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Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos *in vitro*

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Rebecca Kelley is currently undertaking her PhD with Professor David Gardner at the University of Melbourne, Australia. She has over 10 years experience in embryology research at both the University of Melbourne and the University of Adelaide, and in haematology research at the University of Cambridge. Her main interest is in finding ways to improve *in vitro* embryo culture by better understanding mammalian embryo development.

Abstract Embryos are routinely cultured individually, although this can reduce blastocyst development. Culture in atmospheric (20%) oxygen is also common, despite multiple detrimental effects on embryos. Although frequently occurring together, the consequences of this combination are unknown. Mouse embryos were cultured individually or grouped, under physiological (5%) or atmospheric (20%) oxygen. Embryos were assessed by time-lapse and blastocyst cell allocation. Compared with the control group (5% oxygen group culture), 5-cell cleavage (t5) was delayed in 5% oxygen individual culture and 20% oxygen group culture (59.91 ± 0.23, 60.70 ± 0.29, 63.06 ± 0.32 h post-HCG respectively, $P < 0.05$). Embryos in 20% oxygen individual culture were delayed earlier (3-cell cleavage), and at t5 cleaved later than embryos in other treatments (66.01 ± 0.40 h, $P < 0.001$), this delay persisting to blastocyst hatching. Compared with controls, hatching rate and cells per blastocyst were reduced in 5% oxygen single culture and 20% oxygen group culture (134.1 ± 3.4, 104.5 ± 3.2, 73.4 ± 2.2 cells, $P < 0.001$), and were further reduced in 20% oxygen individual culture (57.0 ± 2.8 cells, $P < 0.001$), as was percentage inner cell mass. These data indicate combining individual culture and 20% oxygen is detrimental to embryo development.

KEYWORDS: group culture, IVF, oxidative stress, paracrine factors, single culture, time-lapse

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Introduction

During *in vitro* culture, preimplantation embryos are exposed to various forms of stress, which influence the development of the embryo independently, and can also work in synergy to further compromise viability. These stressors may include culture media formulation, pH, temperature and oxygen, among others (Wale and Gardner, 2016). While a single stressor may perturb embryo development to some degree, it also increases the embryo's vulnerability to a second stressor, as demonstrated in the case of light and temperature (Fischer et al., 1988), single culture and peroxides (Hughes et al., 2010) or oxygen and ammonium (Wale and Gardner, 2013). Understanding the identity and nature of these stressors and their interactions has facilitated significant improvements in the efficacy and safety of embryo culture *in vitro* and the development of more suitable culture conditions. Consequently, embryo culture media have become more physiological over the past two decades, and are no longer simple salt solutions with carbohydrates (Bavister, 1995; Gardner and Lane, 1997; Lane and Gardner, 2007). However, culture media still lack several components of the complex oviduct and uterine environments, including many paracrine signalling molecules (Aviles et al., 2010; Coy and Yanagimachi, 2015; Hannan et al., 2011; Robertson et al., 2015; Salamonsen et al., 2016). Embryos themselves secrete signalling molecules into the media *in vitro*, and these may partially compensate for the absence of signals from the reproductive tract.

The preimplantation embryo is unusual in that it does not have an absolute requirement for signalling from neighbouring cells in order to survive and proliferate (Raff, 1992). Nonetheless, blastocyst development rates and cell numbers *in vitro* are improved by group culture with other embryos (Gardner et al., 1994; Keefer et al., 1994; Lane and Gardner, 1992; Moessner and Dodson, 1995; Paria and Dey, 1990). Recent studies, including those with human embryos, demonstrate that advances in culture systems have not yet replicated the benefits of embryo group culture (Ebner et al., 2010; Isobe et al., 2015; Sun et al., 2014; Vutyavanich et al., 2011). In addition to negative effects on blastocyst development, cell numbers and allocation, individual culture has also been reported to increase apoptosis (Brison and Schultz, 1997), and affect the embryo secretome (Contramaestre et al., 2008; Larson and Kubisch, 1999). It remains unclear whether pregnancy rate in humans is affected by individual culture (Almagor et al., 1996; Ebner et al., 2010; Spyropoulou et al., 1999), and there have been no reports on birth weights or other pregnancy outcomes. The few animal studies that have investigated post-implantation outcomes following individual embryo culture have observed only a trend for decreased implantation rates or live birth rates (Isobe et al., 2015; Kato and Tsunoda, 1994; Lane and Gardner, 1992), and the long term development and health of such offspring has not been monitored.

In recent years, improved technologies for embryo monitoring and diagnosis have become available, such as time-lapse microscopy (Conaghan et al., 2013; Kirkegaard et al., 2014; Pribenszky et al., 2010b; Rubio et al., 2014) and advanced molecular techniques for preimplantation genetic screening (PGS) (Schoolcraft et al., 2010; Scott et al., 2013; Yang et al., 2012), both of which require embryos to be cultured individually (in the case of PGS this is not necessarily required if the biopsy is performed at the blastocyst stage). The widespread adoption of these technologies has therefore resulted in individual embryo culture becoming a standard procedure for many clinics. While some human IVF clinics culture embryos in groups, a recent web survey of 265 clinics from 71 countries indicated that 55% of clinics routinely culture human embryos individually during at least part of the culture period (Christianson et al., 2014). Some of these single embryos will be cultured in microwells, which can improve embryo development compared with single culture in a drop (Chung et al., 2015; Dai et al., 2012; Vajta et al., 2008), but many single embryos are cultured in conventional drops with a wide variety of volumes (Bolton et al., 2014).

In addition to deprivation from paracrine factors, *in vitro* cultured embryos are also frequently exposed to atmospheric (20%) oxygen concentrations. Physiological oxygen concentrations are around 2–8% in the reproductive tract (Fischer and Bavister, 1993; Mastroianni and Jones, 1965), and studies on multiple mammalian species have shown that culture in 20% oxygen is detrimental for embryo development, resulting in slower cleavage timings (Wale and Gardner, 2010; Weinerman et al., 2016), lower blastocyst rates and cell numbers (Batt et al., 1991; Quinn and Harlow, 1978; Tervit et al., 1972; Thompson et al., 1990; Whitten, 1971), increased apoptosis (Van Soom et al., 2002; Yuan et al., 2003), more frequent aneuploidy (Bean et al., 2002), more DNA damage (Kitagawa et al., 2004; Takahashi et al., 2000) and higher hydrogen peroxide concentrations (Goto et al., 1993; Kitagawa et al., 2004; Kwon et al., 1999). Atmospheric oxygen in culture is also associated with differential preimplantation gene expression (Harvey et al., 2004; Kind et al., 2005; Meuter et al., 2014; Rinaudo et al., 2006), histone remodelling and global methylation (Gaspar et al., 2015; Li et al., 2014), alterations of the proteome (Katz-Jaffe et al., 2005), secretome (Kubisch and Johnson, 2007; Rodina et al., 2009) and metabolism (Khurana and Wales, 1989; Wale and Gardner, 2012) compared with 5% oxygen. These changes result in perturbed post-implantation development following culture in 20% oxygen (de Waal et al., 2014; Fischer-Brown et al., 2005; Karagenc et al., 2004). While the detrimental effects of 20% oxygen on human embryos have long been debated, clinical studies have reported that culture in 20% oxygen causes slower cleavage divisions (Kirkegaard et al., 2013), reduced blastocyst rates and blastocyst cell number (Dumoulin et al., 1999; Waldenstrom et al., 2009) and altered gene expression (Mantikou et al., 2016). When embryos cultured in 20% oxygen are transferred to patients, they result in decreased implantation and pregnancy rates (Catt and Henman, 2000; Gomes Sobrinho et al., 2011; Meintjes et al., 2009) and decreased live birth rates (Bontekoe et al., 2012; Meintjes et al., 2009; Waldenstrom et al., 2009) compared with embryos cultured in 5% oxygen. However, in spite of these animal and human data, the use of a reduced oxygen environment for human embryo culture is not common. In a web survey of 265 clinics by Christianson and colleagues (Christianson et al., 2014) only 24% of surveyed clinics reported using reduced oxygen for all of their IVF and embryo culture, and 39% of clinics did not use physiological oxygen at all, meaning that 76% of human embryos are exposed to oxidative stress during some period of their *in vitro* culture.

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While a single stressor may perturb embryo development to some degree, it also reduces the embryo's capacity to cope with a second stressor. The aim of this study was therefore to investigate the potential effect of combining two routinely employed clinical practices, individual embryo culture and atmospheric oxygen, in a mouse model. Time-lapse microscopy, which has previously been employed to investigate the effects of oxygen on the development of single embryos (Wale and Gardner, 2010), was employed to assess cleavage event timings of grouped and singly cultured embryos.

Materials and methods

Animals

F1 hybrid (C57BL/6 × CBA) mice were maintained in a standard animal research facility in individually ventilated cages (Optimice, Animal Care Systems, Centennial, CO, USA) with a 12 h light–dark photoperiod (6.00 h–18.00 h) with controlled temperature and food and water available *ad libitum*.

Fertilized embryos were generated by superovulation of four week old F1 hybrid females with intraperitoneal injections of 5 international units (IU) pregnant mare serum gonadotrophin (PMSG, Folligon; Intervet, Bendigo East, Vic, Australia) at the mid-point of the light phase, followed 48 h later by 5 IU human chorionic gonadotrophin (HCG, Chorulon; Intervet), then mating with males of the same strain overnight. All mice were killed by cervical dislocation. All experiments were approved by The University of Melbourne Animal Ethics Committee on 2 May 2014 (reference number: 1413159).

Embryo culture

Pronucleate oocytes were collected 22 h after HCG injection in G-MOPS PLUS handling medium, containing 5 mg/ml human serum albumin (HSA) (Vitrolife, Göteborg, Sweden) as described previously (Gardner and Lane, 2007, 2014). Embryos were incubated in G-MOPS PLUS containing 550 IU/ml hyaluronidase (bovine testes type IV-S; Sigma-Aldrich, St Louis, MO, USA) until cumulus cells were removed, then washed three times in G-MOPS PLUS and once in pre-incubated G1 culture medium. Embryos were pooled, then allocated to treatments. Embryos were cultured in G1 medium for 48 h, then transferred to G2 medium for a further 24 h or 48 h. All embryo manipulations were performed on a SMZ 1500 microscope with a heated stage (Nikon Instruments, Melville, NY, USA). Embryo development was assessed on the morning of days 3, 4 and 5 of culture (70, 94 and 118 h post-HCG). Developmental stages were defined as follows: 'compacting': the loss of membrane definition between blastomeres; 'early blastocyst': the presence of a blastocoel cavity less than half the volume of the embryo; 'blastocyst': the presence of a cavity occupying at least half the volume of the embryo; 'expanded': the increase in volume of the blastocyst and thinning of the zona; 'hatching': the appearance of cells outside the zona; and 'fully hatched': the complete evacuation of the embryo from the zona (Gardner and Lane, 2014).

G1/G2 media were prepared in the laboratory as described previously (Gardner and Lane, 2007, 2014), except choline chloride, folic acid, inositol, nicotinomide and taurine were omitted from G2. Media were supplemented with hyaluronan (0.125 mg/ml; Vitrolife) and gentomycin (10 μg/ml; Sigma). Media were also supplemented with 2.5 mg/ml recombinant albumin (G-MM, Vitrolife), to eliminate the potential effects of contaminants inherent in serum albumin (Bar-Or et al., 2005; Dyrlund et al., 2014; Morbeck et al., 2014). All additional media chemicals were supplied by Sigma-Aldrich, except essential amino acids (MEM Cellgro; Corning Life Sciences, Tewksbury, MA, USA). All chemicals and plastics were tested in a mouse embryo assay prior to use (Gardner et al., 2005).

Embryo cultures were performed in 35 mm or 60 mm Petri dishes (Falcon Easy-Grip; Corning Life Sciences, Tewksbury, MA, USA) under paraffin oil (Ovoil; Vitrolife) in a humidified multi-gas incubator at 37°C (MCO-5M; Sanyo Electric, Osaka, Japan). For reduced oxygen experiments the incubator atmosphere was 5% O_2, 6% CO_2 and 89% N_2, for 20% oxygen experiments the incubator atmosphere was 6% CO_2 in air. Drops of media were made under oil using an eVol positive displacement pipette (SGE Analytical Science, Ringwood, Vic, Australia) in order to accurately deliver the small volumes employed and prevent evaporation of media during dish preparation (accurate to ± 1.0%). Grouped embryos were cultured in drops of 20 μl (10 embryos per group) and single embryos in 2 μl, in order to maintain the embryo: media volume ratio. Embryos were cultured in groups or individually, in either physiologic (5%) or atmospheric (20%) oxygen, resulting in four treatment groups: 5% oxygen grouped (control group); 5% oxygen single; 20% oxygen grouped; and 20% oxygen single. The treatment of 5% oxygen group culture is referred to as the 'control' because it is considered the current best practise for mammalian embryo culture, but statistical comparisons have been performed between all groups.

To ensure that using two drop volumes did not affect osmolality, the osmolality was measured in both the 2 μl and 20 μl drops after 48 h incubation. There was no difference in osmolality between the two drop sizes, and both were well within normal range for embryo culture media (data not shown). Furthermore, single embryos were cultured in 2 μl or 20 μl drops and the study found that culture in 2 μl did not impair development (data not shown). Consequently, any differences detected in this study cannot be attributed to compromised conditions in the 2 μl drops.

Differential stain

Allocation of cells in the blastocyst to the inner cell mass (ICM) or trophectoderm was assessed using a differential staining protocol (Hardy et al., 1989). All procedures were performed at 37°C, and blastocysts were washed in G-MOPS PLUS between all steps except the last. Simple G1 (sG1) medium without non-essential amino acids, alanyl glutamine, taurine or HSA, containing 4 mg/ml polyvinylpyrrolidone (PVP; Sigma-Aldrich) (sG1 + PVP) was used to dilute trinitrobenzenesulfonic acid (TNBS, Sigma-Aldrich) and anti-DNP produced in rabbit (Sigma Aldrich).

Blastocysts were incubated in 0.5% pronase (Sigma-Aldrich) until the zona was no longer visible, then in 0.5% 2,4,6-TNBS for 10 min. Blastocysts were then transferred to 10% anti-DNP antibody for 10 min, and then into guinea pig serum (IMVS,

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Adelaide, SA, Australia) diluted 50% in 0.02 mg/ml propidium iodide (Sigma-Aldrich) in G-MOPS until blebbing of the cell membranes was observed. The final step was incubation in 0.1 mg/ml bisBenzimide (Hoescht 33258, Sigma) in G-MOPS and 10% ethanol for 30 min. Blastocysts were then washed briefly and mounted in glycerol on a glass microscope slide. Images were captured on an Eclipse TS100 inverted fluorescent microscope with DS-Fi1 camera and Digital Sight control unit (Nikon Instruments). Cells were counted using ImageJ (1.49v, National Institutes of Health, USA).

Morphokinetics

Embryos were monitored using a multi-gas cell imaging incubator fitted with an inverted microscope (MCOK-5M[RC]; Sanyo Electric), as described previously (Binder et al., 2012; Lee et al., 2015; Wale and Gardner, 2010). The embryo culture dishes (which were the same as described in the embryo culture section) rest on a motorised stage, while images are taken through the fixed Olympus microscope with a ×10 objective. The light source is a 0.1 W white LED. Images were obtained every 15 min during culture and the time of cleavage events recorded. Cleavage times were defined as the first time point when new blastomeres were separated by cell membranes. To date, numerous systems for notation of cleavage events have been used, and while there have been proposals for standardization (Ciray et al., 2014; Kaser and Racowsky, 2014), there is still no consensus within the field (Lundin and Ahlstrom, 2015). Ths study has used a common notation system based on that used by Meseguer and colleagues (Meseguer et al., 2011): cleavage time to two cells is represented as t2, and so on for three to eight cells. Syn-t2 is the duration from syngamy to two cells; cc2 is the duration of the second cell cycle of the embryo, i.e. the interval between t2 and t3, likewise cc3 is the third cell cycle, i.e. the interval between t4 and t5; s2 is the duration of the second synchrony, i.e. the interval between t3 and t4, likewise s3 is the duration of the third synchrony, i.e. the interval between t5 and t8. Notation of post-compaction events are as described in the embryo culture section. Cleavage times are expressed as h post-HCG injection, or as h post-t2 (to reduce the potential influence of variation in mating and fertilization times). Time-lapse experiments (but not earlier experiments) in either 5% oxygen or 20% oxygen were conducted in series to compare individual and group culture at each oxygen concentration.

Statistical analysis

For all tests, differences were considered statistically significant when $P < 0.05$. Percentage data (e.g. the number of embryos to reach each stage of development on each day) were compared by Fisher's Exact Test in Prism v5.04 (GraphPad, La Jolla, CA, USA). Comparison of means (e.g. the number of cells per embryo) were compared by General Linear Model Univariate analysis with 'oxygen' and 'grouping' as factors and 'replicate' as a random factor in SPSS Statistics v22 (IBM, Armonk, NY, USA). This was followed by the same test with 'treatment' as a factor and 'replicate' as a random factor. Multiple comparisons were performed with Bonferroni post-hoc analysis. The homogeneity of variance was assessed by visualisation of the residuals on a scatterplot, and if the data were considered skewed then it was transformed by either a square root or logarithmic transformation, depending on which was most appropriate for the data.

Results

Development rates and cell allocation

Single culture in 5% oxygen had no effect on the number of embryos that were compacting on day 3 compared with the control group (5% oxygen group culture) (**Figure 1**), but did reduce the proportion of embryos hatching on day 4 (Supplementary **Table S1**). On day 5, in 5% oxygen, fewer individually cultured embryos were hatching than those in groups, and more were expanded. Atmospheric oxygen did not alter the number of embryos in groups compacting on day 3 compared with controls, but on days 4 and 5 fewer embryos were hatching. While there was no effect of oxygen or individual culture on the proportion of embryos that had developed to the compacting stage on day 3 compared with controls, the combination of both 20% oxygen and individual culture did reduce the number of compacting embryos compared with controls. This pattern continued on days 4 and 5, when the combination of both 20% oxygen and single culture resulted in fewer embryos forming a blastocyst or hatching than embryos cultured in either 20% oxygen in groups, or in 5% oxygen individually.

The general linear model (GLM) univariate analysis showed that there was a significant overall effect of both oxygen ($P < 0.001$) and grouping ($P < 0.001$) on the total number of cells per blastocyst on day 4, and the interaction between the two factors was significant ($P < 0.05$). A Bonferroni post-hoc analysis showed that the mean number of cells per embryo in 5% oxygen was the same in single culture or group culture, but in 20% oxygen embryos cultured individually had fewer cells than those in groups ($P < 0.001$) (**Figure 2A**). All embryos

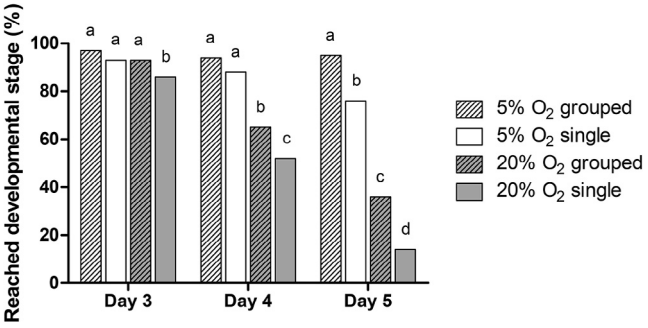

Figure 1 Development of embryos in 5% or 20% oxygen, in groups of 10 or individually. Bars represent the proportion of embryos that reached the nominated developmental stage or beyond on each day of culture (70, 94 and 118 h post-HCG). Stages: day 3 compacting, day 4 blastocyst, day 5 hatching. n = at least 180 embryos per treatment, 11 replicates, half of the embryos were removed from culture on day 4 for staining. Different letters indicate significant differences between treatments; day 3 $P < 0.05$; day 4 $P < 0.01$, day 5 $P < 0.001$. HCG = human chorionic gonadotrophin.

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in 20% oxygen had fewer cells than those in 5% oxygen ($P < 0.001$). The same pattern was observed in the trophectoderm cells (overall effect of oxygen $P < 0.001$, grouping $P < 0.01$, interaction $P < 0.01$), and the post-hoc showed differences between all treatments except the 5% oxygen group and single ($P < 0.001$). The number of ICM cells was affected by both oxygen ($P < 0.001$) and grouping ($P < 0.001$), but there was no significant interaction. The post-hoc analysis showed all treatments were significantly different to each other ($P < 0.001$). Consequently, the proportion of ICM per embryo was affected by both oxygen ($P < 0.05$) and grouping ($P < 0.001$), with a significant interaction ($P < 0.05$). A Bonferroni post-hoc analysis found the percentage ICM was reduced by single culture compared with group culture when embryos were cultured in 5% oxygen ($P < 0.001$) but not 20% oxygen (or, conversely, the percentage ICM was reduced by 20% oxygen compared with 5% oxygen when embryos were in groups [$P < 0.01$] but not when they were cultured individually). All treatments had a lower proportion of ICM compared with the control ($P < 0.01$).

The total number of cells per day 5 blastocyst, the number of cells in the trophectoderm and the number of cells in the ICM (Figure 2B) were analysed by GLM univariate and there was a negative overall effect of both single culture ($P < 0.001$) and 20% oxygen ($P < 0.001$), but the interaction was not significant, implying that single culture had a detrimental effect at both 5% or 20% oxygen (or conversely, that 20% oxygen had a detrimental effect whether embryos were cultured in groups or singly). Post-hoc analysis showed that all treatments were significantly different to each other ($P < 0.001$). The proportion of ICM per embryo was affected by single culture ($P < 0.05$), but not oxygen, and there was no significant interaction.

Morphokinetics

To determine the point at which delayed development was observable, embryos were monitored in a time-lapse system. In 20% oxygen, the timing of syngamy and t2 were not different between individual and group cultured embryos, but mean cc2 was 38 min slower in individually cultured embryos (Table 1). As a result, t3 was delayed in these embryos by nearly 1 h compared with group culture in the same oxygen concentration. cc3 was also slower by 1 h 45 min, resulting in t5 delayed by nearly 3 h. By the time cavitation was reached, single embryos were delayed by 5 h 45 min, and time at hatching was similarly delayed by 4 h 45 min. Although 92% of embryos in single culture in 20% oxygen formed a cavity, only 51% initiated hatching. This was significantly fewer than the control group (95%), single embryos in 5% oxygen (90%) or embryos in groups in 20% oxygen (98%) ($P < 0.001$). Similarly, significantly fewer embryos had completely escaped the zona after individual culture in 20% oxygen (7%) compared with the controls (31%), single culture in 5% oxygen (18%) or group culture in 20% oxygen (20%).

When embryos were cultured in 5% oxygen, no difference was observed between group cultured and single embryos until cc3, when individually cultured embryos were delayed by 43 min. This resulted in a delay of t5 by 47 min. There was

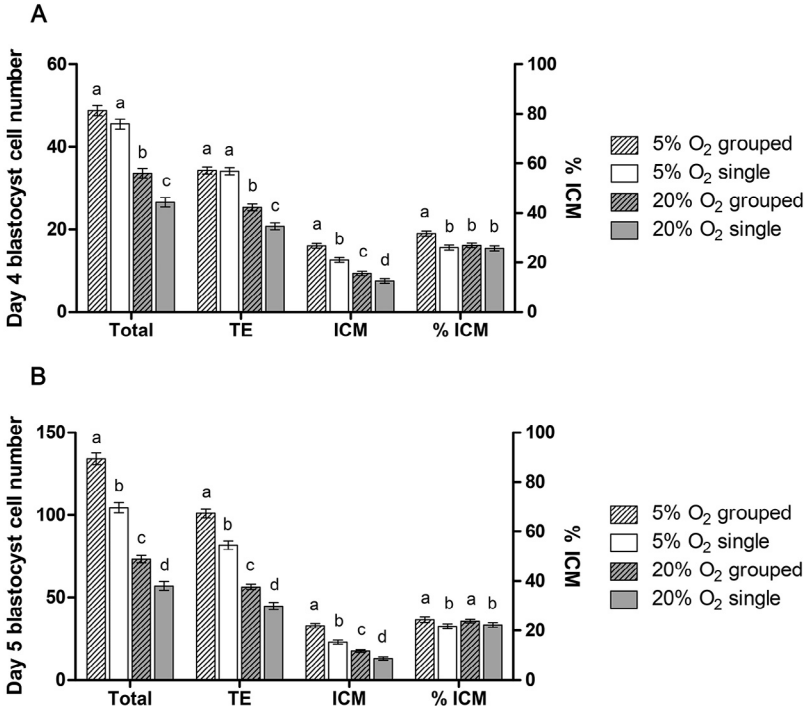

Figure 2 Cell numbers on day 4 (A) and 5 (B) of culture in 5% or 20% oxygen, in groups of 10 or individually. Bars represent mean ± sem; *n* = at least 80 embryos per treatment, day 4 = 5 replicates, day 5 = 6 replicates. Embryos were stained at 94 and 118 h post-HCG, respectively. Different letters represent significant differences between treatments; (A) Total, trophectoderm $P < 0.001$; ICM, percentage ICM $P < 0.01$; (B) Total, trophectoderm, ICM <0.001, percentage ICM <0.05. HCG = human chorionic gonadotrophin; ICM = inner cell mass; TE = trophectoderm.

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Table 1 Time of cleavage events of embryos cultured in 5% or 20%, in groups of 10 or individually.

Parameter		*5% Oxygen*		*20% Oxygen*	
Time of event (h post-HCG)	*Duration between events (h)*	*Grouped*	*Single*	*Grouped*	*Single*
Syngamy		29.69 ± 0.19	30.04 ± 0.24	29.44 ± 0.21	29.63 ± 0.25
	syn – t2	1.60 ± 0.05	1.57 ± 0.06	1.63 ± 0.06	1.61 ± 0.07
1st cleavage (t2)		30.88 ± 0.17	30.93 ± 0.22	30.62 ± 0.18	31.15 ± 0.22
	cc2 (t3 – t2)	19.62 ± 0.16	19.43 ± 0.19	19.94 ± 0.20	20.58 ± 0.24*
2nd cleavage (t3)		50.28 ± 0.18	50.26 ± 0.25	50.28 ± 0.25	51.21 ± 0.31*
	s2 (t4 – t3)	0.90 ± 0.06	0.71 ± 0.08*	0.89 ± 0.10	0.96 ± 0.12
t4		51.17 ± 0.20	50.96 ± 0.28	51.17 ± 0.27	52.17 ± 0.33*
	cc3 (t5 – t4)	9.26 ± 0.13	9.97 ± 0.17***	12.23 ± 0.21	14.01 ± 0.26***
3rd cleavage (t5)		59.91 ± 0.23	60.70 ± 0.29*	63.06 ± 0.32	66.01 ± 0.40***
t6		60.18 ± 0.21	61.25 ± 0.27***	63.80 ± 0.37	66.39 ± 0.49***
4th cleavage (t7)		60.79 ± 0.24	61.66 ± 0.30*	64.37 ± 0.43	67.30 ± 0.58***
t8		61.17 ± 0.24	62.46 ± 0.30***	65.03 ± 0.44	68.26 ± 0.60***
	s3 (t8 – t5)	1.42 ± 0.09	1.46 ± 0.11	2.01 ± 0.13	2.39 ± 0.18
Cavitation		85.38 ± 0.31	85.62 ± 0.43	85.87 ± 0.69	91.65 ± 0.83***
Hatching		94.08 ± 0.71	93.94 ± 0.968	93.20 ± 0.87	97.96 ± 1.12***
Fully hatched		119.78 ± 1.02	119.58 ± 1.60	122.56 ± 0.50	122.57 ± 1.05

Values are mean ± sem of cleavage events (h post-HCG), or time between cleavage events (h). Notation for cleavage events are described in the Methods section. *n* = at least 60 embryos per treatment, 4 replicates at 5% oxygen, 3 replicates at 20% oxygen. cc2 = the duration of the second cell cycle of the embryo; cc3 = the third cell cycle; HCG = human chorionic gonadotrophin; syn-t2 = the duration from syngamy to two cells; s2 = the duration of the second synchrony; s3 = the duration of the third synchrony.
* indicates difference compared with group culture in the same oxygen concentration, *$P < 0.05$, ***$P < 0.001$.

Table 2 Time of cleavage events of embryos cultured in 5% or 20% oxygen, in groups or individually.

Parameter	*5% Oxygen*		*20% Oxygen*	
Time of event (h post-t2)	*Grouped*	*Single*	*Grouped*	*Single*
Syngamy	−1.59 ± 0.05	−1.52 ± 0.07	−1.63 ± 0.06	−1.61 ± 0.07
1st cleavage (t2)	n/a	n/a	n/a	n/a
2nd cleavage (t3)	19.60 ± 0.16	19.43 ± 0.19	19.94 ± 0.20	20.58 ± 0.24*
t4	20.55 ± 0.18	20.15 ± 0.22	20.87 ± 0.22	21.61 ± 0.26*
3rd cleavage (t5)	29.27 ± 0.23	29.91 ± 0.25*	32.78 ± 0.31	35.27 ± 0.40***
t6	29.51 ± 0.20	30.46 ± 0.22**	33.57 ± 0.38	35.74 ± 0.51**
4th cleavage (t7)	30.15 ± 0.25	30.87 ± 0.26*	34.20 ± 0.47	36.92 ± 0.63**
t8	30.27 ± 0.28	31.68 ± 0.29**	34.97 ± 0.48	38.02 ± 0.67***

Values are mean ± sem of cleavage events (h post-t2). Notation for cleavage events are as described in the Methods section. *n* = at least 60 embryos per treatment, 4 replicates at 5% oxygen, 3 replicates at 20% oxygen.
* indicates difference compared with group culture in the same oxygen concentration, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

no significant difference in the time taken for embryos to complete the third synchrony, however t8 was delayed by 1 h 17 min. There was no significant difference in timing of post-compaction events, despite observations in the previous section that fewer individually cultured embryos were hatching on day 5.

When cleavage times were expressed relative to the time each embryo reached t2, the differences between the treatments remained the same as when cleavage times were expressed relative to HCG injection (**Table 2**). As such, the delays in development remained evident irrespective of the normalisation time point used.

The 5% oxygen and 20% oxygen time-lapse experiments were done in series, and so the cleavage times for embryos cultured under the two oxygen concentrations could not be directly compared. It was observed however, that when embryos were cultured in groups, syngamy, t2 and t3 occurred at the same time in 5% and 20% oxygen (**Figure 3**). Then cc3 was approximately 3 h longer for those embryos cultured in groups in 20% oxygen, resulting in a delay of t5 compared with controls. While there appeared to be no difference in cavitation or hatching times, fewer embryos hatched fully in 20% oxygen group culture than controls (20% vs 31%), and it occurred around 3 h later than in 5% oxygen. When embryos were cultured individually, the delay caused by 20% oxygen compared with single embryos in 5% oxygen was observable earlier and was more exaggerated than the delay caused by 20% oxygen when embryos were cultured in groups (1 h delay

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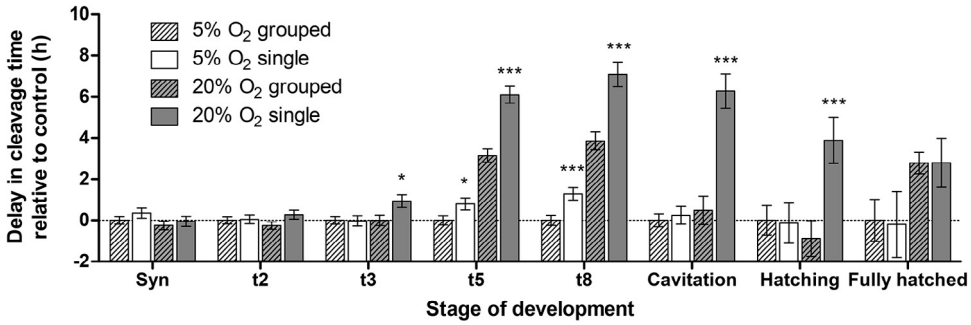

Figure 3 Time of cleavage events relative to group culture at 5% oxygen (control). Data from **Table 1** is represented here as (mean cleavage time) − (mean control cleavage time) ± sem. Notation of cleavage events are described in the Methods section. * indicates difference compared with group culture in the same oxygen concentration, $*P < 0.05$, $***P < 0.001$. n = at least 60 embryos per treatment, 4 replicates at 5% oxygen, 3 replicates at 20% oxygen. Not all embryos reached each developmental stage.

at t3, 6 h delay at t5). There was also an observable delay in cavitation (6 h) and hatching (4 h), and fewer single embryos reached the hatching stage in 20% oxygen than 5% oxygen (51% vs 91%).

Discussion

This study has demonstrated that two common practices of embryo culture, atmospheric oxygen and individual culture, are not only detrimental independently, but that combining these two conditions perturbs embryo development further.

The adverse effects of single culture and 20% oxygen were observed early in culture by time-lapse microscopy. In 20% oxygen, individually cultured embryos were slower to complete the third cell cycle than embryos cultured in groups, which resulted in later cleavage to the 5-cell stage. This delay in cleavage timings persisted to the 8-cell stage, but was not observed later in post-compaction events, although blastocyst cell number was significantly affected. In 20% oxygen, single culture induced a delay compared to group culture that occurred earlier than in 5% oxygen and was more exaggerated. Single embryos at 20% oxygen took longer than embryos in groups at 20% oxygen to complete the second cell cycle, resulting in a delay of cleavage to 3-cells, and this delay increased during the cleavage stages and was maintained post-compaction. These results are in agreement with previous studies that have demonstrated 20% oxygen induces a delay in cleavage timings of individually cultured embryos compared with those in physiological oxygen (Kirkegaard et al., 2013; Wale and Gardner, 2010; Weinerman et al., 2016), but this study is the first to compare morphokinetics of group cultured and individually cultured embryos. Cleavage timings are predictive of embryo viability in multiple species, although debate remains over which morphokinetic parameters are most appropriate to use to select the best embryo for transfer (Conaghan et al., 2013; Hashimoto et al., 2012; Meseguer et al., 2011; Petersen et al., 2016; Somfai et al., 2010; Sugimura et al., 2012; Wong et al., 2010). In the mouse, faster cleaving embryos are more likely to form a blastocyst, have more ICM cells and different mRNA and metabolic profiles at the blastocyst stage, as well as improved fetal development compared with slow-cleaving embryos (Arav et al., 2008; Chung et al., 2015; Lee et al., 2015; Pribenszky et al., 2010a; Weinerman et al., 2016).

In the present study, these delays in cell cleavage timings resulted in a reduction in blastocyst cell numbers on days 4 and 5. On day 4, single culture in 5% oxygen reduced the number of ICM cells and the proportion of ICM per embryo compared with group culture in 5% oxygen. Atmospheric oxygen in groups reduced the total number of cells, trophectoderm, ICM and percentage ICM compared with group culture in 5% oxygen. Combining single culture and 20% oxygen resulted in a further reduction in total cell numbers, trophectoderm and ICM in day 4 blastocysts than either single culture in 5% oxygen or group culture in 20% oxygen. These blastocysts had roughly half the number of cells compared with blastocysts cultured at 5% oxygen in groups. Cell numbers on day 5 followed a similar pattern, except that the total number of cells per embryo was reduced by single culture in 5% oxygen. Also, percentage ICM was reduced by single culture but not 20% oxygen compared with group culture at 5% oxygen. Again, the blastocysts cultured individually in 20% oxygen had roughly half the number of cells compared with blastocysts cultured in groups in 5% oxygen. This reduction in cell numbers is in agreement with the majority of studies on single culture (Dai et al., 2012; Jin and O'Neill, 2014; Kato and Tsunoda, 1994; Lane and Gardner, 1992; O'Neill, 1998; Paria and Dey, 1990; Salahuddin et al., 1995; Stoddart et al., 1996; Vutyavanich et al., 2011) and 20% oxygen (Gardner and Lane, 1996; Karagenc et al., 2004; Quinn and Harlow, 1978; Rinaudo et al., 2006; Wale and Gardner, 2010), and may be caused by an increase in apoptosis that has been observed following single culture (Brison and Schultz, 1997; O'Neill, 1998) and culture in 20% oxygen (Van Soom et al., 2002; Yuan et al., 2003). Alternatively, 20% oxygen can also induce cellular senescence, which would slow the rate of cell divisions, resulting in fewer cells (Meuter et al., 2014). Higher numbers of cells per blastocyst, and particularly a larger ICM, is associated with improved fetal development in the mouse (Lane and Gardner, 1997), which indicates that the most viable embryos were those cultured in groups in 5% oxygen, and the least viable were those cultured individually in 20% oxygen.

The observed higher rate of hatching following group culture is consistent with the majority of previous reports (Contramaestre et al., 2008; Dai et al., 2012; Gardner et al., 1994; Larson and Kubisch, 1999; Lee et al., 1996; Paria and Dey, 1990; Salahuddin et al., 1995; Stoddart et al., 1996). Although hatching is necessary for implantation and is predictive of improved pregnancy rates and live birth in human

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assisted reproductive technologies (Thompson et al., 2013; Yoon et al., 2001), *in vitro* hatching may not be an indicator of post-transfer viability in the mouse (Lane and Gardner, 1997). Blastocyst hatching *in vitro* is brought about by increasing pressure from the expanding blastocyst combined with zona-thinning embryo-secreted proteases, where implantation serine protease 1 (ISP1, also known as strypsin) is thought to play a vital role (Ichikawa et al., 1985; O'Sullivan et al., 2001; Perona and Wassarman, 1986; Sawada et al., 1990; Sharma et al., 2006). An increase in hatching rate may be due to an overall improvement in embryo health, but during culture proteases are secreted into the culture media (Hwang et al., 2000; Martinez-Hernandez et al., 2011; Sawada et al., 1990; Whiteside et al., 2001; Yu et al., 2014), and could assist in the zona degradation and hatching of neighbouring embryos. Also, it has been suggested that protease production for hatching may be stimulated by growth factors and cytokines (Seshagiri et al., 2016). If this were the case, it may partially explain the increased hatching rates reported when embryos are cultured in groups, as embryos secrete many growth factors (Thouas et al., 2015; Wydooghe et al., 2015), which may elicit protease production. The authors are not aware of any direct evidence of a link between growth factors or cytokines and ISP1 production in hatching blastocysts, but leukaemia inhibitory factor (LIF) and epidermal growth factor (EGF) upregulate matrix metalloproteinase (MMP) and urokinase-type plasminogen activator (uPA) activity in blastocyst outgrowths (Harvey et al., 1995), and many other examples exist of proteinase regulation by growth factors and cytokines in other cell types (Lemaire et al., 1997; Zhang et al., 2013). The connection between proteases and growth factors can also operate in reverse, as proteases are involved in protein processing and secretion and thereby can regulate growth factor activity (Westermarck and Kahari, 1999). An alternative theory is that group culture results in increased hatching due to secretion and local concentration of lactate, which may reduce the pH in the immediate microenvironment (Gardner, 2015; Griffiths, 1991). A slightly acidic pH can improve the stability of some proteases and prolong their activity, or can increase their secretion, thereby possibly enhancing zona-degrading activity (Brömme and Wilson, 2011; Kato et al., 2005).

Although the benefits of group culture have been recognized since the 1990s, the exact mechanisms responsible remain unclear. Embryos both secrete and deplete substances in the culture media, and it is likely that combinations of multiple changes to their microenvironment are responsible for the observed improvements in development during group culture (Bavister, 1995). Although embryo-conditioned media has been analysed using advanced technologies such as proteomics and metabolomics (Katz-Jaffe et al., 2006; Seli et al., 2007), the embryo secretome remains only partially defined, and the role of each secreted or depleted substance in contributing to the beneficial effect of group culture is largely unknown. To date, most attention has been given to the potential role of embryo-secreted growth factors and cytokines in promoting growth and/or survival (Hardy and Spanos, 2002; Spanos et al., 2000; Ziebe et al., 2013). Paf, an embryo-secreted ester phospholipid, is also beneficial to singly cultured embryos (O'Neill, 2005). Yet there are other embryo-secreted molecules that alter the microenvironment which have not yet been investigated for their role in the beneficial effect of group culture (Thouas et al., 2015; Wydooghe et al., 2015). One example is lactate, often considered a waste product with no physiological consequence, it can lower pH and act as a signalling molecule, both of which can alter cell behaviour (Gardner, 2015). In addition, as the embryo develops the secretome also changes (Austgulen et al., 1995; Fishel et al., 1984; Hemmings et al., 1992), as can the embryo's response to embryo-secreted factors due to stage-specific expression of some receptors (Sharkey et al., 1995; Thouas et al., 2015). This study observed a delay in cleavage timings from the 3-cell stage in individually cultured embryos, indicating that embryo-mediated alterations to the microenvironment are important from early in culture. This is in keeping with previous evidence in the mouse that the earliest period of development is sensitive to the stress of individual culture (O'Neill, 1998). It is also in keeping with observations that the receptors of some growth factors and cytokines are expressed from the 2-cell stage or earlier (Dadi et al., 2004; Sharkey et al., 1995).

In this study, culture in 20% oxygen resulted in slower cleavage divisions, reduced blastocyst and hatching rates, and fewer cells per blastocyst compared with culture in 5% oxygen (in groups or individually), all of which is in agreement with previous studies in the mouse (Quinn and Harlow, 1978; Rinaudo et al., 2006; Wale and Gardner, 2010; Weinerman et al., 2016). Embryos cultured at 20% oxygen have also been shown to display changes in gene and protein expression, metabolism and reduced post-implantation viability, among other effects (Karagenc et al., 2004; Katz-Jaffe et al., 2005; Khurana and Wales, 1989; Kind et al., 2005; Rinaudo et al., 2006; Wale and Gardner, 2012), but the mechanism by which oxygen perturbs embryo development is not fully understood. Excess oxygen is proposed to induce an increase in intracellular reactive oxygen species (ROS), possibly by changing the activity of NADPH and xanthine oxidases (Guerin et al., 2001). In support of this hypothesis, one form of ROS, hydrogen peroxide (H_2O_2), has been found at higher concentrations in embryos cultured in 20% oxygen compared with 5% oxygen in the mouse (Goto et al., 1993; Kwon et al., 1999), cow (Favetta et al., 2007) and pig (Kitagawa et al., 2004). Although production of ROS is part of normal cell metabolism, elevated ROS can be deleterious, resulting in dysfunction of signalling and other processes (Zhang et al., 2016), and elevated intracellular ROS is associated with poor embryo viability (Esfandiari et al., 2005; Yang et al., 1998). Furthermore, oxygen is a metabolite, and as such changes in its concentration can influence the relative activities of metabolic pathways within the embryo (Khurana and Wales, 1989; Lane and Gardner, 2005; Wale and Gardner, 2012, 2013). In addition to regulating ATP generation and cell growth, changes to cellular metabolism can also influence signalling pathways, including long term alterations of the epigenome (Harvey et al., 2016).

The synergistic effect of combining 20% oxygen and single culture, both of which can be considered stressors, is consistent with previous studies that have treated preimplantation embryos with two stressors (Fischer et al., 1988; Hughes et al., 2010; Wale and Gardner, 2013). Individually cultured embryos may be more sensitive to the additional stress of 20% oxygen, but there may also be an interaction between oxidative stress and the secretome. Changes to oxygen concentration can alter secretions of interferon tau (IFN-τ) from bovine embryos (Kubisch and Johnson, 2007; Rodina et al., 2009). Oxygen is also known to affect embryo metabolism

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(Khurana and Wales, 1989; Wale and Gardner, 2012), resulting in an altered microenvironment through consumption and production of carbohydrates and amino acids. As metabolites also act as signalling molecules, oxygen has the potential to alter paracrine signalling through regulating metabolism. Addition of antioxidants (Truong et al., 2016) or ROS-reducing protein (Isobe et al., 2015) during culture has been demonstrated to improve single embryo development more than when embryos are cultured in groups, indicating that culturing embryos in groups may reduce oxidative stress.

Although mammalian embryos exhibit many species-specific traits, there are also many commonalities in their physiologies and requirements, and hence the mouse has proved to be a good model for human embryo culture (Lane and Gardner, 2007). A possible reservation regarding the use of mouse embryos for experiments investigating group culture is that mice are polyovulatory. We would suggest that the embryotrophic effect of group culture may not necessarily be embryo-specific, but neighbouring embryos may substitute for the lack of paracrine signals from the reproductive tract. This is supported by the observation that embryos from bovine, which is a monovulatory species, also benefit from group culture (Carolan et al., 1996; Doherty et al., 1997; Donnay et al., 1997; Ferry et al., 1994; Fujita et al., 2006; Ikeda et al., 2000; Isobe et al., 2015; Keefer et al., 1994; Larson and Kubisch, 1999; Nagao et al., 2008; Senatore et al., 2010). Interestingly, this effect also occurs between species; mouse embryos can stimulate the development of both cat and cow embryos (Spindler et al., 2006; Stilley et al., 2003). A second issue regarding the use of mouse embryos, particularly hybrid mice, is that the embryos are perceived to be more robust than human embryos. Additionally, mouse embryos are less heterogeneous and obtained from young, fertile donors, in contrast to the heterogeneous, older, infertile human population that undergo IVF treatment, and consequently the mouse embryos used may be of higher quality than many cultured human embryos. The presence of poor quality embryos in a group may reduce the benefits of group culture (Spindler and Wildt, 2002) or even have a negative effect on other embryos (Salahuddin et al., 1995).

The potential combined effects of single culture and oxygen have not been examined in the human; the studies that have compared group and single culture have mostly used 20% oxygen (Almagor et al., 1996; Moessner and Dodson, 1995; Rijnders and Jansen, 1999; Spyropoulou et al., 1999), except Ebner and colleagues (Ebner et al., 2010) and Rebollar-Lazaro and Matson (Rebollar-Lazaro and Matson, 2010), where 5% oxygen was used (personal communications). The range of clinical study designs and culture techniques do not allow for comparison across studies to discern the role of oxygen in determining the outcome of single culture.

Optimisation of each element of the culture system is essential to prevent damage to the embryo from known and unknown stressors, since exposure to one stressor can predispose the embryo to be less resilient to additional stressors. While it is necessary to culture embryos individually in order to facilitate analyses for embryo selection, there is no situation that necessitates the use of atmospheric oxygen for embryo culture. If these results from the mouse are replicated in humans, the combination of atmospheric oxygen and single culture will decrease the number of good quality blastocysts available for transfer. To date, no study has compared the effects of reduced and atmospheric oxygen in individually cultured and group cultured human embryos, but results of this study suggest such an investigation is warranted.

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Appendix: Supplementary material

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References

Almagor, M., Bejar, C., Kafka, I., Yaffe, H., 1996. Pregnancy rates after communal growth of preimplantation human embryos in vitro. Fertil. Steril. 66, 394–397.

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.

Austgulen, R., Arntzen, K.J., Vatten, L.J., Kahn, J., Sunde, A., 1995. Detection of cytokines (interleukin-1, interleukin-6, transforming growth factor-beta) and soluble tumour necrosis factor receptors in embryo culture fluids during in-vitro fertilization. Hum. Reprod. 10, 171–176.

Aviles, M., Gutierrez-Adan, A., Coy, P., 2010. Oviductal secretions: will they be key factors for the future ARTs? Mol. Hum. Reprod. 16, 896–906.

Bar-Or, D., Bar-Or, R., Rael, L.T., Gardner, D.K., Slone, D.S., Craun, M.L., 2005. Heterogeneity and oxidation status of commercial human albumin preparations in clinical use. Crit. Care Med. 33, 1638–1641.

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.

Bean, C.J., Hassold, T.J., Judis, L., Hunt, P.A., 2002. Fertilization in vitro increases non-disjunction during early cleavage divisions in a mouse model system. Hum. Reprod. 17, 2362–2367.

Binder, N.K., Mitchell, M., Gardner, D.K., 2012. Parental diet-induced obesity leads to retarded early mouse embryo development and altered carbohydrate utilisation by the blastocyst. Reprod. Fertil. Dev. 24, 804–812.

Bolton, V.N., Cutting, R., Clarke, H., Brison, D.R., 2014. ACE consensus meeting report: culture systems. Hum. Fertil. 17, 239–251.

Bontekoe, S., Mantikou, E., van Wely, M., Seshadri, S., Repping, S., Mastenbroek, S., 2012. Low oxygen concentrations for embryo culture in assisted reproductive technologies. Cochrane Database Syst. Rev. (7), CD008950.

Brison, D.R., Schultz, R.M., 1997. Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha. Biol. Reprod. 56, 1088–1096.

Brömme, D., Wilson, S., 2011. Role of cysteine cathepsins in extracellular proteolysis. In: Parks, C.W., Mecham, P.R. (Eds.), Extracellular Matrix Degradation. Springer-Verlag, Berlin, Heidelberg, pp. 23–51.

Carolan, C., Lonergan, P., Khatir, H., Mermillod, P., 1996. In vitro production of bovine embryos using individual oocytes. Mol. Reprod. Dev. 45, 145–150.

Catt, J.W., Henman, M., 2000. Toxic effects of oxygen on human embryo development. Hum. Reprod. 15, 199–206.
Christianson, M.S., Zhao, Y., Shoham, G., Granot, I., Safran, A., Khafagy, A., Leong, M., Shoham, Z., 2014. Embryo catheter loading and embryo culture techniques: results of a worldwide Web-based survey. J. Assist. Reprod. Genet. 31, 1029–1036.
Chung, Y.H., Hsiao, Y.H., Kao, W.L., Hsu, C.H., Yao, D.J., Chen, C., 2015. Microwells support high-resolution time-lapse imaging and development of preimplanted mouse embryos. Biomicrofluidics 9, 022407.
Ciray, H.N., Campbell, A., Agerholm, I.E., Aguilar, J., Chamayou, S., Esbert, M., Sayed, S., 2014. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. Hum. Reprod. 29, 2650–2660.
Conaghan, J., Chen, A.A., Willman, S.P., Ivani, K., Chenette, P.E., Boostanfar, R., Baker, V.L., Adamson, G.D., Abusief, M.E., Gvakharia, M., Loewke, K.E., Shen, S., 2013. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. Fertil. Steril. 100, 412–419.
Contramaestre, A.P., Sifontes, F., Marin, R., Camejo, M.I., 2008. Secretion of stem cell factor and granulocyte-macrophage colony-stimulating factor by mouse embryos in culture: influence of group culture. Zygote 16, 297–301.
Coy, P., Yanagimachi, R., 2015. The common and species-specific roles of oviductal proteins in mammalian fertilization and embryo development. Bioscience 65, 973–984.
de Waal, E., Mak, W., Calhoun, S., Stein, P., Ord, T., Krapp, C., Coutifaris, C., Schultz, R.M., Bartolomei, M.S., 2014. In vitro culture increases the frequency of stochastic epigenetic errors at imprinted genes in placental tissues from mouse concepti produced through assisted reproductive technologies. Biol. Reprod. 90, 22.
Dadi, T.D., Li, M.W., Lloyd, K.C., 2004. Expression levels of EGF, TGF-alpha, and EGF-R are significantly reduced in pre-implantation cloned mouse embryos. Cloning Stem Cells 6, 267–283.
Dai, S.J., Xu, C.L., Wang, J., Sun, Y.P., Chian, R.C., 2012. Effect of culture medium volume and embryo density on early mouse embryonic development: tracking the development of the individual embryo. J. Assist. Reprod. Genet. 29, 617–623.
Doherty, E.M.O., Wade, M.G., Hill, J.L., Boland, M.P., 1997. Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. Theriogenology 48, 161–169.
Donnay, I., Van Langendonckt, A., Auquier, P., Grisart, B., Vansteenbrugge, A., Massip, A., Dessy, F., 1997. Effects of co-culture and embryo number on the in vitro development of bovine embryos. Theriogenology 47, 1549–1561.
Dumoulin, J.C., Meijers, C.J., Bras, M., Coonen, E., Geraedts, J.P., Evers, J.L., 1999. Effect of oxygen concentration on human in-vitro fertilization and embryo culture. Hum. Reprod. 14, 465–469.
Dyrlund, T.F., Kirkegaard, K., Poulsen, E.T., Sanggaard, K.W., Hindkjaer, J.J., Kjems, J., Enghild, J.J., Ingerslev, H.J., 2014. Unconditioned commercial embryo culture media contain a large variety of non-declared proteins: a comprehensive proteomics analysis. Hum. Reprod. 29, 2421–2430.
Ebner, T., Shebl, O., Moser, M., Mayer, R.B., Arzt, W., Tews, G., 2010. Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. Reprod. Biomed. Online 21, 762–768.
Esfandiari, N., Falcone, T., Agarwal, A., Attaran, M., Nelson, D.R., Sharma, R.K., 2005. Protein supplementation and the incidence of apoptosis and oxidative stress in mouse embryos. Obstet. Gynecol. 105, 653–660.
Favetta, L.A., St John, E.J., King, W.A., Betts, D.H., 2007. High levels of p66shc and intracellular ROS in permanently arrested early embryos. Free Radic. Biol. Med. 42, 1201–1210.
Ferry, L., Mermillod, P., Massip, A., Dessy, F., 1994. Bovine embryos cultured in serum-poor oviduct-conditioned medium need co-operation to reach the blastocyst stage. Theriogenology 42, 445–453.
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.
Fischer, B., Schumacher, A., Hegele-Hartung, C., Beier, H.M., 1988. Potential risk of light and room temperature exposure to preimplantation embryos. Fertil. Steril. 50, 938–944.
Fischer-Brown, A., Crooks, A., Leonard, S., Monson, R., Northey, D., Rutledge, J.J., 2005. Parturition following transfer of embryos produced in two media under two oxygen concentrations. Anim. Reprod. Sci. 87, 215–228.
Fishel, S.B., Edwards, R.G., Evans, C.J., 1984. Human chorionic gonadotropin secreted by preimplantation embryos cultured in vitro. Science 223, 816–818.
Fujita, T., Umeki, H., Shimura, H., Kugumiya, K., Shiga, K., 2006. Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. J. Reprod. Dev. 52, 137–142.
Gardner, D.K., 2015. Lactate production by the mammalian blastocyst: manipulating the microenvironment for uterine implantation and invasion? Bioessays 37, 364–371.
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., 1997. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Hum. Reprod. Update 3, 367–382.
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.
Gardner, D.K., Lane, M., 2014. Mammalian preimplantation embryo culture. Methods Mol. Biol. 1092, 167–182.
Gardner, D.K., Lane, M., Spitzer, A., Batt, P.A., 1994. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. Biol. Reprod. 50, 390–400.
Gardner, D.K., Reed, L., Linck, D., Sheehan, C., Lane, M., 2005. Quality control in human in vitro fertilization. Semin. Reprod. Med. 23, 319–324.
Gaspar, R.C., Arnold, D.R., Correa, C.A., da Rocha, C.V., Jr., Penteado, J.C., Del Collado, M., Vantini, R., Garcia, J.M., Lopes, F.L., 2015. Oxygen tension affects histone remodeling of in vitro-produced embryos in a bovine model. Theriogenology 83, 1408–1415.
Gomes Sobrinho, D.B., Oliveira, J.B., Petersen, C.G., Mauri, A.L., Silva, L.F., Massaro, F.C., Baruffi, R.L., Cavagna, M., Franco, J.G., Jr., 2011. IVF/ICSI outcomes after culture of human embryos at low oxygen tension: a meta-analysis. Reprod. Biol. Endocrinol. 9, 143.
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.
Griffiths, J.R., 1991. Are cancer cells acidic? Br. J. Cancer 64, 425–427.
Guerin, P., El Mouatassim, S., Menezo, Y., 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum. Reprod. Update 7, 175–189.
Hannan, N.J., Paiva, P., Meehan, K.L., Rombauts, L.J., Gardner, D.K., Salamonsen, L.A., 2011. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. Endocrinology 152, 4948–4956.
Hardy, K., Spanos, S., 2002. Growth factor expression and function in the human and mouse preimplantation embryo. J. Endocrinol. 172, 221–236.

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Hardy, K., Handyside, A.H., Winston, R.M., 1989. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development 107, 597–604.
Harvey, A.J., Kind, K.L., Pantaleon, M., Armstrong, D.T., Thompson, J.G., 2004. Oxygen-regulated gene expression in bovine blastocysts. Biol. Reprod. 71, 1108–1119.
Harvey, A.J., Rathjen, J., Gardner, D.K., 2016. Metaboloepigenetic Regulation of Pluripotent Stem Cells. Stem Cells Int. 2016, 1816525.
Harvey, M.B., Leco, K.J., Arcellana-Panlilio, M.Y., Zhang, X., Edwards, D.R., Schultz, G.A., 1995. Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. Development 121, 1005–1014.
Hashimoto, S., Kato, N., Saeki, K., Morimoto, Y., 2012. Selection of high-potential embryos by culture in poly(dimethylsiloxane) microwells and time-lapse imaging. Fertil. Steril. 97, 332–337.
Hemmings, R., Langlais, J., Falcone, T., Granger, L., Miron, P., Guyda, H., 1992. Human embryos produce transforming growth factors alpha activity and insulin-like growth factors II. Fertil. Steril. 58, 101–104.
Hughes, P.M., Morbeck, D.E., Hudson, S.B., Fredrickson, J.R., Walker, D.L., Coddington, C.C., 2010. Peroxides in mineral oil used for in vitro fertilization: defining limits of standard quality control assays. J. Assist. Reprod. Genet. 27, 87–92.
Hwang, S., Lee, E., Chung, Y., Yoon, B.K., Lee, J.H., Choi, D., 2000. Intactness of zona pellucida does not affect the secretion of a trypsin-like protease from mouse blastocyst. J. Korean Med. Sci. 15, 529–532.
Ichikawa, S., Shibata, T., Takehara, Y., Tamada, H., Oda, K., Murao, S., 1985. Effects of proteinase inhibitors on preimplantation embryos in the rat. J. Reprod. Fertil. 73, 385–390.
Ikeda, K., Takahashi, Y., Katagiri, S., 2000. Effect of medium change on the development of in vitro matured and fertilized bovine oocytes cultured in medium containing amino acids. J. Vet. Med. Sci. 62, 121–123.
Isobe, T., Ikebata, Y., Do, L.T., Tanihara, F., Taniguchi, M., Otoi, T., 2015. In vitro development of OPU-derived bovine embryos cultured either individually or in groups with the silk protein sericin and the viability of frozen-thawed embryos after transfer. Anim. Sci. J. 86, 661–665.
Jin, X.L., O'Neill, C., 2014. Systematic analysis of the factors that adversely affect the rate of cell accumulation in mouse embryos during their culture in vitro. Reprod. Biol. Endocrinol. 12, 35.
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.
Kaser, D.J., Racowsky, C., 2014. Clinical outcomes following selection of human preimplantation embryos with time-lapse monitoring: a systematic review. Hum. Reprod. Update 20, 617–631.
Kato, Y., Tsunoda, Y., 1994. Effects of the culture density of mouse zygotes on the development in vitro and in vivo. Theriogenology 41, 1315–1322.
Kato, Y., Lambert, C.A., Colige, A.C., Mineur, P., Noel, A., Frankenne, F., Foidart, J.M., Baba, M., Hata, R., Miyazaki, K., Tsukuda, M., 2005. Acidic extracellular pH induces matrix metalloproteinase-9 expression in mouse metastatic melanoma cells through the phospholipase D-mitogen-activated protein kinase signaling. J. Biol. Chem. 280, 10938–10944.
Katz-Jaffe, M.G., Linck, D.W., Schoolcraft, W.B., Gardner, D.K., 2005. A proteomic analysis of mammalian preimplantation embryonic development. Reproduction 130, 899–905.
Katz-Jaffe, M.G., Schoolcraft, W.B., Gardner, D.K., 2006. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. Fertil. Steril. 86, 678–685.
Keefer, C.L., Stice, S.L., Paprocki, A.M., Golueke, P., 1994. In vitro culture of bovine IVM-IVF embryos: co-operative interaction among embryos and the role of growth factors. Theriogenology 41, 1323–1331.
Khurana, N.K., Wales, R.G., 1989. Effects of oxygen concentration on the metabolism of [U-14C]glucose by mouse morulae and early blastocysts in vitro. Reprod. Fertil. Dev. 1, 99–106.
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.
Kirkegaard, K., Hindkjaer, J.J., Ingerslev, H.J., 2013. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. Fertil. Steril. 99, 738–744.
Kirkegaard, K., Campbell, A., Agerholm, I., Bentin-Ley, U., Gabrielsen, A., Kirk, J., Sayed, S., Ingerslev, H.J., 2014. Limitations of a time-lapse blastocyst prediction model: a large multicentre outcome analysis. Reprod. Biomed. Online 29, 156–158.
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.
Kubisch, H.M., Johnson, K.M., 2007. The effects of blastomere biopsy and oxygen tension on bovine embryo development, rate of apoptosis and interferon-tau secretion. Reprod. Domest. Anim. 42, 509–515.
Kwon, H.C., Yang, H.W., Hwang, K.J., Yoo, J.H., Kim, M.S., Lee, C.H., Ryu, H.S., Oh, K.S., 1999. Effects of low oxygen condition on the generation of reactive oxygen species and the development in mouse embryos cultured in vitro. J. Obstet. Gynaecol. Res. 25, 359–366.
Lane, M., Gardner, D.K., 1992. Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. Hum. Reprod. 7, 558–562.
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., 2005. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. Reprod. Fertil. Dev. 17, 371–378.
Lane, M., Gardner, D.K., 2007. Embryo culture medium: which is the best? Best Pract. Res. Clin. Obstet. Gynaecol. 21, 83–100.
Larson, M.A., Kubisch, H.M., 1999. The effects of group size on development and interferon-tau secretion by in-vitro fertilized and cultured bovine blastocysts. Hum. Reprod. 14, 2075–2079.
Lee, E.S., Jung, Y.G., Araki, N., Fukui, Y., 1996. Effect of human or murine leukemia inhibitory factor on in vitro development of bovine morulae cultured singly or in a group. J. Mamm. Ova. Res. 13, 19–23.
Lee, Y.S., Thouas, G.A., Gardner, D.K., 2015. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. Hum. Reprod. 30, 543–552.
Lemaire, R., Huet, G., Zerimech, F., Grard, G., Fontaine, C., Duquesnoy, B., Flipo, R.M., 1997. Selective induction of the secretion of cathepsins B and L by cytokines in synovial fibroblast-like cells. Br. J. Rheumatol. 36, 735–743.
Li, W., Goossens, K., Van Poucke, M., Forier, K., Braeckmans, K., Van Soom, A., Peelman, L.J., 2014. High oxygen tension increases global methylation in bovine 4-cell embryos and blastocysts but does not affect general retrotransposon expression. Reprod. Fertil. Dev. doi:10.1071/RD14133; [Epub 17 Dec 2014].
Lundin, K., Ahlstrom, A., 2015. Quality control and standardization of embryo morphology scoring and viability markers. Reprod. Biomed. Online 31, 459–471.
Mantikou, E., Jonker, M.J., Wong, K.M., van Montfoort, A.P., de Jong, M., Breit, T.M., Repping, S., Mastenbroek, S., 2016. Factors affecting the gene expression of in vitro cultured human preimplantation embryos. Hum. Reprod. 31, 298–311.
Martinez-Hernandez, M.G., Baiza-Gutman, L.A., Castillo-Trapala, A., Armant, D.R., 2011. Regulation of proteinases during mouse

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peri-implantation development: urokinase-type plasminogen activator expression and cross talk with matrix metalloproteinase 9. Reproduction 141, 227–239.
Mastroianni, L., Jr., 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.
Meseguer, M., Herrero, J., Tejera, A., Hilligsoe, K.M., Ramsing, N.B., Remohi, J., 2011. The use of morphokinetics as a predictor of embryo implantation. Hum. Reprod. 26, 2658–2671.
Meuter, A., Rogmann, L.M., Winterhoff, B.J., Tchkonia, T., Kirkland, J.L., Morbeck, D.E., 2014. Markers of cellular senescence are elevated in murine blastocysts cultured in vitro: molecular consequences of culture in atmospheric oxygen. J. Assist. Reprod. Genet. 31, 1259–1267.
Moessner, J., Dodson, W.C., 1995. The quality of human embryo growth is improved when embryos are cultured in groups rather than separately. Fertil. Steril. 64, 1034–1035.
Morbeck, D.E., Paczkowski, M., Fredrickson, J.R., Krisher, R.L., Hoff, H.S., Baumann, N.A., Moyer, T., Matern, D., 2014. Composition of protein supplements used for human embryo culture. J. Assist. Reprod. Genet. 31, 1703–1711.
Nagao, Y., Iijima, R., Saeki, K., 2008. Interaction between embryos and culture conditions during in vitro development of bovine early embryos. Zygote 16, 127–133.
O'Neill, C., 1998. Autocrine mediators are required to act on the embryo by the 2-cell stage to promote normal development and survival of mouse preimplantation embryos in vitro. Biol. Reprod. 58, 1303–1309.
O'Neill, C., 2005. The role of paf in embryo physiology. Hum. Reprod. Update 11, 215–228.
O'Sullivan, C.M., Rancourt, S.L., Liu, S.Y., Rancourt, D.E., 2001. A novel murine tryptase involved in blastocyst hatching and outgrowth. Reproduction 122, 61–71.
Paria, B.C., Dey, S.K., 1990. Preimplantation embryo development in vitro: co-operative interactions among embryos and role of growth factors. Proc. Natl. Acad. Sci. U.S.A. 87, 4756–4760.
Perona, R.M., Wassarman, P.M., 1986. Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophectoderm. Dev. Biol. 114, 42–52.
Petersen, B.M., Boel, M., Montag, M., Gardner, D.K., 2016. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. Hum. Reprod. In press.
Pribenszky, C., Losonczi, E., Molnar, M., Lang, Z., Matyas, S., Rajczy, K., Molnar, K., Kovacs, P., Nagy, P., Conceicao, J., Vajta, G., 2010a. Prediction of in-vitro developmental competence of early cleavage-stage mouse embryos with compact time-lapse equipment. Reprod. Biomed. Online 20, 371–379.
Pribenszky, C., Matyas, S., Kovacs, P., Losonczi, E., Zadori, J., Vajta, G., 2010b. Pregnancy achieved by transfer of a single blastocyst selected by time-lapse monitoring. Reprod. Biomed. Online 21, 533–536.
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.
Raff, M.C., 1992. Social controls on cell survival and cell death. Nature 356, 397–400.
Rebollar-Lazaro, I., Matson, P., 2010. The culture of human cleavage stage embryos alone or in groups: effect upon blastocyst utilization rates and implantation. Reprod. Biol. 10, 227–234.
Rijnders, P.M., Jansen, C.A., 1999. Influence of group culture and culture volume on the formation of human blastocysts: a prospective randomized study. Hum. Reprod. 14, 2333–2337.
Rinaudo, P.F., Giritharan, G., Talbi, S., Dobson, A.T., Schultz, R.M., 2006. Effects of oxygen tension on gene expression in preimplantation mouse embryos. Fertil. Steril. 86, 1252–1265.
Robertson, S.A., Chin, P.Y., Schjenken, J.E., Thompson, J.G., 2015. Female tract cytokines and developmental programming in embryos. In: Leese, H.J., Brison, D.R. (Eds.), Cell Signaling During Mammalian Early Embryo Development. Springer-Verlag, New York, pp. 173–213.
Rodina, T.M., Cooke, F.N., Hansen, P.J., Ealy, A.D., 2009. Oxygen tension and medium type actions on blastocyst development and interferon-tau secretion in cattle. Anim. Reprod. Sci. 111, 173–188.
Rubio, I., Galan, A., Larreategui, Z., Ayerdi, F., Bellver, J., Herrero, J., Meseguer, M., 2014. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. Fertil. Steril. 102, 1287–1294.
Salahuddin, S., Ookutsu, S., Goto, K., Nakanishi, Y., Nagata, Y., 1995. Effects of embryo density and co-culture of unfertilized oocytes on embryonic development of in-vitro fertilized mouse embryos. Hum. Reprod. 10, 2382–2385.
Salamonsen, L.A., Evans, J., Nguyen, H.P., Edgell, T.A., 2016. The microenvironment of human implantation: determinant of reproductive success. Am. J. Reprod. Immunol. 75, 218–225.
Sawada, H., Yamazaki, K., Hoshi, M., 1990. Trypsin-like hatching protease from mouse embryos: evidence for the presence in culture medium and its enzymatic properties. J. Exp. Zool. 254, 83–87.
Schoolcraft, W.B., Fragouli, E., Stevens, J., Munne, S., Katz-Jaffe, M.G., Wells, D., 2010. Clinical application of comprehensive chromosomal screening at the blastocyst stage. Fertil. Steril. 94, 1700–1706.
Scott, R.T., Jr., Upham, K.M., Forman, E.J., Hong, K.H., Scott, K.L., Taylor, D., Tao, X., Treff, N.R., 2013. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. Fertil. Steril. 100, 697–703.
Seli, E., Sakkas, D., Scott, R., Kwok, S.C., Rosendahl, S.M., Burns, D.H., 2007. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. Fertil. Steril. 88, 1350–1357.
Senatore, E.M., Xu, J., Suarez Novoa, M.V., Gong, G., Lin, T., Bella, A., Moreno, J.F., Mannino, M.E., Tian, X., Presicce, G.A., Wu, S.C., Du, F., 2010. Improved in vitro development of OPU-derived bovine (Bos taurus) embryos by group culture with agarose-embedded helper embryos. Theriogenology 74, 1643–1651.
Seshagiri, P.B., Vani, V., Madhulika, P., 2016. Cytokines and blastocyst hatching. Am. J. Reprod. Immunol. 75, 208–217.
Sharkey, A.M., Dellow, K., Blayney, M., Macnamee, M., Charnock-Jones, S., Smith, S.K., 1995. Stage-specific expression of cytokine and receptor messenger ribonucleic acids in human preimplantation embryos. Biol. Reprod. 53, 974–981.
Sharma, N., Liu, S., Tang, L., Irwin, J., Meng, G., Rancourt, D.E., 2006. Implantation Serine Proteinases heterodimerize and are critical in hatching and implantation. BMC Dev. Biol. 6, 61.
Somfai, T., Inaba, Y., Aikawa, Y., Ohtake, M., Kobayashi, S., Konishi, K., Imai, K., 2010. Relationship between the length of cell cycles, cleavage pattern and developmental competence in bovine embryos generated by in vitro fertilization or parthenogenesis. J. Reprod. Dev. 56, 200–207.
Spanos, S., Becker, D.L., Winston, R.M., Hardy, K., 2000. Anti-apoptotic action of insulin-like growth factor-I during human preimplantation embryo development. Biol. Reprod. 63, 1413–1420.
Spindler, R.E., Wildt, D.E., 2002. Quality and age of companion felid embryos modulate enhanced development by group culture. Biol. Reprod. 66, 167–173.
Spindler, R.E., Crichton, E.G., Agca, Y., Loskutoff, N., Critser, J., Gardner, D.K., Wildt, D.E., 2006. Improved felid embryo

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development by group culture is maintained with heterospecific companions. Theriogenology 66, 82–92.
Spyropoulou, I., Karamalegos, C., Bolton, V.N., 1999. A prospective randomized study comparing the outcome of in-vitro fertilization and embryo transfer following culture of human embryos individually or in groups before embryo transfer on day 2. Hum. Reprod. 14, 76–79.
Stilley, K.S., Lane, M., Gardner, D.K., 2003. Heterologous co-culture of bovine embryos with mouse embryos stimulates blastocyst development and differentiation. Theriogenology 59, 459.
Stoddart, N.R., Wild, A.E., Fleming, T.P., 1996. Stimulation of development in vitro by platelet-activating factor receptor ligands released by mouse preimplantation embryos. J. Reprod. Fertil. 108, 47–53.
Sugimura, S., Akai, T., Hashiyada, Y., Somfai, T., Inaba, Y., Hirayama, M., Yamanouchi, T., Matsuda, H., Kobayashi, S., Aikawa, Y., Ohtake, M., Kobayashi, E., Konishi, K., Imai, K., 2012. Promising system for selecting healthy in vitro-fertilized embryos in cattle. PLoS ONE 7, e36627.
Sun, B., Yu, W., Wang, F., Song, W., Jin, H., Sun, Y., 2014. Effects of group culture on the development of discarded human embryos and the construction of human embryonic stem cell lines. J. Assist. Reprod. Genet. 31, 1369–1376.
Takahashi, M., Keicho, K., Takahashi, H., Ogawa, H., Schultz, R.M., Okano, A., 2000. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. Theriogenology 54, 137–145.
Tervit, H.R., Whittingham, D.G., Rowson, L.E., 1972. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil. 30, 493–497.
Thompson, J.G., 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.
Thompson, S.M., Onwubalili, N., Brown, K., Jindal, S.K., McGovern, P.G., 2013. Blastocyst expansion score and trophectoderm morphology strongly predict successful clinical pregnancy and live birth following elective single embryo blastocyst transfer (eSET): a national study. J. Assist. Reprod. Genet. 30, 1577–1581.
Thouas, G.A., Dominguez, F., Green, M.P., Vilella, F., Simon, C., Gardner, D.K., 2015. Soluble ligands and their receptors in human embryo development and implantation. Endocr. Rev. 36, 92–130.
Truong, T.T., Soh, Y.M., Gardner, D.K., 2016. Antioxidants improve mouse preimplantation embryo development and viability. Hum. Reprod. doi:10.1093/humrep/dew098; [Epub 10 May 2016].
Vajta, G., Korosi, T., Du, Y., Nakata, K., Ieda, S., Kuwayama, M., Nagy, Z.P., 2008. The Well-of-the-Well system: an efficient approach to improve embryo development. Reprod. Biomed. Online 17, 73–81.
Van Soom, A., Yuan, Y.Q., Peelman, L.J., de Matos, D.G., Dewulf, J., Laevens, H., de Kruif, A., 2002. Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition. Theriogenology 57, 1453–1465.
Vutyavanich, T., Saeng-Anan, U., Sirisukkasem, S., Piromlertamorn, W., 2011. Effect of embryo density and microdrop volume on the blastocyst development of mouse two-cell embryos. Fertil. Steril. 95, 1435–1439.
Waldenstrom, U., Engstrom, 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.
Wale, P.L., Gardner, D.K., 2010. Time-lapse analysis of mouse embryo development in oxygen gradients. Reprod. Biomed. Online 21, 402–410.
Wale, P.L., Gardner, D.K., 2012. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. Biol. Reprod. 87, 21–28.
Wale, P.L., Gardner, D.K., 2013. Oxygen affects the ability of mouse blastocysts to regulate ammonium. Biol. Reprod. 89, 75.
Wale, P.L., Gardner, D.K., 2016. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. Hum. Reprod. Update 22, 2–22.
Weinerman, R., Feng, R., Ord, T.S., Schultz, R.M., Bartolomei, M.S., Coutifaris, C., Mainigi, M., 2016. Morphokinetic evaluation of embryo development in a mouse model: functional and molecular correlates. Biol. Reprod. doi:10.1095/biolreprod.115.134080; [Epub 24 Feb 2016].
Westermarck, J., Kahari, V.M., 1999. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J. 13, 781–792.
Whiteside, E.J., Kan, M., Jackson, M.M., Thompson, J.G., McNaughton, C., Herington, A.C., Harvey, M.B., 2001. Urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9) expression and activity during early embryo development in the cow. Anat. Embryol. 204, 477–483.
Whitten, W.K., 1971. Nutrient requirements for the culture of preimplantation embryos in vitro. Adv. Biosci. 6, 129–139.
Wong, C.C., Loewke, K.E., Bossert, N.L., Behr, B., De Jonge, C.J., Baer, T.M., Reijo Pera, R.A., 2010. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. Nat. Biotechnol. 28, 1115–1121.
Wydooghe, E., Vandaele, L., Heras, S., De Sutter, P., Deforce, D., Peelman, L., De Schauwer, C., Van Soom, A., 2015. Autocrine embryotropins revisited: how do embryos communicate with each other in vitro when cultured in groups? Biol. Rev. Camb. Philos. Soc. doi:10.1111/brv.12241; [Epub 26 Nov 2015].
Yang, H.W., Hwang, K.J., Kwon, H.C., Kim, H.S., Choi, K.W., Oh, K.S., 1998. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum. Reprod. 13, 998–1002.
Yang, Z., Liu, J., Collins, G.S., Salem, S.A., Liu, X., Lyle, S.S., Peck, A.C., Sills, E.S., Salem, R.D., 2012. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study. Mol. Cytogenet. 5, 24.
Yoon, H.J., Yoon, S.H., Son, W.Y., Im, K.S., Lim, J.H., 2001. High implantation and pregnancy rates with transfer of human hatching day 6 blastocysts. Fertil. Steril. 75, 832–833.
Yu, S., Yehia, G., Wang, J., Stypulkowski, E., Sakamori, R., Jiang, P., Hernandez-Enriquez, B., Tran, T.S., Bonder, E.M., Guo, W., Gao, N., 2014. Global ablation of the mouse Rab11a gene impairs early embryogenesis and matrix metalloproteinase secretion. J. Biol. Chem. 289, 32030–32043.
Yuan, Y.Q., Van Soom, A., Coopman, F.O., Mintiens, K., Boerjan, M.L., Van Zeveren, A., de Kruif, A., Peelman, L.J., 2003. Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. Theriogenology 59, 1585–1596.
Zhang, H., Hou, L., Li, C.M., Zhang, W.Y., 2013. The chemokine CXCL6 restricts human trophoblast cell migration and invasion by suppressing MMP-2 activity in the first trimester. Hum. Reprod. 28, 2350–2362.
Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., Dong, W., 2016. ROS and ROS-mediated cellular signaling. Oxid. Med. Cell. Longev. 2016, 4350965.
Ziebe, S., Loft, A., Povlsen, B.B., Erb, K., Agerholm, I., Aasted, M., Gabrielsen, A., Hnida, C., Zobel, D.P., Munding, B., Bendz, S.H., Robertson, S.A., 2013. A randomized clinical trial to evaluate the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization. Fertil. Steril. 99, 1600–1609.

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