Impact of oxygen concentration on adult murine pre-antral follicle development in vitro and the corresponding metabolic profile

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ABSTRACT: Oxygen concentration during in vitro culture has a significant effect on the physiology of embryos, altering metabolic profile and developmental outcome. Although atmospheric oxygen has been used routinely for the culture of ovarian follicles, oxygen concentration may also be critical for follicle growth but the optimal concentration has not been determined. In this study, mechanically isolated primary and secondary follicles (80–140 μm diameter) from adult mouse ovaries were cultured in serum-free conditions for 8 days in either 5 or 20% oxygen to determine growth (follicular diameter), morphology and viability. For each oxygen concentration, half of the medium was replaced on Days 2, 4 and 6 or on Day 4 only. In the latter group, metabolic analysis of spent follicular culture media was performed by 1H-NMR. The proportion of viable, growing follicles was significantly (> 0.0001) higher in 5% than in 20% oxygen (59% versus 8%). Reducing the frequency of medium replacement during culture in 5% oxygen resulted in significantly (> 0.001) more viable follicles (79 versus 46%). In 20% oxygen, poor follicular viability was observed irrespective of the frequency of medium replacement (8 and 10% respectively). Metabolic profiles showed marked differences in amino acid and carbohydrate utilization with respect to both oxygen concentration and between Days 4 and 8 of development. Metabolites which significantly discriminated between oxygen concentration at both time points were glucose consumption, lactate utilization, alanine, alanyl-glutamine, leucine and proline. In conclusion, the poor in vitro follicular development previously observed in minimal culture conditions may reflect the use of 20% oxygen. Frequent medium replenishment is not necessary and does not overcome the detrimental effect of high oxygen on follicle viability. Further optimization of culture conditions would benefit from metabolic analyses and the use of 5% oxygen should be tested further for impact on functional aspects of follicle culture such as steroid production which is currently unknown.

Key words: follicles / granulosa cells / metabolism / murine / oocyte / oxygen

Introduction

Autografting of cryopreserved ovarian tissue has achieved restoration of fertility in a number of women post-cancer treatment, but the high risk of re-introducing malignant cells contraindicates this approach for women with leukaemia. In vitro follicle culture may reduce this risk. In vitro maintenance of human ovarian cortex has been established for over 30 years (Baker and Neal, 1974) but no appreciable follicle growth had been achieved until a recent report using a two-stage culture system of individual follicles in the presence of Activin A (Telfer et al., 2008). Although endocrine requirements during in vitro follicle development have been relatively well-established (Nayudu and Osborn, 1992; Hartshorne et al., 1994; Adriaens et al., 2004), the same cannot be said for fundamental nutritional requirements. Recent improvements in the culture of human embryos for assisted reproductive technology are a consequence of a greater understanding of optimal culture conditions (Gardner and Lane, 2003) and similar improvements in understanding and culture system design could have major implications for in vitro follicle culture.

Atmospheric oxygen during oocyte maturation (Eppig and Wigglesworth, 1995) and early embryonic cleavage has a detrimental effect on developmental competence (Wale and Gardner, 2010), and subsequent fetal development (Harlow and Quinn, 1979; Feil et al., 2006). In contrast to the avascular environment within the ovary, isolated follicles are generally cultured in atmospheric oxygen (20%). A 20% oxygen concentration during follicle culture results in a reduction in growth (Xu et al., 2011) and antrum formation (Silva et al., 2010) relative to 5% oxygen.
A consequence of exposure to 20% oxygen during follicular culture is the loss of cell junctions between granulosa cells and the oocyte, resulting in a loss of communication culminating in impaired oocyte maturation (Heise et al., 2009). In contrast, others have shown atmospheric oxygen to be superior over 5% oxygen during follicle culture in terms of an increase in antral formation and oocyte maturation (Smiz et al., 1996). However, both studies which report a benefit of 20% oxygen include either fetal calf serum or maternal serum in the culture (Smiz et al., 1996; Wycherley et al., 2004), whereas no serum was used in the studies which showed superior results with 5% oxygen (Heise et al., 2009; Xu et al., 2011).

Oxygen at a concentration of 20% has been shown to alter gene expression (Rinaudo et al., 2006), the embryonic proteome (Katz-Jaffe et al., 2006) and the metabolome, as quantified by amino acid turnover, carbohydrate uptake by embryos and transamination (Wale and Harris, 1993; Lane and Gardner, 1994) has been greatly alleviated by in-

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carboxylic acid cycle (TCA) and oxidative phosphorylation (Wycherley et al., 2005).

Amino acids are important for numerous cellular functions: as substrates for synthesis of proteins and nucleotides, as energy sources, in the maintenance of intracellular pH, as antioxidants (Gardner and Wale, 2013) for regulation of metabolic pathways and as signalling molecules (Washington et al., 2010). Apart from an increase in leucine uptake (Chand and Legge, 2011), little is known of the metabolism of amino acids during follicle development. Specific patterns of amino acid utilization are associated with oocyte quality (Hemmings et al., 2012), embryo developmental stage (Houghton et al., 2002) and implantation potential (Brison et al., 2004), indicating a role in supporting development.

Successful pre-antral follicle culture has traditionally included serum (Hartshorne, 1997). However, such an approach leads to increasing variability and inhibits meaningful assessment of metabolic activity. In a serum-free culture environment, suboptimal conditions may have major consequences which may be masked by the presence of serum. Therefore, the development of a serum free defined culture environment has major implications for the understanding of follicular physiology.

To facilitate more accurate measurement of hormone production during follicular culture, oil overlay is typically omitted; however, this in turn leads to greater fluctuations in medium osmolarity (Swain et al., 2012), pH and temperature (Swain, 2012). This variability is compounded by frequent medium changes; generally every 2 days, although the necessity to replenish medium frequently due to the instability of glutamine [and consequent generation of toxic ammonium (Gardner and Lane, 1993; Lane and Gardner, 1994)] has been greatly alleviated by inclusion of a more stable form of glutamine (L-alanyl-L-glutamine) in culture media.

The relevance of an animal model is dependent on its ability to identify factors necessary for follicular development. Significantly, all previous mouse follicle culture systems have used prepubertal follicles. Although a more uniform population of follicles is present in the prepubertal ovary (Peters, 1969), the growth rate of these follicles is different from that of similar stage follicles in adult ovaries (Pedersen, 1970a, b).

The aim of this study was, therefore, to establish a defined culture environment for adult mouse primary and secondary follicles in which the impact of oxygen concentration could be assessed at both the morphological and metabolic levels. A secondary aim was to determine whether frequent medium replenishment is necessary with the use of a more stable form of glutamine.
Follicle diameters were measured on Days 0 and 8 for each follicle and the difference reported as an increase in diameter. On Day 8 follicles were evaluated morphologically and considered intact if containing an oocyte. To assess viability following mechanical isolation, a representative group of follicles was evaluated for each experiment on Day 0 using the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes). Viability was also assessed on Day 8 together with detailed morphology with H&E staining of serial sections, development of the zona pellucida, cell proliferation and viability was confirmed with apoptosis staining.

Viability
Calcine AM and ethidium homodimer-1 (Molecular Probes) were prepared in appropriate pre-gassed medium at 37°C, added to the culture wells (both 4 μM) and incubated for 3 h. Follicles were assessed under a Leica DM 2500 fluorescent microscope with a N2 filter (580–650 nm) and GFP filter (470–510 nm) and photographed with a digital camera. Hydrolysis of calcine AM by intracellular esterases results in bright green cytoplasm in living cells, whereas dead cells with lysed membrane allow ethidium homodimer to bind to DNA resulting in red nuclei.

Kit was validated by treatment of follicles with 10% ethanol.

Morphological assessment and immunohistochemistry
After live/dead staining follicles were fixed in 4% paraformaldehyde overnight and subsequently processed and embedded in paraffin wax followed by serial sectioning (4 μm thick). At least two sections were stained with haematoxylin and eosin. Additional sections were assessed as follows.

Zona pellucida protein detection has been previously described (Gook et al., 2008). Briefly, sections were dewaxed followed by incubation for 1 h at 37°C with polyclonal rabbit antibodies raised against zona pellucida protein 1 and 2 (gift from Jeff Harris). Sections were washed to remove unbound antibody, then incubated with peroxidase labelled polymer conjugated to goat anti-rabbit IgG (EnVision®; DakoCytomation) for 30 min followed by removal and incubation with chromogen diaminobenzidine (DAB; DakoCytomation). Sections were counterstained with Harris haematoxylin. Primary antibody was replaced with rabbit IgG in negative control slides and the method validated with mouse oocytes.

Sections were assessed for apoptosis using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore). After deparaffinizing, sections were incubated with protease K (20 μg/ml) followed by brief incubation in 3% hydrogen peroxide. Nucleotides and terminal deoxynucleotidyl transferase were added to sections, the reaction was halted after 1 h at 37°C and the anti-digoxigenin conjugate was added for 30 min. Digoxigenin nucleotides were subsequently detected with an anti-digoxigenin antibody conjugated to peroxidase. Chromogenic substrate was added and slides were counter stained with 1% methyl green (Merck). Sections of breast tissue were used as a positive control.

Cell proliferation
Sections were stained using a Ventana Benchmark ULTRA, a fully automated staining instrument. Sections were deparaffinized and incubated at 95°C for 8 min for antigen retrieval and subsequently incubated with rabbit monoclonal Ki-67 antibody (Ventana; Roche). The antibody was detected using a DAB detection system (ultraView Universal DAB Detection Kit; Ventana, Roche) in an automated staining module. Briefly, slides were washed to remove excess primary antibody followed by incubation with horseradish peroxidase conjugated secondary antibody. Again unbound antibody was removed by washing and antibody antigen complex detected by addition of the DAB chromogen together with hydrogen peroxide and copper sulphate. Finally, sections were counterstained with Gill’s 2 haematoxylin (Australian Biostain). A positive control section of tonsil was included with each follicle section.

In all of the above situations, subsequent immunochemistry staining was unaffected by preceding live/dead staining.

Metabolomic assessment
Metabolites in the culture medium were quantified from follicles grown individually and not those grown in the corral wells using 1H-NMR spectroscopy.

Preparation for 1H-NMR analysis
Removal of proteins, macromolecules and lipids from the medium was achieved by methanol-based liquid–liquid solvent extraction (LLE), using a 2:1 ratio of methanol:culture medium sample, to prepare samples for 1H-NMR quantitative metabolite analysis (Roessner and Dias, 2013). Culture medium samples were thawed for 30 min at 25°C before 40 μl of ice-chilled methanol (Sigma) for LLE and 5μl imidazole (5 mmol/l; Sigma) was added to each 20 μl aliquot of culture medium to correct for variation in metabolite recovery after sample preparation and spectral acquisition. Samples were centrifuged (3000g, 4°C, 30 min) and 70 μl of protein-free supernatant was recovered. Solvent was evaporated from each sample under speed vacuum (55°C for 10 h) and reconstituted in 540 μl of sodium phosphate in deuterium oxide (buffered to pH 7 using deuterium chloride; Sigma) and a further 60 μl of deuterium oxide (Cambridge Isotope Laboratories, Inc., UK) containing 5 mmol/l 3-(Trimethylsilyl)-1-propanesulfonic acid-d3 sodium salt (DSS, NMR reference compound; Sigma) and 0.2% w/v sodium azide (Sigma), as reported in Sheedy et al. (2010) and Temmerman et al. (2012).

1H-NMR spectroscopy
Prepared samples were added to NMR tubes (7 inch, 507 grade with a 5.5 mm diameter from Wilmad LabGlass) and run at 25°C on an 800 MHz Avance US2 spectrometer (Bruker Biospin) equipped with a 5 mm triple resonance cryoprobe. All samples were locked to deuterium oxide and gradient shimmmed. The 90° pulse width was calculated for each sample, and receiver gain value optimized for each sample. All data were collected over 32 k data points and 1536 scans. The one-dimensional noesyd 1d (NOESY with presaturation for water suppression, consisting of recycle delay-90°–90°–90°–90°, acquire FID) was used with a recycle delay of 1.5 s and mixing time (τm) of 50 ms. The transmitter frequency offset was optimized to coincide with the HDO signal. The NMR experimental conditions were chosen according to suggested parameters outlined in the Chenomx 6.1 NMR Suite software package. A 1.5 s recycle delay was chosen to minimize some effect of T1 relaxation. A short recycle delay was used to maintain compatibility with the compound libraries in the Chenomx software (Webb-Robertson et al., 2005; Saude et al., 2006; Sheedy et al., 2010). Spectra were pre-processed in Bruker’s Topspin NMR software, using an exponential window function and manual phase correction. Samples were processed with a linear baseline correction and referenced to (CH3)2Si-singlet of DSS at 0.00 ppm in the Chenomx NMR Suite.

Data processing and statistical analysis
The proportions of growing intact follicles were compared using a two-tailed Fisher’s exact test. Growth as determined by the average increase in diameter ± SEM over the culture period was compared using an unpaired t-test.

Metabolites were identified and quantified from culture medium through 1H-NMR spectra using the 800 MHz compound library in the Chenomx NMR Suite (for samples in the pH range of 6–8) (Temmerman et al., 2012). Prior to multivariate data analysis, a non-Gaussian density function was applied for each spectrum (due to left/right data skewness). Negative values (denoting metabolite consumption) were scaled by adding the
concentrations are expressed as the mean ± standard error of the mean. Logarithm (log10) transformation was applied to transform data to a non-skewed Gaussian or t-distribution. Homoscedasticity was overcome by log10-transformation, and examined by plotting the sample mean (x-axis) and standard deviation (y-axis) in the R statistical software package (R Development Core Team, 2008). A median normalization procedure was applied to NMR data after log10 transformation (level scaling) (van den Berg et al., 2006; Temmerman et al., 2012) in R (R Development Core Team, 2008).

The Unscrambler 10.1 (CAMO Software, Norway) software was used to conduct Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) of the spectral data to identify differences in amino acid and carbohydrate utilization by Days 4 and 8 follicles after culturing at 5 and 20% oxygen. The two principal components/factors (for PCA/PLS-DA respectively) accounting for the greatest amount of variance in the quantified data matrix of metabolites were used to create two-dimensional scores plots (representing the total spectral area) and loadings plots (representative of utilization of each metabolite by the ovarian follicle). Differences in amino acid, glucose, lactate and pyruvate utilization were analyzed by the non-parametric Kruskal–Wallis test using Dunn’s post-test for multiple comparisons, where $P < 0.05$ denotes a significant change in metabolite utilization. Metabolite concentrations are expressed as the mean ± standard error of the mean.

Results

Follicle culture

Live/dead staining of follicles on Day 0 showed that 97% (90/93) were viable after the mechanical isolation. Follicles on Day 0 had ≤2 layers of granulosa cells with a mean diameter of 110 μm (range of 80–140 μm, Fig. 1a and b). The majority of follicles cultured in the presence of FBS (positive control) in both oxygen concentrations [5% oxygen; 100% (24/24), and 20% oxygen; 96% (23/24)] showed similar morphology to that previously reported (Adriaens et al., 2004); breakdown of the three-dimensional structure with migration of the theca cells across the dish and proliferation of the mural granulosa cells. In contrast, the three-dimensional structure was maintained during the culture period for the majority of follicles [5% oxygen; 89% (196/223) and 20% oxygen; 93% (209/223)] in the defined medium (serum free). Occasionally in the defined medium the basement membrane connection with the granulosa cells was lost and, as a consequence, the oocyte was released from the follicle, these were subsequently removed from any further analysis.

Viability

Assessment of viability at the end of the culture period in the defined medium showed three different patterns of staining; those in which the majority of granulosa cells and theca were dead (non-viable, Fig. 2a), a mixture of dead and live granulosa cells with predominantly dead cells (Fig. 2b) and those in which the majority of granulosa and theca cells were alive (viable, Fig. 2c). Viability was verified by morphological appearance (Fig. 1c), presence of apoptosis (Fig. 1e) and intensity of zona protein production (Fig. 1g).

The number of viable follicles in the defined medium in each group is presented in Table 1. Very few follicles had the majority of live cells throughout the follicle in 20% oxygen. In contrast a higher proportion was viable in 5% oxygen. The majority of the viable follicles showed an increase in diameter over the 8 days of culture (Table 1, growth). Active growth at the end of the culture was evident by the presence of...
Ki67 antigen in numerous granulosa and theca cells within these growing follicles (Fig. 11). In 5% oxygen the average increase in diameter was similar irrespective of the frequency of medium change (one medium change; 62.9 ± 5.7 μm and three medium changes; 64.9 ± 4.2 μm). The increase in diameter over the 8 days in culture is due to an increase in the number of granulosa cell layers from a maximum of two on Day 0 (Fig. 1a) to ≥6 layers with the start of antrum formation in some follicles (Fig. 1g; arrow head). Twenty percent oxygen resulted in very few viable follicles which grew (n = 16) in the three medium change condition the average increase in diameter (10 follicles; 50.5 ± 13.6 μm) was similar to that in 5% oxygen (43 follicles; 64.9 ± 4.2 μm). However, reduced growth was observed in the 20% oxygen with a single medium change (6 follicles; 15.8 ± 3.8 μm) compared with the 5% oxygen conditions (72 follicles; 62.9 ± 5.7 μm, P < 0.01). A highly significant reduction in the proportion of viable growing follicles was observed in the 20% compared with the 5% oxygen within the same medium conditions (Table I, P < 0.0001). Although both 5% oxygen conditions resulted in more viable growing follicles, the single medium change resulted in the highest proportion of growing viable follicles (76.6%) which was significantly higher than in the three medium change condition (42.2%, P < 0.0001).

Overall metabolism

Utilization of amino acids and carbohydrates was only assessed in the single medium change condition. PCA (Supplementary data, Fig. S1) and PLS-DA (Fig. 3) of the total consumption and utilization of both carbohydrates and amino acids together with viability assessed on Day 8 show a distinct population for the 5% oxygen group on Day 8 compared with all of the other groups. A tight cluster is observed for Day 4 in 5% oxygen which overlaps both the measurements for 20% oxygen but the non-viable follicles tend to fan out from this cluster indicating more variable metabolism associated with loss of viability (Fig. 3a). The contribution of metabolites to discriminate the groups is best depicted by the PLS-DA loadings plot (Fig. 3b). There is a clear change in metabolism for glucose and lactate from Days 4 to 8 in 5% oxygen (Figs 3b and 4) and also for the amino acids alanine, alanyl-glutamine, leucine and proline (Figs 3b and 6). The total amino acid turnover differs from Days 4 to 8 for follicles in the presence of 5 and 20% oxygen (Fig. 5), complimenting the change observed with carbohydrate utilization (Fig. 4). In contrast, the few follicles which maintained viability in 20% oxygen showed little change in metabolism (glucose, lactate and proline) from Days 4 to 8 (Fig. 3b). A large change in glucose and lactate metabolism is observed in the non-viable follicles in 20% oxygen between Days 4 and 8 (Figs 3b and 4).

Carbohydrate metabolism

In the first 4 days of culture in 5% oxygen almost no glucose was consumed but by Day 8 consumption of glucose dramatically increased (P < 0.001, Fig. 4a). Correspondingly, little pyruvate is produced at both time points in 5% oxygen (Fig. 4b). The inverse is observed in 20% oxygen with respect to glucose consumption. In contrast, in 20% oxygen pyruvate production is high on Day 4 but, as with glucose, significantly decreases by Day 8. Although no lactate utilization is observed initially in 5% oxygen, by Day 8 there is a significant production of lactate (Day 4 compared with Day 8; P < 0.05, Fig. 4c). In contrast to 5% oxygen, lactate was consumed at a constant rate at both time points in 20% oxygen.

Amino acid metabolism

Amino acid turnover (Fig. 5) parallels the trend of carbohydrate metabolism, showing a low level of turnover initially in 5% oxygen followed by a significant increase in turnover (P < 0.001), mainly due to consumption, by Day 8. The alignment of carbohydrate metabolism and amino acid turnover is also observed in 20% oxygen, showing a higher turnover on Day 4 compared with 5% oxygen (P < 0.05) and a reduction in turnover by Day 8 (comparison with 5% oxygen; P < 0.05), both of which are a
consequence of significant changes in consumption (compared with 5% oxygen; $P < 0.01$). Changes in consumption (−) and production (+) for each amino acid is reported in Table II. A switch from production to a significant level of consumption was observed from Days 4 to 8 in 5% oxygen for alanine ($P < 0.001$), alanyl-glutamine ($P < 0.001$) and proline ($P < 0.0001$). An increased consumption in the latter half of the culture period was observed for leucine ($P < 0.01$), phenylalanine ($P < 0.01$) and valine ($P < 0.05$) in 5% oxygen. Initial consumption at Day 4 switched to production at Day 8 for arginine ($P < 0.001$) and cystine ($P < 0.05$) in 5% oxygen.

Greater variation was observed for amino acid metabolism in 20% oxygen relative to 5% oxygen. In 20% oxygen the inverse of the result in 5% was observed, i.e. a switch from consumption to production of proline ($P < 0.01$) and an increase production of alanine ($P < 0.001$) from Days 4 to 8 (Fig. 6). Again, in contrast to the consumption of alanyl-glutamine in 5% oxygen, net production was observed at both time

Figure 3 Differences in carbohydrate and amino acid utilization by Days 4 and 8 follicles cultured in 5% and 20% oxygen illustrated by Partial Least Squares Discriminant Analysis (PLS-DA). The PLS-DA scores plot (a) revealed that follicles at Day 8 cultured in 5% oxygen are metabolically distinct from Day 8 follicles cultured in 20% and all Day 4 follicles. The corresponding loadings plot (b) confirms that glucose, lactate, alanine and alanyl-glutamine and proline utilization distinguish Day 8 follicles in 5% oxygen from all other groups. The weighted contribution of variance of each follicle group is denoted in red, and each metabolite in blue. The differences in metabolite utilization profiles were initially confirmed by PCA (Supplementary data, Fig. S1).
points in 20% oxygen (compared with 5% oxygen at both time points, \( P < 0.0001 \)). Although both leucine and valine were consumed on Day 8 in both oxygen concentrations a significantly lower consumption was observed in 20% oxygen (leucine, \( P < 0.001 \); valine, \( P < 0.01 \)).

**Discussion**

The study establishes that viable follicles with histologically normal appearance can develop in the absence of serum under 5% oxygen. These follicles developed a theca layer, increased the number of granulosa cell layers, were actively proliferating and showed evidence of a zona pellucida. In contrast, although granulosa cell layers appear to have increased in 20% oxygen, follicles were non-viable with predominantly apoptotic cells, an over abundance of zona pellucida proteins (indicative of atresia) and, by the completion of culture, showed no increase in diameter due to granulosa cell lysis. Although depletion of energy-producing metabolites may be implicated, replenishment of the medium every second day did not alter the outcome in 20% oxygen. In 5% oxygen, more frequent replenishment of medium is not necessary and may actually be detrimental, possibly removing autocrine/paracrine factors which may benefit follicular development.

An adverse affect of atmospheric oxygen on oocytes and embryos during culture (reviewed by Gardner and Wale (2013)) has been established. The consequences include inability to resume meiosis (Eppig and Wigglesworth, 1995), delayed cleavage (Wale and Gardner, 2010) and reduced cell numbers in blastocysts (Karagenc et al., 2004). Conflicting results relating to the impact of atmospheric oxygen on pre-antral follicle development have been reported. Smitz et al. (1996) reported that 20% oxygen resulted in higher follicle survival and a higher proportion with both antrum formation and mature oocytes compared with 5% oxygen. However, a detrimental effect of 20% oxygen has been reported by others using similar criteria for follicle growth and oocyte quality in secondary follicles from rhesus monkey (Xu et al., 2011), sheep (Cecconi et al., 1999) and rat (Heise et al., 2009).
The impact of 20% oxygen on follicular health reported in the present study has not been observed previously. This may be due to the previous inclusion of serum in culture (Nayudu and Osborn, 1992; Boland et al., 1994b; Smitz et al., 1996), which may provide growth factors and/or metabolic energy sources to maintain follicle development.

This study has established a unique follicle culture system. First, we have used adult primary and secondary follicles in contrast to previously established systems with prepubertal or juvenile follicles. Secondly, we have employed a successful serum-free culture milieu. Thirdly, we have established the superiority of a physiological oxygen environment in terms of follicular growth. The effect on functionality such as steroid production remains to be tested.

**Metabolomic profile**

\(^1\)H-NMR is a highly reproducible and quantitative platform for quantifying sub-micromole changes in utilization of the 20 amino acids, glucose, lactate and pyruvate in a single assay. \(^1\)H-NMR \(^1\)NHM metabolomics has been applied successfully in a murine follicle culture system for this study.

Generally, follicles in 20% oxygen are metabolically more active in the initial 4 days of culture, consuming more glucose and lactate, whereas in 5% oxygen the follicle is relatively inactive during the initial period of culture. Although diameters were not measured on Day 4, this slower metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a). The high oxygen environment accelerates in vivo time (110 h) for granulosa cells in these early secondary (type 4) follicles metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a). The high oxygen environment accelerates in vivo time (110 h) for granulosa cells in these early secondary (type 4) follicles metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a). The high oxygen environment accelerates in vivo time (110 h) for granulosa cells in these early secondary (type 4) follicles metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a). The high oxygen environment accelerates in vivo time (110 h) for granulosa cells in these early secondary (type 4) follicles metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a). The high oxygen environment accelerates in vivo time (110 h) for granulosa cells in these early secondary (type 4) follicles metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a). The high oxygen environment accelerates in vivo time (110 h) for granulosa cells in these early secondary (type 4) follicles metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a). The high oxygen environment accelerates in vivo time (110 h) for granulosa cells in these early secondary (type 4) follicles metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a). The high oxygen environment accelerates in vivo time (110 h) for granulosa cells in these early secondary (type 4) follicles metabolism is consistent with previous demonstration of a slow doubling time (110 h) for granulosa cells in these early secondary (type 4) follicles in vivo (Pedersen, 1970a).
Although similar glucose consumption was observed in 20% oxygen on Day 4 in the present study (34 nmol/follicle/day), in contrast lactate was rather consumed (−44 nmol/follicle/day) and a minor amount of pyruvate produced (7 nmol/follicle/day). This indicates that, energy sources are predominantly directed into the TCA cycle in our serum-free 20% oxygen environment. In contrast, in serum lipids may provide an alternative energy source. Additionally, this is the first study using adult follicles with all previous studies using prepubertal follicles. 


In the present study, a production of lactate on Day 8 through glycolysis was observed in the healthy follicles cultured in 5% oxygen, indicating this may be preferred metabolism. However, the stoichiometric ratio of glucose to lactate (1:1) suggests that half of the glucose was directed to lactate, with the remainder being directed into either the TCA cycle or pentose phosphate pathway (PPP). The utilization of both glycolysis and the TCA cycle has been reported in serum-free culture of pre-antral follicles in the presence of gonadotrophins (Roy and Terada, 1999). A notable benefit of directing metabolism down the PPP is the generation of nucleotide precursors and NADPH for biosynthesis, and provision for a supply of reduced glutathione, a key intracellular antioxidant (Rieger et al., 1992; Gardner and Wale, 2013). A similar stoichiometric relationship has been reported for oocyte–cumulus complexes during in vitro maturation (Sutton-McDowall et al., 2010) directing an increased utilization of glucose through PPP (Downs and Utecht, 1999) and glucosamine biosynthesis pathways (Sutton-McDowall et al., 2006).

Amino acids

Generally, in all groups assessed there is a parallel between carbohydrate utilization and amino acid turnover. Although carbohydrate metabolism suggests that the follicles are relatively inactive initially in 5% oxygen, the consumption and production of amino acids (alanine, leucine and proline) clearly shows that these follicles are actively metabolizing amino acids, but at a lower rate than on Day 8. The switch from production to consumption over the duration of the culture in 5% oxygen (alanine, alanyl-glutamine, leucine and proline) reflects a dynamic multicellular unit that alters utilization with associated proliferation and increased carbohydrate metabolism. In contrast, production of the same amino acids in 20% oxygen over the latter stage of culture is associated with apoptosis.

The most striking amino acid switch in 20% oxygen is the production of a high level of alanine compared with a high level of consumption in 5% oxygen in the latter stage of culture. A similar correlation between poor prognosis and production of alanine has been observed with failure to cleave (Hemnings et al., 2012) and arrested cleavage (Houghton et al., 2002; Stokes et al., 2007). Alternatively, the high level of alanine in the medium at 20% oxygen may represent a higher utilisation and breakdown of alanyl-glutamine.

A switch from production to consumption was observed for proline in 5% oxygen. Again, the opposite was observed in 20% oxygen. Proline influx has been shown to act as a signalling molecule in differentiation of embryonic stem cells through the mTOR pathway (Washington et al., 2010), a known activator of follicle development (Telfer and Zelinski, 2013).

Conclusions

The use of a serum-free culture system in the present study has established the importance of physiological oxygen during follicular development. The metabolic consequences of an elevated oxygen concentration, which may perturb normal development, have been characterized.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors’ roles

D.G. and K.L. performed the follicle culture and analysis of data and prepared figures pertaining to this component of the study. J.R.S. conducted metabolomics experiments and statistical analysis including preparation of the figures and table. All authors contributed to the study design and preparation of the manuscript.

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Conflict of interest

None declared.

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