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

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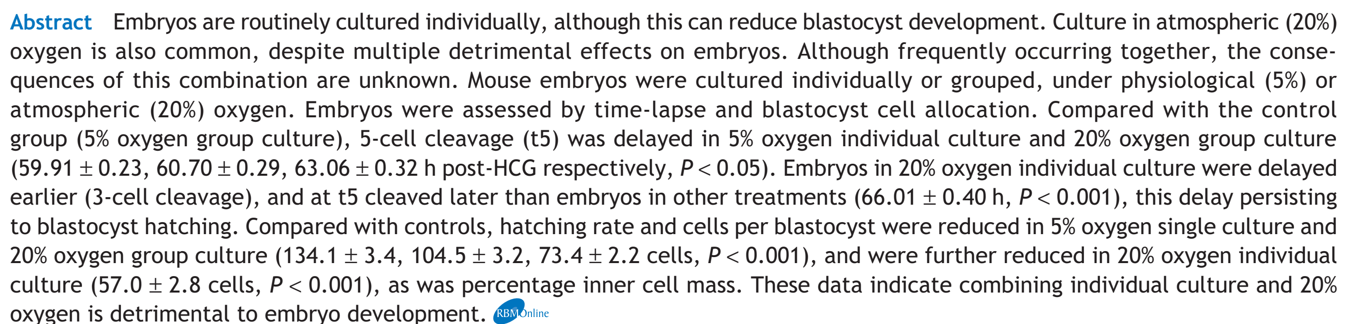
Abstract Embryos are routinely cultured individually, although this can reduce blastocyst development. Culture in atmospheric (20%) oxygen is also common, despite multiple detrimental effects on embryos. Although frequently occurring together, the consequences of this combination are unknown. Mouse embryos 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. RBMOnline

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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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