Reproductive BioMedicine Online (2010) **21**, 402–410

www.sciencedirect.com
www.rbmonline.com

ARTICLE

Time-lapse analysis of mouse embryo development in oxygen gradients

PL Wale, DK Gardner *

Department of Zoology, University of Melbourne, Parkville, Vic. 3010, Australia
* Corresponding author. *E-mail address:* david.gardner@unimelb.edu.au (DK Gardner).

Petra Wale has worked in clinical IVF for over a decade both in Australia, at Melbourne IVF, and in the USA, at the Colorado Center for Reproductive Medicine. She is currently undertaking her PhD with Professor David Gardner at the University of Melbourne, Australia. Her main interests include embryo culture technologies and the development of embryo viability assays.

Abstract Atmospheric oxygen (~20%) in culture significantly impairs preimplantation embryo development. However, it is not known whether all stages of preimplantation embryo development are susceptible to oxygen toxicity. This study investigated the temporal responses of preimplantation embryos to oxygen conditions *in vitro*. Mouse embryos were cultured in atmospheric (~20%) or lower (5%) oxygen concentrations for the first 48 h, followed by culture in the same or reciprocal oxygen concentrations for another 48 h: group 1 (control, 5 and 5%); group 2 (5 and 20%); group 3 (20 and 5%); and group 4 (20 and 20%). Time-lapse microscopy was performed with imaging of individual embryos at 15-min intervals. Compared with embryos cultured in 5% oxygen, embryos cultured in 20% oxygen were delayed at the 1st cleavage by 0.45 h ($P < 0.05$), at the 2nd cleavage by 0.84 h ($P < 0.01$) and at the 3rd cleavage by 3.19 h ($P < 0.001$). Switching from 20% to 5% oxygen after 48 h did not completely alleviate earlier induced perturbations. Partial or complete culture in atmospheric oxygen resulted in significantly fewer blastocyst cell numbers compared with control ($P < 0.05$). Oxygen can influence mouse embryo development at both the cleavage and post-compaction stages.

KEYWORDS: blastocyst culture, development kinetics, oxygen concentration, time-lapse

Introduction

Historically, preimplantation embryos of the human and other eutherian mammals have been cultured under atmospheric oxygen (~20%) (Edwards et al., 1981), a protocol derived from earlier somatic tissue culture techniques (Ham and Puck, 1962). However, experimental analysis of oxygen concentrations within the oviduct and uterus of different mammalian species has revealed that oxygen concentration typically lies in the range of 2–8% (Fischer and Bavister, 1993; Maas et al., 1976; Mastroianni and Jones, 1965). Lower oxygen culture, at around 5–7%, has been shown to improve embryo development in several species including the sheep (Thompson et al., 1990), cow (Thompson et al., 1990), goat (Batt et al., 1991) and human (Catt and Henman, 2000; Ciray et al., 2009; Kovacic and Vlaisavljevi, 2008; Kovacic et al., 2009; Meintjes et al., 2009; Waldenström et al., 2009). Furthermore, increased blastocyst cell number and improved

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2010.04.028

fetal development have been reported from mouse embryos cultured in reduced oxygen concentrations (Quinn and Harlow, 1978; Harlow and Quinn, 1979; Gardner and Lane, 1996; Karagenc et al., 2004).

Several studies have indicated that the preimplantation embryo exhibits temporal sensitivity to oxygen. Pabon et al. (1989) observed delays in development to the morula stage when embryos had been exposed to 20% oxygen as pronucleate oocytes, for as little as 1 h, followed by cultured in 5% oxygen. Similarly, Gardner and Lane (1996) determined that culture media equilibrated for 6 h in 20% oxygen prior to culture in 5% oxygen was detrimental to subsequent embryo development, while Karagenc et al. (2004) more recently reported that exposure of pronucleate oocytes to 20% oxygen for 23 h resulted in a significant decrease in blastocyst cell number. Of interest, several studies have reported no difference in blastocyst development rates when the preimplantation embryo was only exposed to atmospheric oxygen concentration at post-compaction stages (Karagenc et al., 2004; Kind et al., 2005; Feil et al., 2006). Collectively, such works suggest that the pronucleate oocyte has a greater sensitivity to atmospheric oxygen, the consequences of which may only be apparent with more accurate assessment over the complete period of preimplantation development.

Morphological assessment of the preimplantation embryo typically involves observations at discrete time points, usually once every 12—24 h, generally during work hours, in line with standard laboratory practices. Furthermore, the proportion of embryos that reach a specific stage at these discrete time points may be heavily influenced by the particular culture system employed and as a result the significance of specific time points will vary between laboratories (Bavister, 1995; Shoukir et al., 1997). A novel way to increase the sensitivity of morphological evaluation is through the use of high-frequency temporal assessment of embryo development with time-lapse microscopy. Using such an approach, this study aimed to determine the temporal effect of atmospheric oxygen on embryo development by assessing the embryos response to either a static or changing concentration of oxygen.

Materials and methods

Animals and hormonal stimulation

Pronucleate oocytes were obtained from F1 hybrid (C57BL/6 × CBA/Ca) mice. Animals were housed in a 12 h light and 12 h dark photoperiod with food and water *ad libidum*. Six-week old females were superovulated with intraperitoneal injections of 5 IU pregnant mare's serum gonadotrophin (Folligon; Intevet, UK) followed 48 h later by 5 IU human chorionic gonadotrophin (HCG, Chorulon; Intervet). Females were mated with F1 males overnight following the HCG injection. The presence of a vaginal plug the following morning was used as an indicator of successful mating.

Embryo collection and culture

Pronucleate oocytes were collected at 22 h post HCG in G-MOPS handling medium (Lane and Gardner, 2004) supplemented with 5 mg/ml human serum albumin, followed by cumulus removal in G-MOPS containing 300 IU/ml hyaluronidase (bovine testes, type IV; Sigma Aldrich). Pronucleate oocytes were removed from the hyaluronidase immediately once the cumulus cells had detached, washed twice in G-MOPS and then once in G1 medium before culture. All embryos were cultured individually in 2 μl drops of G1/G2 medium (Gardner and Lane, 2002, 2007) supplement with 5 mg/ml human serum albumin under paraffin oil (Ovoil; Vitrolife) at 37°C in a multi-gas incubator set to 6% CO_2, 5% O_2, 89% N_2 (lower oxygen) or 6% CO_2 in air (atmospheric oxygen). After 48 h, all embryos were transferred into G2 medium with half of the embryos cultured in the same gas phase, and the remaining half cultured under the alternate gas phase. This resulted in a two-by-two cross-over experiment, with a total of four treatments consisting of a control group, group 1 (5 and 5%) and three test groups, group 2 (5 and 20%), group 3 (20 and 5%) and group 4 (20 and 20%). A comparison to in-vivo development was made by obtaining 2-cell embryos flushed from the female reproductive tract at a pre-designated time.

Time-lapse analysis

All embryos were cultured in a multi-gas cell imaging incubator (MCOK-5M[RC]; Sanyo) containing an inverted microscope through which an image of each individual embryo could be obtained every 15 min without disturbance of culture. Blastocysts were morphologically defined as expanded or hatching/hatched. Blastocyst cell number was determined on the morning of day 5 immediately following the final morphological assessment at 118 h post HCG. Blastocyst cell numbers were analysed by individually staining embryos with 0.1 mg/ml bisbenzimide (Hoescht, 33342; Sigma Chemical Co) in 10% ethanol for 1 h followed by rinsing in G-MOPS with 5 mg/ml human serum albumin. All steps were preformed at 37°C. The embryos were mounted in glycerol under individual cover slips on a clean glass slide before being observed under fluorescent light (Eclipse TS100-F; Nikon). The number of cells was counted at ×200 magnification.

Experimental design

Pronucleate oocytes were collected from four or five individual females per treatment replicate and cultured separately in order to track embryo populations from each female. Analysis between female donors within each treatment group revealed no differences. The four treatment groups were examined in succession, i.e. group 1, then group 2, then group 3 and finally group 4. This was then repeated a further two times to give three replicates. Embryo development of at least 100 embryos per treatment group was assessed.

Statistical analysis

A two-tailed *t*-test was used to analyse the interaction between oxygen concentration in the first 48 h and cell cycle length. Differences between all treatments and the timing of blastocyst development, as well as cell number, were subjected to a one-way analysis of variance (ANOVA)

followed by Bonferroni multiple comparisons test for parametric data and a Kruskal–Wallis test followed by Dunn multiple comparison test for non-parametric data. The differences in morphological development from each treatment were analysed using the Chi-squared test.

Results

Embryos cultured in atmospheric oxygen for the first 48 h were delayed by 0.45 h ($P < 0.05$) at the first cleavage event and this delay was extended further to 0.84 h ($P < 0.05$) at the second cleavage event (**Table 1**). The length of the second cell cycle for embryos cultured in 20% oxygen was 20.79 ± 0.11 h compared with 20.40 ± 0.08 h for embryos cultured in 5% oxygen ($P < 0.001$) and the third cell cycle length was 14.30 ± 0.39 h for 20% and 11.95 ± 0.09 h for 5% ($P < 0.001$). At the third cleavage event, embryos cultured in 20% oxygen for the initial 48 h were delayed 3.19 h ($P < 0.001$) compared with embryos cultured in 5% oxygen. The timing of cleavage to 5, 6, 7 and 8 cells for embryos cultured in 20% oxygen was significantly different ($P < 0.001$) from embryos cultured in 5% oxygen (**Table 1**).

The range of time over which the first three cleavage events occurred increased as embryo development proceeded. However, there was a disproportionate increase in this range for embryos cultured in 20% oxygen for the first 48 h period compared with embryos cultured for the same period in 5% oxygen. For the first cleavage event, the range was 10 h for both 20% and 5% (**Figure 1**a). Then, for the second cleavage event the range was 14 h for 20% and 12 h for 5% (**Figure 1**b). By the third cleavage event, the range over which embryos cultured in 20% oxygen cleaved to the 8-cell stage was 21 h, whereas for the embryo population cultured in 5% the range was 15 h (**Figure 1**c). Analysis of cumulative development clearly shows that for any given time period the total percentage of embryos cleaved is less with 20% oxygen (**Figure 2**).

Oxygen concentration also appeared to influence the timing of the blastocoel appearance, full blastocoel expansion and the first sign of hatching (**Table 2**). Embryos cultured for the initial 48 h in 20% oxygen demonstrated further delay in their development with the blastocoel appearing at 2.84 h (group 3) and 3.49 h (group 4), after the control ($P < 0.001$). For embryos initially cultured at 5% and then switched to 20% oxygen (group 2), blastocoel appearance was also delayed, occurring 1.20 h after the control.

Overall, the proportions of pronucleate oocytes reaching the expanded blastocyst stage on day 5 were 90% (control), 87% (group 2), 68% (group 3) and 73% (group 4) (**Table 3**). There was a significant decrease in the portion of embryos reaching the blastocyst stage in groups 3 and 4 compared with both the control ($P < 0.001$) and group 2 ($P < 0.05$). Exposure of embryos to 20% oxygen for the first 48 h (groups 3 and 4) resulted in a significant decrease in the total number of cells in the resultant blastocysts (**Table 3**) compared with either the control ($P < 0.001$) or group 2 ($P < 0.01$). Similar blastocyst cell numbers were observed in group 3 compared with group 4. Blastocysts that were exposed to 20% oxygen at any stage (groups 2–4) had significantly fewer cells compared with the control ($P < 0.05$) (**Table 3**).

In the control group (group 1), the median of the first cleavage event was 31.3 h post HCG. When in-vivo-derived embryos were flushed at the same time point, 56.8% were at the 2-cell stage. The status of the first cell cycle at 31.3 h post HCG was assessed in all four treatments, with embryos that cleaved to the 2-cell stage before 31.3 h designated as early cleaving embryos and those that cleaved after 31.3 h were defined as late cleaving embryos. Embryos cultured in 5% oxygen had a significantly larger portion of early cleaving embryos with 52.8% compared to 42.8% for embryos cultured in 20% oxygen ($P < 0.05$). **Figure 3** represents the association between the first cleavage event with the proportion of embryos reaching the blastocyst stage (**Figure 3**a) and the total number of cells in the resultant blastocysts (**Figure 3**b). Across all four treatment groups, there was a relationship between the timing of the first cleavage event and blastocyst cell numbers, with late cleaving embryos resulting in blastocysts with significantly fewer cell numbers ($P < 0.01$, **Figure 3**a).

Table 1 The effect of oxygen for the first 48 h of culture on the timing of 1st, 2nd and 3rd cleavage events.

Cleavage event	*Stage*	*Treatment group*	
		1 and 2 (5% O_2)	*3 and 4 (20% O_2)*
1st cleavage	2 cell	31.44 ± 0.13[a]	31.89 ± 0.15[a]
	3 cell	51.01 ± 0.15[b]	51.68 ± 0.20[b]
2nd cleavage	4 cell	51.84 ± 0.16[b]	52.68 ± 0.20[b]
	5 cell	62.37 ± 0.17[c]	65.54 ± 0.28[c]
	6 cell	62.96 ± 0.17[c]	66.07 ± 0.28[c]
	7 cell	63.39 ± 0.17[c]	66.57 ± 0.30[c]
3rd cleavage	8 cell	63.79 ± 0.19[c]	66.98 ± 0.30[c]

Values are mean ± SEM hours after administration of human chorionic gonadotrophin.
[a–c]Same letters within a row represent significant differences between treatment groups: a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$.

Discussion

This study has revealed that the detrimental effects of atmospheric oxygen on mouse embryos during in-vitro culture are evident from the first cleavage division. Continuous assessment of embryo development, using time-lapse microscopy, revealed that oxygen plays an important physiological role throughout the preimplantation period. Oxygen had a biphasic effect on embryo development with the greatest effect during the cleavage stages, plausibly reflecting a greater sensitivity of embryos to oxygen toxicity prior to compaction. Overall, atmospheric oxygen resulted in slower cleaving embryos, decreased blastocyst development and decreased blastocyst cell numbers. Importantly, the detrimental effects of atmospheric oxygen on the early embryo were irreversible, as switching the embryo to a

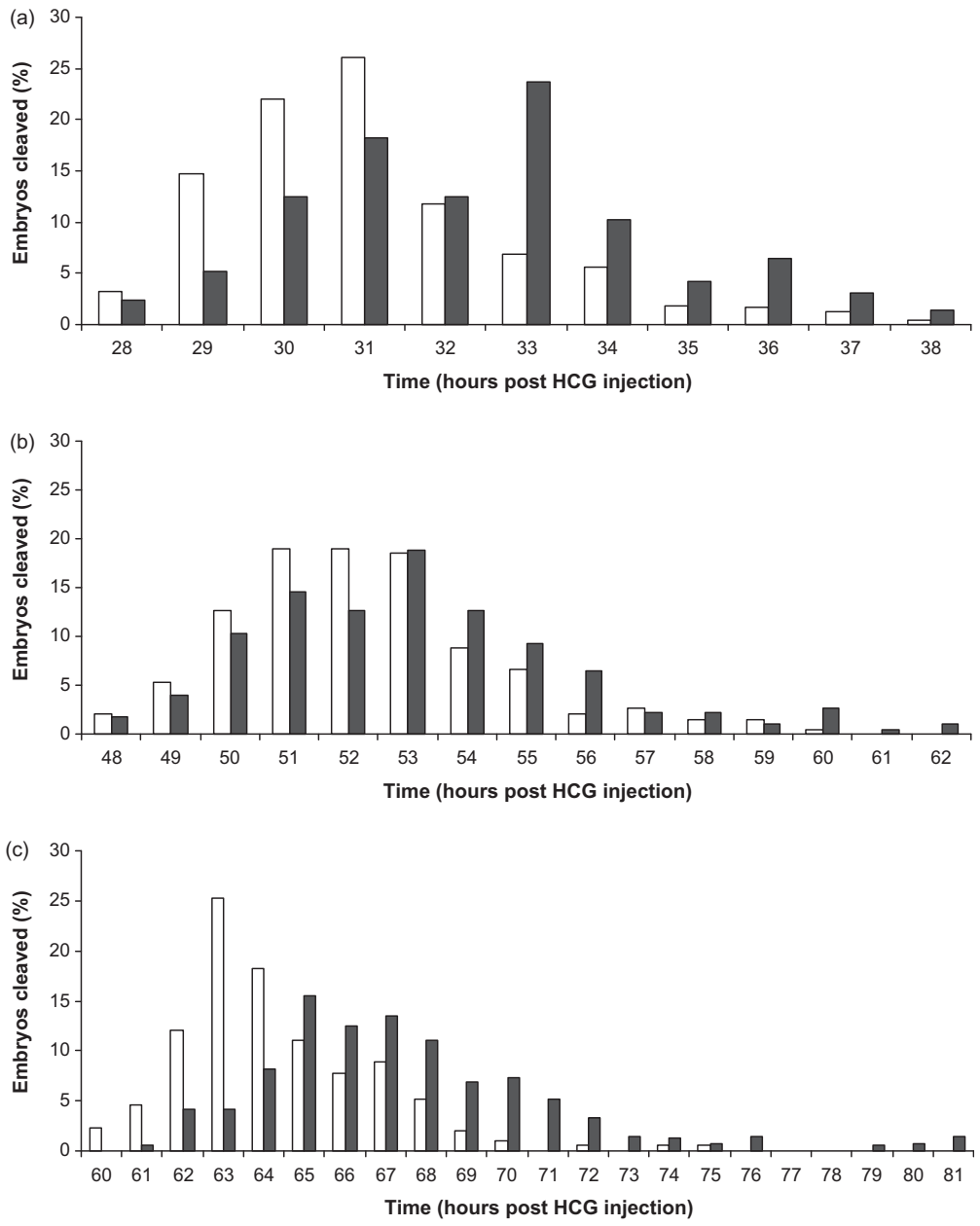

Figure 1 Distribution of cleavage timing for 1st, 2nd and 3rd cleavage divisions. (**a**) 1st cleavage (2 cells). (**b**) 2nd cleavage (4 cells). (**c**) 3rd cleavage (8 cells). White bars represent 5% oxygen concentration (groups 1 and 2) and black bars represent 20% oxygen concentration (groups 3 and 4).

reduced oxygen concentration for the second 48 h of development (post compaction) was not successful in alleviating the developmental perturbations induced during the initial 48 h.

The negative effect of culturing mouse embryos in 20% oxygen, observed in this study, is in agreement with several other animal studies (Batt et al., 1991; Gardner and Lane, 1996; Karagenc et al., 2004; Kitagawa et al., 2004; Leoni et al., 2007; Quinn and Harlow, 1978; Thompson et al., 1990; Umaoka et al., 1992). Furthermore, the results of this study are in agreement with those of Pabon et al. (1989), Gardner and Lane (1996) and Karagenc et al. (2004), in highlighting the sensitivity of the pronucleate oocyte to atmospheric oxygen. It has previously been reported that culturing embryos in 20% oxygen reduces blastocyst cell numbers if culture commences at the pronucleate oocyte or 2-cell stage, but not at the 8-cell stage (Karagenc et al., 2004). Similarly, Kind et al. (2005) reported no difference in blastocyst cell numbers from embryos cultured in 7% or 20% oxygen after the post-compaction period. However, in this study, in which embryos were cultured individually, switching from 5% to 20% oxygen for the second 48 h of development had a significant effect on blastocyst cell numbers. A plausible explanation for this difference is that group culture assists embryo development, in atmospheric oxygen, either through paracrine factors or a reduced local

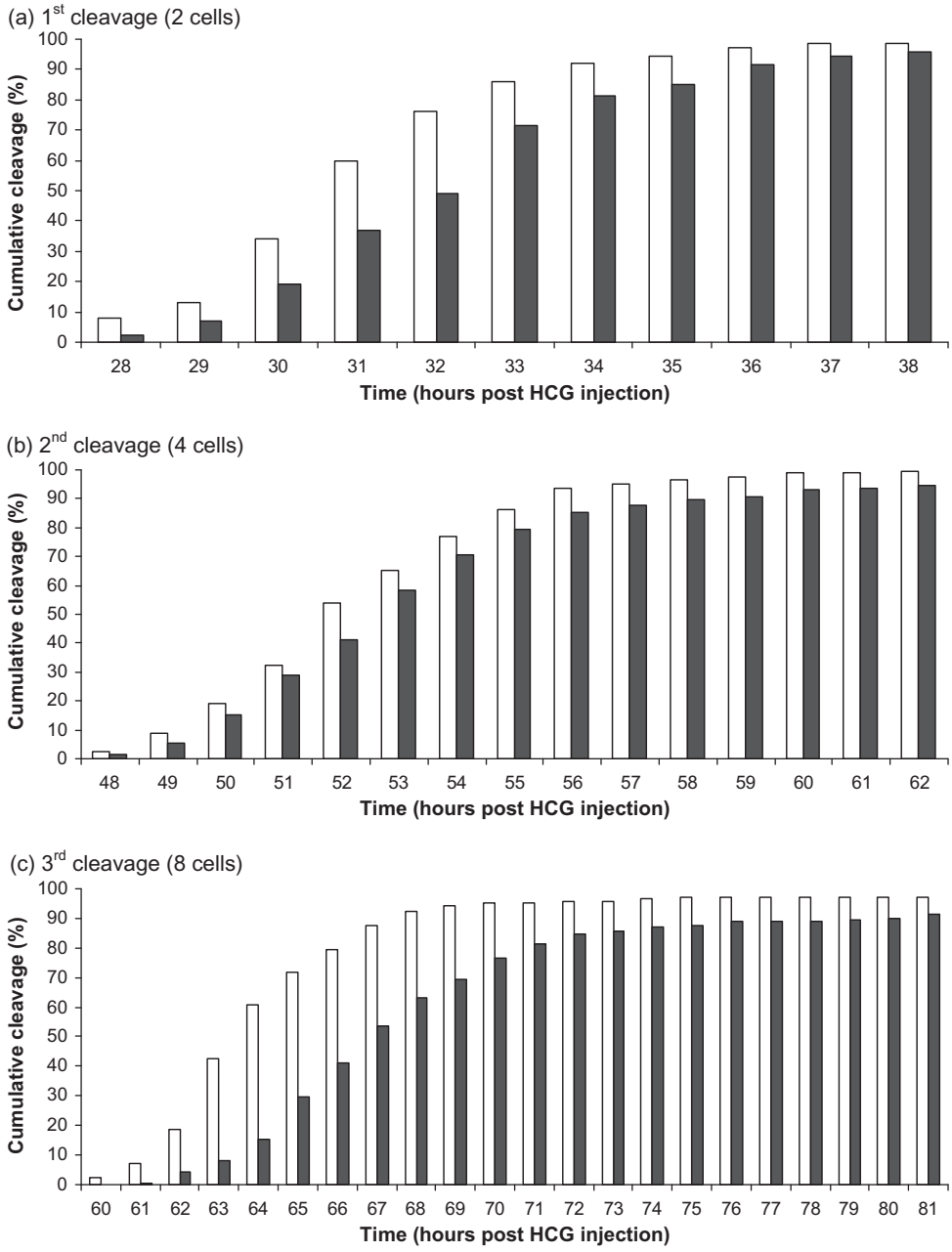

Figure 2 The cumulative percentage of embryos which had progressed through the 1st, 2nd and 3rd cleavage divisions. (**a**) 1st cleavage (2 cells). (**b**) 2nd cleavage (4 cells). (**c**) 3rd cleavage (8 cells). White bars represent 5% oxygen concentration (groups 1 and 2) and black bars represent 20% oxygen concentration (groups 3 and 4).

Table 2 The effect of oxygen on the timing of blastocyst development and hatching.

Development event	*Treatment group*			
	1 (5 and 5%)	*2 (5 and 20%)*	*3 (20 and 5%)*	*4 (20 and 20%)*
Appearance of the blastocoel	89.11 ± 0.29^{c,d}	90.31 ± 0.44^{a,b}	91.95 ± 0.55^{a,c}	92.60 ± 0.57^{b,d}
Full blastocoel expansion	95.75 ± 0.48^{a,c}	96.41 ± 0.46^{b}	98.21 ± 0.63^{a}	98.93 ± 0.60^{b,c}
First sign of hatching	102.50 ± 0.73	102.72 ± 0.87	104.70 ± 1.19	105.02 ± 0.85

Values are mean ± SEM hours after administration of human chorionic gonadotrophin.
$^{a-d}$Same letters within a row represent significant differences between treatment groups: a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$; d = $P < 0.001$.

Table 3 The effect of oxygen on blastocyst development rates and total cell numbers.

Parameter	*Treatment group*			
	1 (5 and 5%) (n = 126)	*2 (5 and 20%) (n = 107)*	*3 (20 and 5%) (n = 116)*	*4 (20 and 20%) (n = 107)*
Development to 2 cells (%)	99.2	99.1	96.6	95.1
Blastocyst development (%)	$90.4^{d,e}$	$86.8^{a,b}$	$68.2^{b,d}$	$72.5^{a,e}$
Blastocyst cell number (mean ± SEM)	$95.51 \pm 2.66^{a,d,e}$	$85.43 \pm 2.25^{a,b,c}$	$71.22 \pm 3.01^{b,d}$	$72.51 \pm 3.04^{c,e}$

[a–d]Same letters within a row represent significant differences between treatment groups: a = $P < 0.05$; b = $P < 0.1$; c = $P < 0.01$; d = $P < 0.001$; e = $P < 0.01$.

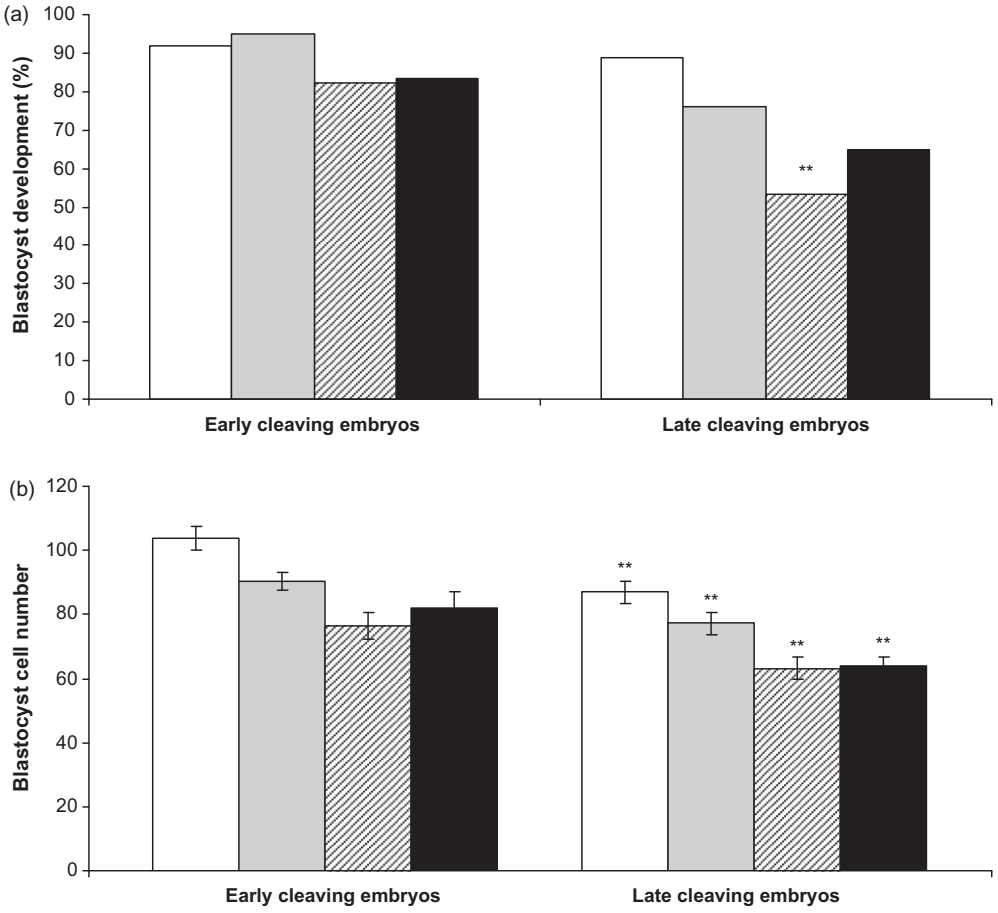

Figure 3 Association between timing of first cleavage event and blastocyst development rates and blastocyst cell numbers. (**a**) Percentage blastocyst development; ** = significant difference between early cleaving embryos within the same treatment ($P < 0.05$). (**b**) Mean ± SEM blastocyst cell number; ** = significant difference between from early cleaving embryos within the same treatment ($P < 0.01$). Embryos that cleaved to the 2-cell stage before the median time (31.3 h post HCG) for the control group (5 and 5% O_2) were designated as early cleaving embryos and those that cleaved after the median were defined as late cleaving embryos. White bars = control group; grey bars = group 2 (5 and 20% O_2), hashed bars = treatment 3 (20 and 5% O_2); black bars = group 4 (20 and 20% O_2).

oxygen concentration as a result of the increased embryo density.

Furthermore, results from this study, revealed that the detrimental effects of atmospheric oxygen are cumulative. As a result of culture in 20% oxygen, the delay in the timing of cleavage events became more pronounced as embryo development progressed. In addition, embryos cultured exclusively in 20% oxygen had the most delayed development, with the longest time from HCG injection to appearance of the blastocoel, full blastocoel expansion and first sign of hatching. Similarly, Karagenc et al. (2004) reported a chronic effect of atmospheric oxygen, with a significant reduction in cell numbers when embryos were exposed to 20% oxygen for 95 h when compared with 23 h or 46 h.

The timing of the first cleavage is considered a non-invasive marker of embryo developmental potential and is routinely used in human IVF programmes to identify embryos with higher implantation potential (Lawler et al., 2007). Several studies have observed that embryos cleaving early are more likely to develop into blastocysts (Arav et al., 2008; Sakkas et al., 1998; Shoukir et al., 1997). In this study, a significantly larger portion of early cleaving embryos was reported from culture in 5% oxygen, with this portion more analogous to the portion of early cleaving embryos observed from in-vivo flushing at the same time point. Although with all four treatments, blastocysts resulting from embryos cleaving to the 2-cell stage early had significantly greater cell numbers. Since, blastocyst cell number has previously been correlated with subsequent fetal development in the mouse (Lane and Gardner, 1997), this study's data support the analysis of early cleavage as a predictor of developmental capacity irrespective of culture conditions.

It has been reported that the transfer of blastocysts developed in 20% oxygen for the entire culture period, does not affect the implantation potential process *per se* (Harlow and Quinn, 1979; Karagenc et al., 2004). However, both Harlow and Quinn (1979) and Karagenc et al. (2004) reported that the transfer of blastocysts cultured in 20% oxygen had severe consequences on fetal development. Feil et al. (2006) further reported no difference in implantation rates between embryos which were cultured in either 7% or 20% oxygen during the post-compaction period, but likewise, there was a significant difference in the numbers of viable fetuses per blastocyst implanted.

To date, the majority of human embryos are routinely cultured in atmospheric oxygen, which highlights the relevance of the present study to clinical IVF. Clinical data from studies comparing human embryos cultured in atmospheric and reduced oxygen concentrations have been somewhat inconclusive. Associations between the use of reduced oxygen throughout the preimplantation culture period and outcomes such as improved embryo development and viability have been reported previously for human embryos (Catt and Henman, 2000; Ciray et al., 2009; Kovacic and Vlaisavljevi, 2008; Kovacic et al., 2009; Meintjes et al., 2009; Waldenström et al., 2009). In contrast, other studies have failed to demonstrate a beneficial effect of lower oxygen on the development of human embryos at both cleavage and blastocyst stages (Bahceci et al., 2005; Behr et al., 1999; Dumoulin et al., 1999; Kea et al., 2007; Nanassy et al., 2010).

Several studies have focused on the impact of oxygen on cleavage-stage development (Bahceci et al., 2005; Dumoulin et al., 1999; Kea et al., 2007). An early study by Dumoulin et al. (1999) investigated the impact of oxygen on cleavage-stage embryo development and found no differences in morphometric parameters. However, the culture media employed lacked amino acids and, hence, embryo development was severely compromised as manifest by the reported low implantation rates of 13.4% and 14.0% for 5% and 20% oxygen, respectively. In contrast, both Bahceci et al. (2005) and Kea et al. (2007) reported significantly better embryo quality on day 3, for embryos cultured in 5% oxygen. However, this did not translate into improved clinical outcomes possibly due to the selection of the highest quality embryos for transfer, which does not reflect the overall viability of the cohort. Analysis of cumulative pregnancy rate would be required to address this apparent discrepancy.

The results from this present study offer an explanation as to why previous studies have not observed differences in developmental outcomes with extended culture. Embryos cultured in 20% oxygen had significant delays in cleavage divisions, thus confirming the sensitivity of the pronucleate oocyte to atmospheric oxygen. Furthermore, the detrimental effects, as a result of culturing embryos in 20% oxygen during the first 48 h, were irreversible. Nanassy et al. (2010) compared the effect of 5% and 20% oxygen during the second 48 h of development; however, all embryos were cultured for the first 48 h period in 20% oxygen. Similar to the data presented herein, Nanassy et al. (2010) reported no difference in clinical outcomes, conceivably because all embryos had been exposed to atmospheric oxygen at the highly sensitive cleavage stage.

In contrast, in a small groups of patients, Kea et al. (2007) evaluated the impact of two oxygen concentrations (5% and 20%) on development to day 3, followed by culture exclusively in 5% oxygen from day 3 to day 5. Further supporting the need for a lower oxygen concentration for the first 48 h, the resultant pregnancy rates were 70% from blastocyst development in the continual presence of 5% oxygen and only 33% for the embryos which had been exposed to 20% oxygen during the cleavage stages. However, due to the small number of patients, 22 in total, this difference did not achieve statistical significance.

Of particular importance in the current study is the observation that all stages of embryo development are sensitive to atmospheric oxygen. When Behr et al. (1999) investigated the effect of oxygen on human blastocyst development, surplus embryos were cultured for the first 48 h period in either 5% and 20% oxygen and then exclusively in 20% oxygen for the second 48 h period. Behr and colleagues found no difference in the proportions of embryos forming blastocysts in either group. In contrast, the present study determined that exposing embryos to atmospheric oxygen post compaction was detrimental and resulted in blastocysts with significantly fewer cells. Unfortunately, Behr et al. (1999) did not analyse blastocyst cell number. Alternatively, the disparity in results may be attributed to species difference.

With regard to aetiology, the detrimental affect of atmospheric oxygen to embryonic development could be the result of delay at cell cycle checkpoints. Zheng et al. (2005) suggested that DNA repair may be required by the early embryo to correct damage that arises during DNA replication and/or in response to exposure to genotoxic agents. Reactive oxygen species are genotoxic agents and their production in mouse embryos increases during culture in atmospheric oxygen (Goto et al., 1993). DNA repair in the pronucleate oocyte is thought to rely entirely on the maternal mRNA and proteins deposited and stored in the oocyte before ovulation (Jaroudi and SenGupta, 2007). Furthermore, DNA repair genes have been shown to be expressed in the early stages of mammalian embryo development (Zheng et al., 2005). Consequently, the delay in the timing of cleavage events and the corresponding increase in the length of cell cycles, when the embryo was exposed to 20% for the initial 48 h period, may be the result of delays at cell cycle checkpoints to repair an increased amount of damaged DNA.

However, longer cell cycles, as a result of delay to correct DNA damage, do not fully explain the aetiology of embryos cultured in atmospheric oxygen, as even those embryos cleaving early but exposed to 20% oxygen had compromised cell numbers. This suggests, with respect to embryonic development, the insult of a non-physiological oxygen concentration extends beyond delayed cleavage events. Impaired metabolism may be a mechanism through which atmospheric oxygen further perturbs embryonic development and influences embryonic viability. Lane and Gardner (2005) reported a significant increase in the glycolytic activity of flushed 2-cell mouse embryos that were cultured in 20% oxygen compared with embryos cultured in 5% oxygen. Perturbed metabolism, as a result of culture in atmospheric oxygen, may influence subsequent development as alternations in metabolic activity are associated with impaired development in culture and loss of viability (Gardner, 1998; Lane and Gardner, 2005).

This study successfully employed time-lapse microscopy to investigate the effect of oxygen concentration on the kinetics of embryo cleavage events and blastocyst development. The use of an imaging incubator facilitated a high-frequency temporal analysis of embryo development rather than a limited number of discrete time-point observations. Similarly, Arav et al. (2008) evaluated the efficacy of time-lapse imaging, during the culture of mouse embryos, to predict embryonic developmental competence and concluded that time-lapse imaging facilitates non-invasive monitoring of embryos. Arav et al. (2008) reported that 50% of the embryo population cleaved to 2 cells within a 2-h window (33.5—35.5 h post HCG), of which 53% went on to form blastocyst. Interestingly, Arav et al. (2008) cultured embryos under 5% CO_2 in air and the embryo population will have been detrimentally affected by atmospheric oxygen. In the present study, when embryos were cultured in atmospheric oxygen, 50% of the embryo population had cleaved by 31.9 h and for those embryos cultured in the reduced oxygen concentration, 50% had cleaved by 31.3 h. Worthy of note, when in-vivo-derived embryos were flushed at 31.3 h, a portion (56.8%) similar to the embryo population cultured in reduced oxygen had cleaved to 2 cells.

In conclusion, the early manifestation of oxygen toxicity, evident from the first cleavage event, is reported here for the first time. Furthermore, this study demonstrates that culture in atmospheric oxygen up to the 8-cell stage causes irreversible damage to the preimplantation embryo. It is, therefore, advisable to culture embryos at a reduced oxygen concentration throughout the preimplantation period in order to maintain the viability of more embryos within a cohort. Maintaining the viability of more embryos should therefore lead to a greater cumulative pregnancy rate. The use of a reduced oxygen concentration for all stages of preimplantation embryo development (pronucleate oocyte to blastocyst) is therefore an important consideration for clinical IVF laboratories.

Acknowledgements

The authors wish to thank Dr George Thouas for his comments on the manuscript.

References

Arav, A., Aroyo, A., Yavin, S., Roth, Z., 2008. Prediction of embryonic developmental competence by time-lapse observation and 'shortest-half' analysis. Reprod. Biomed. Online 17, 669—675.

Bahceci, M., Ciray, H.N., Karagenc, L., Ulug, U., Bener, F., 2005. Effect of oxygen concentration during the incubation of embryos of women undergoing ICSI and embryo transfer: a prospective randomized study. Reprod. Biomed. Online 11, 438—443.

Batt, P.A., Gardner, D.K., Cameron, A.W., 1991. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. Reprod. Fertil. Dev. 3, 601—607.

Bavister, B.D., 1995. Culture of preimplantation embryos: facts and artifacts. Hum. Reprod. Update 1, 91—148.

Behr, B., Pool, T.B., Milki, A.A., Moore, D., Gebhardt, J., Dasig, D., 1999. Preliminary clinical experience with human blastocyst development in vitro without co-culture. Hum. Reprod. 14, 454—457.

Catt, J.W., Henman, M., 2000. Toxic effects of oxygen on human embryo development. Hum. Reprod. 15, 199—206.

Ciray, H.N., Aksoy, T., Yaramanci, K., Karayaka, I., Bahceci, M., 2009. In vitro culture under physiologic oxygen concentration improves blastocyst yield and quality: a prospective randomized survey on sibling oocytes. Fertil. Steril. 91, 1459—1461.

Dumoulin, J.C.M., Meijers, C.J.J., Bras, M., Coonen, E., Geraedts, J.P.M., Evers, J.L.H., 1999. Effect of oxygen concentration on human in-vitro fertilization and embryo culture. Hum. Reprod. 14, 465—469.

Edwards, R.G., Purdy, J.M., Steptoe, P.C., Walters, D.E., 1981. The growth of human preimplantation embryos in vitro. Am. J. Obstet. Gynecol. 141, 408—416.

Feil, D., Lane, M., Roberts, C.T., Kelley, R.L., Edwards, L.J., Thompson, J.G., Kind, K.L., 2006. Effect of culturing mouse embryos under different oxygen concentrations on subsequent fetal and placental development. J. Physiol. 572, 87—96.

Fischer, B., Bavister, B.D., 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J. Reprod. Fertil. 99, 673—679.

Gardner, D.K., 1998. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology 49, 83—102.

Gardner, D.K., Lane, M., 1996. Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. Hum. Reprod. 11, 2703—2712.

Gardner, D.K., Lane, M., 2002. Development of viable mammalian embryos in vitro: evolution of sequential media. In: Cibelli, J., Lanza, R., Campbell, K.H.S., West, M.D. (Eds.), Principles of Cloning. Academic Press, New York, pp. 187—213.

Gardner, D.K., Lane, M., 2007. Embryo culture systems. In: Gardner, D.K. (Ed.), In Vitro Fertilization — A Practical Approach. Informa Healthcare, New York, pp. 221—282.

Goto, Y., Noda, Y., Mori, T., Nakano, M., 1993. Increased generation of reactive oxygen species in embryos cultured in vitro. Free Radic. Biol. Med. 15, 69—75.

Ham, R.G., Puck, T.T., 1962. A regulated incubator controlling CO_2 concentration, humidity and temperature for use in animal cell culture. Proc. Soc. Exp. Biol. Med. 111, 67—71.

Harlow, G.M., Quinn, P., 1979. Foetal and placenta growth in the mouse after pre-implantation development in vitro under oxygen concentrations of 5 and 20%. Aust. J. Biol. Sci. 32, 363—369.

Jaroudi, S., SenGupta, S., 2007. DNA repair in mammalian embryos. Mutat. Res. 635, 53—77.

Karagenc, L., Sertkaya, Z., Ciray, N., Ulug, U., Bahceci, M., 2004. Impact of oxygen concentration on embryonic development of mouse zygotes. Reprod. Biomed. Online 9, 409–417.

Kea, B., Gebhardt, J., Watt, J., Westphal, L.M., Lathi, R.B., Milki, A.A., Behr, B., 2007. Effect of reduced oxygen concentrations on the outcome of in vitro fertilization. Fertil. Steril. 87, 213–216.

Kind, K.L., Collett, R.A., Harvey, A.J., Thompson, J.G., 2005. Oxygen-regulated expression of GLUT-1, GLUT-3, and VEGF in the mouse blastocyst. Mol. Reprod. Dev. 70, 37–44.

Kitagawa, Y., Suzuki, K., Yoneda, A., Watanabe, T., 2004. Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology 62, 1186–1197.

Kovacic, B., Vlaisavljevi, V., 2008. Influence of atmospheric versus reduced oxygen concentration on development of human blastocysts in vitro: a prospective study on sibling oocytes. Reprod. Biomed. Online 17, 229–236.

Kovacic, B., Sajko, M.C., Vlaisavljevic, V., 2009. A prospective, randomized trial on the effect of atmospheric versus reduced oxygen concentration on the outcome of intracytoplasmic sperm injection cycles. Fertil. Steril. doi:10.1016/j.fertnstert.2009.03.077.

Lane, M., Gardner, D.K., 1997. Differential regulation of mouse embryo development and viability by amino acids. J. Reprod. Fertil. 109, 153–164.

Lane, M., Gardner, D.K., 2004. Preparation of gametes, in vitro maturation, in vitro fertilization, embryo recovery and transfer. In: Gardner, D.K., Lane, M., Watson, A.J. (Eds.), A Laboratory Guide to the Mammalian Embryo. Oxford Press, New York, pp. 24–40.

Lane, M., Gardner, D.K., 2005. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. Reprod. Fertil. Dev. 17, 371–378.

Lawler, C., Baker, H.W.G., Edgar, D.H., 2007. Relationships between timing of syngamy, female age and implantation potential in human in vitro-fertilised oocytes. Reprod. Fertil. Dev. 19, 482–487.

Leoni, G.G., Rosati, I., Succu, S., Bogliolo, L., Bebbere, D., Berlinguer, F., Ledda, S., Naitana, S., 2007. A low oxygen atmosphere during IVF accelerates the kinetic of formation of in vitro produced ovine blastocysts. Reprod. Domest. Anim. 42, 299–304.

Maas, D.H., Storey, B.T., Mastroianni Jr., L., 1976. Oxygen tension in the oviduct of the rhesus monkey (*Macaca mulatta*). Fertil. Steril. 27, 1312–1317.

Mastroianni Jr., L., Jones, R., 1965. Oxygen tension within the rabbit fallopian tube. J. Reprod. Fertil. 9, 99–102.

Meintjes, M., Chantilis, S.J., Douglas, J.D., Rodriguez, A.J., Guerami, A.R., Bookout, D.M., Barnett, B.D., Madden, J.D., 2009. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. Hum. Reprod. 24, 300–307.

Nanassy, L., Peterson, C.A., Wilcox, A.L., Peterson, C.M., Hammoud, A., Carrell, D.T., 2010. Comparison of 5% and ambient oxygen during days 3-5 of in vitro culture of human embryos. Fertil. Steril. 93, 579–585.

Pabon, J., Findley, W., Gibbons, W., 1989. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. Fertil. Steril. 51, 896–900.

Quinn, P., Harlow, G.M., 1978. The effect of oxygen on the development of preimplantation mouse embryos in vitro. J. Exp. Zool. 206, 73–80.

Sakkas, D., Shoukir, Y., Chardonnens, D., Bianchi, P.G., Campana, A., 1998. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum. Reprod. 13, 182–187.

Shoukir, Y., Campana, A., Farley, T., Sakkas, D., 1997. Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. Hum. Reprod. 12, 1531–1536.

Thompson, J.G.E., Simpson, A.C., Pugh, P.A., Donnelly, P.E., Tervit, H.R., 1990. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. J. Reprod. Fertil. 89, 573–578.

Umaoka, Y., Noda, Y., Narimoto, K., Mori, T., 1992. Effects of oxygen toxicity on early development of mouse embryos. Mol. Reprod. Dev. 31, 28–33.

Waldenström, U., Engström, A.-B., Hellberg, D., Nilsson, S., 2009. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. Fertil. Steril. 91, 2461–2465.

Zheng, P., Schramm, R.D., Latham, K.E., 2005. Developmental regulation and in vitro culture effects on expression of DNA repair and cell cycle checkpoint control genes in rhesus monkey oocytes and embryos. Biol. Reprod. 72, 1359–1369.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 10 February 2010; refereed 29 March 2010; accepted 22 April 2010.