/j.fertnstert.2013.03.030 PMID: 23635349

3. Kondapalli LA, Dillon KE, Sammel MD, Ray A, Prewitt M, Ginsberg JP, et al. Quality of life in female cancer survivors: is it related to ovarian reserve? Qual Life Res. 2013; 23:585–592. https://doi.org/10.1007/s11136-013-0473-y PMID: 23881516

4. Donnez J, Martinez-Madrid B, Jadoul P, Van Langendonckt A, Demylle D, Dolmans MM. Ovarian tissue cryopreservation and transplantation: a review. Hum Reprod Update. 2006; 12:519–535. https://doi.org/10.1093/humupd/dml032 PMID: 16849817

5. Wallace WHB, Anderson RA, Irvine DS. Fertility preservation for young patients with cancer: who is at risk and what can be offered? Lancet Onco. 2005; 6:209–218.

6. Schmidt KT, Larsen EC, Andersen CY, Andersen AN. Risk of ovarian failure and fertility preserving methods in girls and adolescents with a malignant disease. BJOG: An Int J Obstet Gynaecol. 2009; 117:163–174.

7. Silber S. Ovarian tissue cryopreservation and transplantation: scientific implications. J Assist Reprod Genet. 2016; 33:1595–1603. https://doi.org/10.1007/s10815-016-0814-1 PMID: 27722934

8. Jensen AK, Macklon KT, Fedder J, Ernst E, Humaidan P, Andersen CY. 86 successful births and 9 ongoing pregnancies worldwide in women transplanted with frozen-thawed ovarian tissue: focus on birth and perinatal outcome in 40 of these children. J Assist Reprod Genet. 2017; 34:325–336. https://doi.org/10.1007/s10815-016-0843-9 PMID: 28028773

9. Dolmans MM, Marinescu C, Saussoy P, Van Langendonckt A, Amorim C, Donnez J. Reimplantation of cryopreserved ovarian tissue from patients with acute lymphoblastic leukemia is potentially unsafe. Blood. 2010; 116:2908–2914. https://doi.org/10.1182/blood-2010-01-265751 PMID: 20595517

10. Abir R, Aviram A, Feinmesser M, Stein J, Yaniv I, Parnes D. Ovarian minimal residual disease in chronic myeloid leukaemia. Reprod Biomed Online. 2014; 28:255–260. https://doi.org/10.1016/j.rbmo.2013.10.011 PMID: 24365024

11. Abir R, Nitke S, Haroush AB, Fisch B. In vitro maturation of human primordial ovarian follicles: Clinical significance, progress in mammals, and methods for growth evaluation. Histol Histopathol. 2006; 21:887–898. https://doi.org/10.14670/HH-21.887 PMID: 16691541

12. O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biol Reprod. 2003; 68:1682–1686. https://doi.org/10.1095/biolreprod.102.013029 PMID: 12606400

13. Hornick JE, Duncan FE, Shea LD, Woodruff TK. Isolated primate primordial follicles require a rigid physical environment to survive and grow in vitro. Hum Reprod. 2012; 27:1801–1810. https://doi.org/10.1093/humrep/der468 PMID: 22456922

14. Laronda MM, Duncan FE, Hornick JE, Xu M, Pahnke JE, Whelan KA, et al. Alginate encapsulation supports the growth and differentiation of human primordial follicles within ovarian cortical tissue. J Assist Reprod Genet. 2014; 31:1013–1028. https://doi.org/10.1007/s10815-014-0252-x PMID: 24845158

15. Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. Hum Reprod. 2008; 23:1151–1158. https://doi.org/10.1093/humrep/den070 PMID: 18326514

16. Telfer EE, Zelinski MB. Ovarian follicle culture: advances and challenges for human and nonhuman primates. Fertil Steril. 2013; 99:1523–1533. https://doi.org/10.1016/j.fertnstert.2013.03.043 PMID: 23635350

17. West ER, Shea LD, Woodruff TK. Engineering the follicle microenvironment. Semin Reprod Med. 2007; 25:287–299. https://doi.org/10.1055/s-2007-980222 PMID: 17594609

18. Guzel Y, Oktem O. Understanding follicle growth in vitro: Are we getting closer to obtaining mature oocytes from in vitro-grown follicles in human? Mol Reprod Dev. 2017; 84:544–559. https://doi.org/10.1002/mrd.22822 PMID: 28452156

19. Yin H, Kristensen SG, Jiang H, Rasmussen A, Andersen CY. Survival and growth of isolated pre-antral follicles from human ovarian medulla tissue during long-term 3D culture. Hum Reprod. 2016; 31:1531–1539. https://doi.org/10.1093/humrep/dew049 PMID: 27112699

20. Adriaens I, Cortvrindt R, Smitz J. Differential FSH exposure in preantral follicle culture has marked effects on folliculogenesis and oocyte developmental competence. Hum Reprod. 2004; 19:398–408. PMID: 14747188

21. Smitz J, Dolmans MM, Donnez J, Fortune JE, Hovatta O, Jewgenow K, et al. Current achievements and future research directions in ovarian tissue culture, in vitro follicle development and transplantation: implications for fertility preservation. Hum Reprod Update. 2010; 16:395–414. https://doi.org/10.1093/humupd/dmp056 PMID: 20124287

22. McLaughlin M, Telfer EE. Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. Reproduction. 2010; 139:971–978. https://doi.org/10.1530/REP-10-0025 PMID: 20207724

23. Morimoto Y, Oku Y, Sonoda M, Haruki A, Ito K, Hashimoto S, et al. High oxygen atmosphere improves human follicle development in organ cultures of ovarian cortical tissues in vitro. Hum Reprod. 2007; 22:3170–3177. https://doi.org/10.1093/humrep/dem314 PMID: 17932085

24. Jorssen EP, Langbeen A, Fransen E, Martinez EL, Leroy JL, Bols PE. Monitoring preantral follicle survival and growth in bovine ovarian biopsies by repeated use of neutral red and cultured in vitro under low and high oxygen tension. Theriogenology. 2014; 82:387–395. https://doi.org/10.1016/j.theriogenology.2014.04.019 PMID: 24877724

25. Xu J, Lawson MS, Yeoman RR, Pau KY, Barrett SL, Zelinski MB, et al. Secondary follicle growth and oocyte maturation during encapsulated three-dimensional culture in rhesus monkeys: effects of gonadotrophins, oxygen and fetuin. Hum Reprod. 2011; 26:1061–1072. https://doi.org/10.1093/humrep/der049 PMID: 21362681

26. Gook DA, Edgar DH, Lewis K, Sheedy JR, Gardner DK. Impact of oxygen concentration on adult murine pre-antral follicle development in vitro and the corresponding metabolic profile. Mol Hum Reprod. 2014; 20:31–41. https://doi.org/10.1093/molehr/gat062 PMID: 24013158

27. Connolly JM, Kane MT, Quinlan LR, Dockery P, Hynes AC. Hypoxia limits mouse follicle growth in vitro. Reprod Fertil Dev. 2016; 28: 1570–1579.

28. Talevi R, Barbato V, Mollo V, Fiorentino I, De Stefano C, Guarino FM, et al. Replacement of sodium with choline in slow-cooling media improves human ovarian tissue cryopreservation. Reprod Biomed Online. 2013; 27:381–389. https://doi.org/10.1016/j.rbmo.2013.07.003 PMID: 23953587

29. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev. 1996; 17:121–155. https://doi.org/10.1210/edrv-17-2-121 PMID: 8706629

30. Talevi R, Barbato V, Fiorentino I, Braun S, De Stefano C, Ferraro R, et al. Successful slush nitrogen vitrification of human ovarian tissue. Fertil Steril. 2016; 105:1523–1531. https://doi.org/10.1016/j.fertnstert.2016.01.043 PMID: 26902858

31. Ke MT, Fujimoto S, Imai T. SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. Nat Neurosci. 2013; 16:1154–1161. https://doi.org/10.1038/nn.3447 PMID: 23792946

32. Gougeon A. Dynamics of follicular growth in the human: a model from preliminary results. Hum Reprod. 1986; 1:81–87. PMID: 3558758

33. Xu M, Barrett SL, West-Farrell ER, Kondipalli LA, Kieswetter SE, Shea LD, et al. In vitro grown human ovarian follicles from cancer patients support oocyte growth. Hum Reprod. 2009; 24:2531–2540. https://doi.org/10.1093/humrep/dep228 PMID: 19597190

34. Higuchi CM, Maeda Y, Horiuchi T, Yamazaki Y. A simplified method for three-dimensional (3-D) ovarian tissue culture yielding oocytes competent to produce full-term offspring in mice. PLoS One. 2015; 10: e0143114 https://doi.org/10.1371/journal.pone.0143114 PMID: 26571501

35. Wycherley G, Downey D, Kane MT, Hynes AC. A novel follicle culture system markedly increases follicle volume, cell number and oestradiol secretion. Reproduction. 2004; 127:669–677. https://doi.org/10.1530/rep.1.00040 PMID: 15175503

36. Larrea X, Buchler P. A transient diffusion model of the cornea for the assessment of oxygen diffusivity and consumption. IOVS 2009; 50:1076–1080.

37. Kim SS, Yang HW, Kang HG, Lee HH, Lee HL, Ko DS, et al. Quantitative assessment of ischemic tissue damage in ovarian cortical tissue with or without antioxidant (ascorbic acid) treatment. Fertil Steril. 2004; 82:679–685. https://doi.org/10.1016/j.fertnstert.2004.05.022 PMID: 15374714

38. Langbeen A, De Porte HF, Bartholomeus E, Leroy JL, Bols PE. Bovine in vitro reproduction models can contribute to the development of (female) fertility preservation strategies. Theriogenology. 2015; 84:477–489. https://doi.org/10.1016/j.theriogenology.2015.04.009 PMID: 25981885

39. Fortune JE, Kito S, Wandji S-A, Srsen V. Activation of bovine and baboon primordial follicles in-vitro. Theriogenology. 1998; 49:441–449. PMID: 10732025

40. Fortune JE, Kito S, Byrd DD. Activation of primordial follicles in-vitro. J Reprod Fertil. 1999; 54:439–448.

41. Fortune JE, Cushman RA, Wahl CM, Kito S. The primordial to primary follicle transition. Mol Cell Endocrinol. 2000; 163:53–60. PMID: 10963874

42. Gigli I, Byrd DD, Fortune JE. Effects of oxygen tension and supplements to the culture medium on activation and development of bovine follicles in-vitro. Theriogenology. 2006; 66:344–353. https://doi.org/10.1016/j.theriogenology.2005.11.021 PMID: 16442155

43. Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. Hum Reprod. 2008; 23:1151–1158. https://doi.org/10.1093/humrep/den070 PMID: 18326514

44. McLaughlin M, Kelsey TW, Wallace WH, Anderson RA, Telfer EE. Non-growing follicle density is increased following adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) chemotherapy in the adult human ovary. Hum Reprod. 2017; 32:165–174 https://doi.org/10.1093/humrep/dew260 PMID: 27923859

45. Anderson RA, McLaughlin M, Wallace WH, Albertini DF, Telfer EE. The immature human ovary shows loss of abnormal follicles and increasing follicle developmental competence through childhood and adolescence. Hum Reprod. 2014; 29:97–106 https://doi.org/10.1093/humrep/det388 PMID: 24135076

46. Carlsson IB, Laitinen MP, Scott JE, Louhio H, Velentzis L, Tuuri T, et al. Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture. Reproduction. 2006; 131:641–649. https://doi.org/10.1530/rep.1.00868 PMID: 16595715

47. Ishikawa T, Kyoya T, Nakamura Y, Sato E, Tomiyama T, Kyono K. Oxygen consumption rate of early pre-antral follicles from vitrified human ovarian cortical tissue. J Reprod Dev. 2014; 60:460–467. https://doi.org/10.1262/jrd.2014-009 PMID: 25262776