Oxygen concentration during mouse oocyte in vitro maturation affects embryo and fetal development

K.M. Banwell, M. Lane, D.L. Russell, K.L. Kind and J.G. Thompson

The Research Centre for Reproductive Health, Discipline of Obstetrics and Gynaecology, The School of Paediatrics and Reproductive Health, The University of Adelaide, South Australia 5005, Australia

BACKGROUND: Little is known of how the oxygen environment in the ovarian follicle affects oocyte and embryo development, but this has an important impact on the conditions used for in vitro maturation (IVM) of oocytes. We investigated the effect of varying oxygen concentrations during IVM on subsequent pre and post-implantation development. METHODS: IVM of mouse cumulus-oocyte complexes (COCs) was performed under 2, 5, 10 or 20% O2 (6% CO2, balance N2). In vivo-matured COCs were collected post ovulation. Embryos were generated by IVF and culture. Blastocyst development, cell number and apoptosis were assessed, and fetal and placental outcomes analysed following embryo transfer at day 18 of pregnancy. RESULTS: Oxygen concentration during IVM did not affect oocyte maturation or subsequent fertilization, cleavage and blastocyst development rates. Maturation of oocytes under 2% O2 increased blastocyst trophectoderm cell number compared with all groups and numbers at 5% were higher than 20% (both P<0.05). Percentage of apoptotic cells was increased in blastocysts developed from 2% O2-matured oocytes, compared with maturation at 5% O2 or in vivo (P<0.05). Rates of embryo implantation and development into a viable fetus were not altered by IVM oxygen. However, fetal weight was reduced following oocyte maturation at 5% O2 compared with 20% O2 and maturation at 5% O2 also reduced placental weight, when compared with in vivo-matured oocytes (both P<0.05). CONCLUSIONS: Level of O2 exposure during oocyte maturation can alter the cellular composition of blastocysts, but these changes in cell number do not correlate with the altered fetal and placental outcomes after transfer.

Keywords: in vitro maturation; oocyte maturation; oxygen; trophectoderm

Introduction

Oocyte in vitro maturation (IVM) has been proposed as a possible adjunct to traditional IVF therapies for infertile couples, especially for women who respond inappropriately to gonadotrophin stimulation or as an alternative to stimulation for all patients. However, few pregnancies resulting from human oocyte IVM have been recorded and the process requires further improvement and assessment before becoming routine clinical practice (Trounson et al., 1994; Barnes et al., 1996; Cha and Chian, 1998; Cha et al., 2000; Kim et al., 2000; Smith et al., 2000). This is mirrored by results in most other mammalian species, where it is widely accepted that oocytes matured in vitro have a reduced developmental capacity when compared with in vivo-matured oocytes (Eppig and Schroeder, 1989; Combelles et al., 2002). In the large pre-ovulatory follicle, where the oocyte undergoes maturation in vivo, the oocyte is removed from any direct oxygen supply, with the follicular vasculature being totally excluded by the basal lamina (Hazzard and Stouffer, 2000; Plendl, 2000; Tamanini and De Ambrogi, 2004). As a result, the oocyte must gain oxygen from the surrounding vasculature by the process of diffusion across both the cellular layers of the follicle (mural granulosa cells and cumulus cells) and the fluid-filled antrum. The oxygen content of the follicular fluid has been measured in several species and has been shown in both bovine and humans to vary both between subjects and between follicles in the same subject (Van Blerkom et al., 1997; Huey et al., 1999; Berg et al., 2003).

The dissolved oxygen content of human follicular fluid ranges between 1.3 and 5.5% oxygen, with studies showing that this oxygen content has no association with frequency of oocyte meiotic maturation or fertilization, embryo cleavage or morphology (Van Blerkom et al., 1997; Huey et al., 1999). However, following fertilization, the ability of oocytes from low oxygen follicles (<1.5% O2) to develop to the 6- to 8-cell stage is reduced (Van Blerkom et al., 1997). It has also been shown that a high grade of follicular vascularity (as assessed by Doppler ultrasonography) correlates with a higher rate of pregnancy and live births following embryo transfer in women (Chui et al., 1997), suggesting that a threshold oxygen supply is important.
The optimal oxygen level for human IVM has not been determined and normally standard oxygen concentrations used for embryo culture [i.e. 5% O2 or air (21% O2)] have been applied during IVM. Little is known about how the in vivo follicular oxygen environment during the final stages of oocyte maturation or the oxygen environment during IVM influences the subsequent developmental potential of the oocyte. Where oxygen effects have been investigated there are conflicting results. In the bovine model, IVM at 5% O2 (5% CO2) dramatically decreased the viability of oocytes in several studies, when compared with 20% O2 (Pinyopummuntr and Bavister, 1995; Hashimoto et al., 2000), whereas oxygen levels appear to have no effect on pig oocytes (Park et al., 2005). Furthermore, blastocyst development rates have been shown to both improve (Park et al., 2005) or remain unchanged when porcine oocytes are matured in 20% oxygen (Kikuchi et al., 2002). Media composition may play a role in these discrepancies, as Hashimoto et al. (2000) also reported that 5% O2 during IVM supports bovine embryo development if the concentration of glucose is increased.

Currently, no studies examining the long-term developmental effects on embryos produced from oocytes matured under different oxygen environments have been performed. Accordingly, we investigated the impact of oxygen concentration during mouse IVM on oocyte maturation, subsequent preimplantation embryo development and post-transfer outcomes. The range of oxygen concentrations studied included 2, 5, 10 and 20% O2 mixtures. The parameters used to assess maturation and preimplantation embryo development were nuclear maturation, pronucleus formation, cleavage to 2-cell, blastocyst formation, blastocyst cell number and allocation as well as blastocyst cell apoptosis. Post-transfer measures included implantation and development, fetal crown-rump length and fetal and placental weights.

**Materials and Methods**

**COC collection and IVM**

All experiments were conducted according to the National Health and Medical Research Council of Australia guidelines for the use of animals and following approval from The University of Adelaide and Institute of Medical and Veterinary Science ethics committees. Unless otherwise specified, all chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, USA). For all IVM experiments, cumulus-oocyte complexes (COCs) were isolated from female hybrid CBAB6F1 mice (21 days old) that received 5 IU equine chorionic gonadotrophin (eCG) (Folligon; Serono, Rome, Italy) injected s.c. 44 h prior to oocyte collection. COCs were isolated in HEPES buffered +MEM media supplemented with 50 µg/ml Streptomycin, 75 µg/ml Penicillin G and 3 mg/ml fatty acid free bovine serum albumin (BSA) (ICPbio, Auckland, New Zealand) and fertilized in the same media using fresh sperm obtained from a CBAB6F1 male donor. After incubation with the sperm for 4 h, the eggs were washed three times in potassium simplex optimized media (KSOM) without amino acids but containing L-alanyl-L-glutamine (1.0 mM) (Glutamax; Invitrogen), placed in fresh 20 µl drops of KSOM under oil and cultured overnight. Embryos that cleaved to the 2-cell stage were transferred to a fresh drop of KSOM under oil and cultured for 72 h. Regardless of IVM conditions, all fertilization steps and embryo culture were carried out under an atmosphere of 5% O2, 6% CO2 and balance of N2 at 37°C.

**Fertilization assessment**

After the 4 h incubation for fertilization and following another 3 h of culture in fresh medium, a subset of presumptive zygotes was assessed for presence of the male pronucleus using orcein staining as previously described.

**Diff erential nuclear staining**

The number of cells within the inner cell mass (ICM) and trophectoderm (TE) of blastocysts was assayed by differential staining. Blastocysts were placed in 0.05% pronase solution at 37°C for up to 5 min to remove the zona pellucida before being washed in HEPES buffered version of G1 media (H-SG1) (Lane and Gardner, 2003). This was followed by incubation with ice-cold 0.5% (w/v) 2,4,6-trinitrobenzene sulfonic acid solution for 10 min at 4°C in the dark followed by a wash in H-SG1 media. Embryos were then incubated in a 0.1 mg/ml solution of anti-dinitrophenyl-BSA antibody (Becton-Dickinson Labware, Franklin Lakes, USA). Maturation of COCs under different oxygen concentrations was performed for 17 h at 37°C in modular incubation chambers (Billups-Rothenburg, Del Mar, USA) filled with test gas mixtures. The gas mixtures used were 2, 5, 10 or 20% O2 (6% CO2 and balance of N). Hereafter, these gas mixtures are referred to as simply 2, 5, 10 and 20% O2. For some experiments the 10% oxygen treatment group was not included. Culture dishes were prepared a day ahead and were allowed to equilibrate in the modulators at 37°C overnight.

**Maturation status assessment**

After IVM, a subset of COCs from each treatment group were treated with 50 µl/ovine hyaluronidase and all cumulus cells were removed with the aid of gentle pipetting. Denuded oocytes were collected, washed through fresh media, followed by phosphate-buffered saline (PBS) and were then fixed in ethanol: glacial acetic acid (1:3). Oocytes were fixed for a minimum of 48 h before staining with 1% aceto-orcein solution. Nuclear structures were visualized under phase contrast microscopy using a Leica DM IRB microscope (Leica, Germany).

**In vivo maturation**

For some experiments, in vivo-matured oocytes were generated. CBAB6F1 mice (21 days old) received a dose of 5 IU eCG and 5 IU HCG (Pregnyl; Organon) injected subcutaneously 61 and 13 h prior to fertilization, respectively. This ensured the in vivo-matured oocytes were at the same developmental stage as the IVM oocytes and were receptive to fertilization. In vivo-matured ova were collected from the oviduct into HEPES buffered αMEM media.

**In vitro fertilization**

Ova were washed twice in bicarbonate buffered αMEM media supplemented with 50 µg/ml Streptomycin, 75 µg/ml Penicillin G and 3 mg/ml fatty acid free bovine serum albumin (BSA) (ICPbio, Auckland, New Zealand) and fertilized in the same media using fresh sperm obtained from a CBAB6F1 male donor. After incubation with the sperm for 4 h, the eggs were washed three times in potassium simplex optimized media (KSOM) without amino acids but containing L-alanyl-L-glutamine (1.0 mM) (Glutamax; Invitrogen), placed in fresh 20 µl drops of KSOM under oil and cultured overnight. Embryos that cleaved to the 2-cell stage were transferred to a fresh drop of KSOM under oil and cultured for 72 h. Regardless of IVM conditions, all fertilization steps and embryo culture were carried out under an atmosphere of 5% O2, 6% CO2 and balance of N2 at 37°C.
for 10 min at 37°C. After a wash in H-SG1 media, complement-mediated lysis was induced by incubation with complement solution [guinea pig serum diluted in H-SG1 media with 20 μg/ml propidium iodide (PI)] at 37°C for 5 min. Finally, embryos were briefly washed in H-SG1 media, transferred to 25 μg/ml bisbenzimide (Hoechst 33342) in ethanol and stored overnight at 4°C in the dark. Embryos were stored in 100% alcohol in the dark at 4°C until visualized. Stained embryos were mounted in glycerol on a microscope slide and overlayed with a coverslip. The differential colour of the nuclei was examined using an Olympus AH-3 microscope (Olympus, Tokyo, Japan) whereby under ultraviolet light the ICM (stained with Hoechst 33342) appeared blue and the dual stained TE cells appeared pink/red. Number of ICM and TE cells was obtained for each embryo, and the ratio of ICM to total number of cells calculated.

**Embryo apoptosis assessment**

Apoptotic DNA was detected in blastocyst stage embryos using a terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labelling (TUNEL) assay (Roche Diagnostic, Penzberg, Germany) according to the manufacturer’s instructions. Briefly, blastocysts were washed in PBS supplemented with 3 mg/ml polyvinyl pyrrolidone (PVP) twice before being fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4°C. Embryos were then washed twice in PBS/PVP, permeabilized in 0.5% Triton X-100 for 1 h at room temperature and washed twice in PBS/PVP. Positive controls were then created by incubation with DNase I (0.005 U/μl) for 20 min at 37°C, which cleaves all DNA, after which these embryos were washed in PBS/PVP twice. All embryos were incubated in fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (TdT) (TUNEL reagents, Roche Diagnostic) for 1 h at 37°C in the dark. Negative controls were incubated in fluorescein-dUTP in the absence of TdT. After TUNEL, embryos were washed twice in PBS/PVP twice. Male pronucleus formation was assessed over a minimum of 5% Triton X-100 and once in PBS/PVP and mounted in glycerol for immediate analysis by fluorescence microscopy.

**Embryo transfer**

Naturally ovulating female Swiss mice aged between 8 and 12 weeks were mated with vasectomized CBAB6F1 males to induce pseudopregnancy. On day 4 of pseudopregnancy, the female recipients were anaesthetized with 2% Avertin (2,2,2-tribromoethanol in 2-methyl-2-butanol, diluted to 2% in sterile saline; 0.015 ml/g body weight) and six embryos transferred to each uterine horn (~90 h post-fertilization). Treatment groups were randomly allocated to mouse and horn by random number selection. The absence of transuterine migration following embryo transfer in mice has been verified in recent studies (Rulicke et al., 2006).

**Fetal and placental analysis at day 18 of pregnancy**

Embryo transfer recipients were sacrificed on day 18 of pregnancy by cervical dislocation. A post-mortem examination was carried out to determine the number of implanations and fetuses, as well as fetal and placental weight and fetal crown-rump length.

**Analysis of results**

Maturation status and pronuclear formation data is presented as mean and all other data are presented as mean ± SEM. All statistical analyses were carried out using either SigmaStat for Windows Version 2.03 or Statistical Package for the Social Sciences (SPSS) for Windows (SPSS Inc, Chicago, USA). Maturation status and pronuclear formation (fertilization) data was analysed using chi-squared analysis. Cleavage and blastocyst development data was subjected to arcsine transformation and analysed by two-way Analysis of Variance (ANOVA). Fetal and placental measures were analysed using a General Linear Model Univariate analysis with litter size as a covariate with Bonferroni post-hoc test. All other data was assessed using a one-way ANOVA unless stated. In addition to this, the ICM numbers were log transformed prior to analysis. A probability level of 5% (P < 0.05) was taken as significant for all analyses.

**Results**

**Maturation status**

Oocyte nuclear maturation status was assessed across five experimental replicates (total number of oocytes per group ≥34). The number of oocytes that reached metaphase II (MII) stage of maturation after IVM ranged 80.2–97.4%, but there were no significant differences in maturation rate following IVM under 2, 5, 10 or 20% O2 (Table 1).

**Fertilization rate**

Male pronucleus formation was assessed over a minimum of six replicates (n ≥ 16). The ability of oocytes to be fertilized by fresh sperm was not significantly affected by the oxygen concentration during IVM, as indicated by male pronucleus formation (Table 1). All treatment groups showed an average rate of fertilization between 87.5 and 95.8%.

**In vitro embryo development**

Development to 2-cell stage was determined across 20 replicates (n ≥ 419) and blastocyst development was examined over 14 replicates (n ≥ 240). Oxygen concentration during IVM had no effect on the number of oocytes that cleaved to the 2-cell stage (Table 1). Across the groups an average of 82.2–86.7% cleavage rate was observed. There was also no significant difference in the number of cleaved embryos that went on to form blastocysts (Table 1). However, there was a

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<tr>
<th>Table 1: The effect of IVM O2 concentration on maturation, fertilization, cleavage and blastocyst development rates in mice</th>
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<td>2% O2</td>
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<tr>
<td>Oocytes reaching MII (%)</td>
</tr>
<tr>
<td>Male pronucleus formation (%)</td>
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<tr>
<td>Cleavage to 2-cell (%)</td>
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<td>Blastocyst development from 2-cell (%)</td>
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MII, metaphase II.
trend for blastocyst development rates to decrease as oxygen concentration during IVM increased ($P = 0.08$).

**Differential nuclear staining**

Blastocyst total cell number decreased as oxygen during IVM increased (Fig. 1A). Blastocysts derived from the 2% oxygen-matured oocytes had more total cells, when compared with blastocysts from oocytes matured at 10 or 20% oxygen. Total blastocyst cell number also tended to be higher following oocyte IVM at 2% oxygen, compared with 5% O$_2$ ($P < 0.06$, Fig. 1A). The numbers of TE cells in the blastocysts also decreased as IVM oxygen level increased (Fig. 1B). Blastocysts from 2% oxygen-matured oocytes had increased numbers of TE cells, compared with all other treatment groups. TE cell number was also higher in blastocysts from 5% oxygen-matured oocytes, when compared with the 20% group. Oxygen concentration during IVM did not alter the numbers of ICM cells present in blastocysts (Fig. 1C). ICM-to-total cell ratio (expressed as a percentage) was lower in blastocysts derived from the 2%, compared with 20% oxygen-treated oocytes (Fig. 1D).

**Embryo apoptosis**

The average percentage of apoptotic cells was increased in blastocysts derived from oocytes matured at all oxygen levels compared with those derived from oocytes matured in vivo (Fig. 2). The blastocysts from the 2% oxygen oocytes also had significantly higher levels of apoptosis than those from oocytes matured at 5% oxygen.

**Fetal and placental outcomes**

Embryos were transferred to a total of 33 recipients with 6 embryos transferred per horn. A total of 22 of the recipients were pregnant in at least one horn at day 18 of pregnancy. Non-pregnant animals contained embryos from all treatment groups (2%: $n = 7$ horns; 5%: $n = 6$ horns; 20%: $n = 5$ horns; in vivo: $n = 2$ horns). These animals were not included in further analysis. A total of 43 uterine horns were studied (2%: $n = 11$; 5%: $n = 10$; 10%: $n = 9$; 20%: $n = 9$; in vivo: $n = 6$).

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**Figure 1**: The effects of O$_2$ treatment during IVM on (A) total number of blastocyst cells, (B) number of TE cells, (C) number of ICM cells and (D) percentage ICM cells of total cell number. Data are represented as mean ± SEM. A minimum of 24 blastocysts were stained for each treatment group across 10 experiments. Different superscripts denote a significant difference between treatment groups ($P < 0.05$).
n = 12; 20%: n = 10; in vivo: n = 10; one horn in one animal did not receive transfer due to uterine horn malformation). Blastocysts were generated from at least five experiments. No implantation sites were detected in three of the horns that received an embryo transfer (2%: n = 1; 5%: n = 2). Implantation rate and development of a viable fetus following implantation (viable fetuses/blasto cyst implanted) was not different between oocyte maturation groups or when compared with blastocysts derived from in vivo-matured oocytes (Table 2). Viable conceptuses present within a uterine horn at day 18 of pregnancy ranged from 1 to 5 (mean, 1.7 ± 0.2). Total number of fetuses carried by the recipient mothers ranged from 1 to 5 (mean, 1.7 ± 0.2). The number of viable fetuses at day 18 of pregnancy within a horn did not vary with treatment [2%, 1.7 ± 0.5 (n = 11 horns); 5%, 1.8 ± 0.4 (n = 12 horns); 20%, 1.9 ± 0.4 (n = 10 horns); in vivo, 1.6 ± 0.5 (n = 10 horns)]. Total number of fetuses carried by the mother was also calculated to provide an indication of total maternal load (mothers carrying a horn containing fetuses from 2% matured oocytes: fetal load, 3.4 ± 0.4, 5%: 3.4 ± 0.4, 20%: 3.4 ± 0.3, in vivo: 3.6 ± 0.4).

IVM at 2, 5 or 20% oxygen did not alter fetal weight, compared with those oocytes matured in vivo (Table 2). Fetal weight was reduced following IVM at 5% oxygen, compared with the 20% oxygen group (Table 2). This difference in fetal weight was independent of the number of fetuses or implantations within the treatment horn, or the total number of fetuses or implantations within the mother. Fetal crown-rump length was not altered following maturation at any oxygen concentration, when compared across treatment groups, or to those matured in vivo.

Placental weight was reduced following IVM at 5% oxygen, compared with placentae derived from in vivo-matured oocytes (Table 2). This difference in placental weight was independent of the number of implantations within the treatment horn, or total litter size, but was not significant when corrected for the number of fetuses within the treatment horn. The fetal: placental weight ratio was not altered by maturation oxygen environment.

**Discussion**

Since the 1970s, the study of oxygen concentration during mouse oocyte maturation has almost exclusively involved the use of 5 or 20% oxygen mixtures (Eppig and Wigglesworth, 1995; Hu et al., 2001; Adam, 2004). This is largely due to previous studies showing that a 5% oxygen concentration is optimal for oocyte nuclear maturation (as measured by extrusion of a polar body) in both mouse and hamster (Haidri et al., 1971; Gwatkin and Haidri, 1974) and that mouse embryo culture is widely performed under 5% O₂ (Harlow and Quinn, 1979). In the current study, we have demonstrated that the oxygen concentration under the conditions used during mouse IVM of COCs, including 2% oxygen, has no effect on the ability of those oocytes to reach the MII stage of nuclear maturation, fertilize, cleave or reach the blastocyst stage of embryo development. However, the oxygen environment during IVM does affect the quality of resulting blastocysts, revealing for the first time that oxygen concentration during IVM affects subsequent blastocyst cell lineage in the mouse. Total blastocyst cell number decreased as oxygen concentration during oocyte maturation increased. This change in blastocyst total cell number can be attributed to a reduction in the number of TE cells with increasing O₂ levels. Although apoptosis rates were also higher in blastocysts derived from

**Table 2:** The effect of IVM O₂ concentration on implantation rates and subsequent fetal and placental development

<table>
<thead>
<tr>
<th></th>
<th>2% O₂</th>
<th>5% O₂</th>
<th>20% O₂</th>
<th>In vivo</th>
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<tr>
<td>Implantation rate</td>
<td>69.7 ± 10.5</td>
<td>56.9 ± 9.0</td>
<td>80.0 ± 5.4</td>
<td>65.0 ± 10.1</td>
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<tr>
<td>Viable fetuses/blasto cyst implanted</td>
<td>45.0 ± 10.3</td>
<td>49.3 ± 8.8</td>
<td>42.0 ± 9.0</td>
<td>39.7 ± 10.4</td>
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<tr>
<td>Fetal weight (mg)</td>
<td>870.2 ± 26.7</td>
<td>823.3 ± 28.1</td>
<td>928.5 ± 26.1</td>
<td>879.3 ± 32.3</td>
</tr>
<tr>
<td>Fetal crown-rump length (mm)</td>
<td>18.4 ± 1.0</td>
<td>19.9 ± 0.3</td>
<td>20.1 ± 0.3</td>
<td>19.3 ± 0.4</td>
</tr>
<tr>
<td>Placental weight (mg)</td>
<td>98.7 ± 5.5</td>
<td>87.4 ± 4.0</td>
<td>100.1 ± 5.5</td>
<td>104.5 ± 5.4</td>
</tr>
<tr>
<td>Fetal: placental weight ratio</td>
<td>9.1 ± 0.4</td>
<td>9.8 ± 0.5</td>
<td>9.6 ± 0.5</td>
<td>8.8 ± 0.5</td>
</tr>
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</table>

Data for in vivo-matured oocytes are also shown. Number of blastocysts transferred, 2% n = 60; 5% n = 72; 20% n = 60; in vivo n = 60. aImplantation rate: number of fetal/placental units or resorptions present as a percentage of the number of embryos transferred. bViable fetuses/blasto cyst implanted: percentage of implanted embryos that developed into a viable fetus (2%, n = 19; 5%, n = 21; 20%, n = 19; in vivo, n = 16 viable fetuses). Different superscripts within a row represent statistically significant differences, P < 0.05.
oocytes matured at 2% oxygen, this increase in total cell number was not fully accounted for by the presence of apoptotic cells, as these only represented a small number of cells. Higher total cell number was also observed in porcine blastocysts following IVM of oocytes under 5% oxygen and electrical pulse activation or fertilization with frozen sperm (Kikuchi et al., 2002; Iwamoto et al., 2005). However, an increased activation rate in the 5% oxygen-matured oocytes was also observed (Iwamoto et al., 2005). This is in contrast to our study, which found no significant effect of oxygen environment during oocyte maturation on fertilization rates with fresh sperm. Recently, work by Preis et al. (2007) also found similar rates of blastocyst formation regardless of IVM oxygen level, with increased cell number following IVM under 5% oxygen compared with 20%.

The mechanism by which this increase in TE cell number occurs is not known and may not be a direct consequence of the oxygen concentration used to mature oocytes. Furthermore, we have not characterized TE cell numbers following in vivo oocyte maturation followed by IVF. TE cells form the placental interface and are therefore involved in oxygen and nutrient transport to the fetus. Furthermore, oxygen concentration is a significant regulator of TE cell differentiation and proliferation (Caniggia and Winter, 2002). Nevertheless, blastocyst cell numbers are lower when embryos are cultured in 2% O2 compared with 7% O2, which is also reflected in the differential expression of O2-sensitive genes in resulting blastocysts (Kind et al., 2004). However, whether 2% oxygen represents a hypoxic stimulus for the mouse oocyte is uncertain, as no studies have investigated the in vivo follicular oxygen concentration in the mouse. Data from human, bovine and porcine follicles, suggests 2% oxygen would be considered in the low range (Knudsen et al., 1978; Van Blerkom et al., 1997; Huey et al., 1999; Haddad, 2002; Berg et al., 2003). Conversely, 20% oxygen represents a non-physiological oxygen condition, and it is also possible that an increase in oxidative stress during IVM in the 20% group may contribute to the alteration in cell fate programming.

We have shown that ICM number was not significantly altered following IVM at different oxygen concentrations; however, the decrease in total cell number resulted in an alteration of the ICM-to-total cell ratio. The total blastocyst cell number and the number of ICM cells have been reported as positively correlated with fetal development after embryo transfer (Lane and Gardner, 1997). The cell number results in the current study therefore suggested that embryos derived from high oxygen-matured oocytes may have a reduced developmental capacity once transferred. However, this was not found to be so. Regardless of the oxygen environment during maturation, the subsequent blastocysts were able to implant and develop at the same rate as those derived from oocytes matured in vivo. The number of total cells present in the blastocyst at the time of transfer did not, in this case, correlate with implantation success.

Maturation under 5% oxygen gave rise to fetuses that were significantly lighter than those derived from 20% oxygen-matured oocytes. This suggests a more adverse outcome following IVM under 5% oxygen in terms of fetal weight, however, these weights were not different from those resulting from fetuses derived from in vivo oocyte maturation. The placentae from 5% oxygen-matured oocytes also weighed less and in this instance they were significantly different compared with in vivo controls. Although the structure and function of the placentae has not been investigated here, there was no alteration in the fetal-to-placental weight ratio, suggesting that placental function may not have been altered.

Across all our comparisons to the in vivo-matured oocyte and subsequent embryo and conceptus development, we could find no evidence that any of our treatments resulted in poorer outcomes when compared with oocytes matured in vivo. Contrary to the current dogma, we found little evidence that a low level of oxygen during the final stages of oocyte maturation has any sustained detrimental effect on outcomes for the conceptus. Nevertheless, although 2% O2 is considered a moderate hypoxic state, lower O2 concentrations may indeed induce more detrimental short- and long-term outcomes. Longer-term post-natal outcomes following IVM are not addressed by this study, and have been little studied by others. The longer-term outcome for offspring following IVM should be investigated as it is possible the short-term differences seen in this study will translate to effects in adulthood as is the case with other reproductive technologies (Thompson et al., 2007).

In vivo, the oocyte is buffered from the external environment by other cellular follicular components. During IVM, the surrounding cumulus cells also buffer the oocyte from the external environment. Using mathematical modelling, it has been calculated that little oxygen is lost through diffusion across the cumulus cell layer that surrounds the oocyte, with the mouse cumulus consuming ~0.5% oxygen when the follicular fluid oxygen concentration is in the range of 3–20% (Clark et al., 2006). Therefore, it is unlikely that an anoxic state was observed at the surface of the oocyte under the conditions utilized here. However, we have yet to determine, under these incubation conditions, if O2 concentration is rate-limiting for oxidative phosphorylation, especially at the lower concentrations. Further studies will be aimed at investigating ATP production and mitochondrial function and location within oocytes matured under differing O2 concentrations.

Low oxygen availability in the follicle has been shown to correlate with increased oocyte chromosomal abnormalities and decreased pregnancy rate (Chui et al., 1997; Van Blerkom et al., 1997). The exposure of an oocyte to a low oxygen environment during follicular growth is likely to be longer than the relatively short exposure during IVM (17 h). Therefore, it remains to be determined if detrimental effects on long-term outcomes may be more dramatic from a chronic exposure to low oxygen. Another consideration that Hashimoto et al. (2000) revealed is that cattle oocyte developmental potential when matured under different O2 concentrations depended on the level of glucose availability. We cannot rule out that our results may have differed if an alternative IVM system had been utilized.

Finally, all of the oocytes used in this study were in vitro fertilized and the resulting zygotes cultured. It is known that the embryo culture environment can affect the subsequent long-term outcomes (Fleming et al., 2004; Thompson et al.,
In this study, relatively small differences were observed between embryos derived from \textit{in vivo}-matured oocytes and those from IVM. Therefore it is plausible that developmental potential of blastocyst-stage embryos is primarily determined by the culture environment during embryo development in comparison with conditions used during IVM. This could be examined if we had compared the survival amongst \textit{in vivo}-derived blastocysts with the treatments outlined here. For IVM to become routine clinical practise as an addition to human infertility treatment, such as IVF, the effects of this additional manipulation must be fully understood and the environment optimized. Indeed, even when used in an experimental setting, it is important to comprehend the changes induced in outcomes by environmental factors. The results of this study demonstrate that the oxygen environment under which the oocyte is matured can alter cell number in resulting blastocysts, which appear to not correlate well with the subtle changes in subsequent fetal and placental parameters. This suggests that changes to the embryo development induced by IVM oxygen concentration are not necessarily reflected in altered fetal outcomes following transfer. If this study had followed the usual paradigm of simply comparing 5 versus 20\% O\textsubscript{2} (or air) atmospheres during IVM, very different conclusions would have been reached. The data obtained under this circumstance would have led to conclusions that differences in cell allocation in blastocysts correlated to differences in fetal and placental weight, suggesting in general that low O\textsubscript{2} (i.e. 5\%) during IVM is not suitable for subsequent development. This study shows the value of an expanded comparison when determining the effect of environmental parameters on developmental outcomes.

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