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In-vitro culture of individual mouse preimplantation embryos: the role of embryo density, microwells, oxygen, timing and conditioned media

Rebecca L Kelley, David K Gardner *

School of BioSciences, The University of Melbourne, Parkville, Victoria 3010, Australia

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

KEY MESSAGE

Single embryo culture is typical for time-lapse systems but, at least in the mouse, it reduces development compared with group culture. Embryo development during individual culture was improved by using reduced oxygen, reduced media volume or microwell dishes, and conditioned medium. The latter could possibly lead to modified media formulations.

A B S T R A C T

Single embryo culture is suboptimal compared with group culture, but necessary for embryo monitoring, and culture systems should be improved for single embryos. Pronucleate mouse embryos were used to assess the effect of culture conditions on single embryo development. Single culture either before or after compaction reduced cell numbers (112.2 ± 3.1; 110.2 ± 3.5) compared with group culture throughout (127.0 ± 3.4; $P < 0.05$). Reduction of media volume from 20 µl to 2 µl increased blastocyst cell numbers in single embryos cultured in 5% oxygen (84.4 ± 3.2 versus 97.8 ± 2.8; $P < 0.05$), but not in 20% oxygen (55.2 ± 2.9 versus 57.1 ± 2.8). Culture in microwell plates for the EmbryoScope and Primo Vision time-lapse systems changed cleavage timings and increased inner cell mass cell number (24.1 ± 1.0; 23.4 ± 1.2) compared with a 2 µl microdrop (18.4 ± 1.0; $P < 0.05$). Addition of embryo-conditioned media to single embryos increased hatching rate and blastocyst cell number (91.5 ± 4.7 versus 113.1 ± 4.4; $P < 0.01$). Single culture before or after compaction is therefore detrimental; oxygen, media volume and microwells influence single embryo development; and embryo-conditioned media may substitute for group culture.

Introduction

Individual culture of in-vitro fertilized embryos has become standard practice in many clinics, and will likely become more common with the application of new technologies for embryo monitoring and selection, such as time-lapse microscopy (Conaghan et al., 2013; Kirkegaard et al., 2014; Rubio et al., 2014). Compared with embryos cultured in groups, individual culture in multiple species results in slower cleavage divisions, fewer embryos developing to the blastocyst

* Corresponding author.
E-mail address: david.gardner@unimelb.edu.au (DK Gardner).

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stage, blastocysts with reduced cell number and altered cell allocation, and increased levels of apoptosis (Brison and Schultz, 1997; Gardner et al., 1994; Isobe et al., 2015; Keefer et al., 1994; Kelley and Gardner, 2016; Lane and Gardner, 1992; Paria and Dey, 1990). Studies in humans have also reported lower cell numbers, blastocyst rates and pregnancy rates after single culture (Almagor et al., 1996; Ebner et al., 2010; Moessner and Dodson, 1995; Rebollar-Lazaro and Matson, 2010), although some studies have shown that single and group culture produce the same results (Rijnders and Jansen, 1999; Spyropoulou et al., 1999). The reduced development rates and cell numbers in embryos cultured individually can be explained by deprivation from paracrine signalling molecules (Paria and Dey, 1990). It is estimated that about 55% of clinics worldwide routinely culture human embryos individually (Christianson et al., 2014); therefore, it is important to understand the consequences of this practice, and to determine if adjustments should be made to the culture system to improve outcomes for individually cultured embryos.

The embryo is more sensitive to stress *in vitro* during the precompaction stages than postcompaction (Gardner and Lane, 2005; Lane and Gardner, 2005), but it is not known if this paradigm applies to the stress of single culture. It is not uncommon for clinics to culture embryos individually for part of the preimplantation period; for example, single culture to day 3, followed by group culture to day 5 and 6 (Christianson et al., 2014). Little evidence is available on the effects of this practice, but two studies suggest that single culture may be more detrimental in the precompaction stages of human blastocyst development than postcompaction (Rebollar-Lazaro and Matson, 2010; Rijnders and Jansen, 1999). Stokes et al. (2005) made the same observation in the pig, and,in the mouse, O'Neill (1998) found that any period of single culture was detrimental to blastocyst development. Further investigation is needed to determine if preimplantation embryos can be exposed to a period of single culture without compromising development.

The volume of culture medium per embryo (referred to as embryo density) is an important consideration in single embryo culture, but it is unclear how optimum embryo density may be influenced by other culture conditions, such as oxygen. Embryo density during single embryo culture can have a dramatic effect on cell numbers, as well as cleavage, blastocyst and hatching rates in the mouse (Lane and Gardner, 1992; Melin et al., 2009; O'Neill, 1997; Paria and Dey, 1990). In humans, one study observed no effect of embryo density on blastocyst rate (Rijnders and Jansen, 1999), but evidence from two recent studies show that embryo density is also an important factor in human blastocyst development, although their conclusions on optimal density were inconsistent (De Munck et al., 2015; Minasi et al., 2015). The beneficial effect of increasing embryo density may be explained by the concentration of autocrine factors in the media. Media volume per embryo is currently inconsistent between clinics, and the practice of individual clinics may be a consequence of habit or convenience, rather than an optimized part of the embryo culture system (Bolton et al., 2014; Reed, 2012).

Oxygen is a key element of embryo culture systems, and atmospheric (20%) oxygen causes multiple perturbations in embryo development, including increases in apoptosis (Van Soom et al., 2002), DNA damage (Takahashi et al., 2000), aneuploidy (Bean et al., 2002), and hydrogen peroxide levels (Goto et al., 1993), as well as changes to the transcriptome (Harvey et al., 2004), proteome (Katz-Jaffe et al., 2005), metabolome (Wale and Gardner, 2010), secretome (Kubisch and Johnson, 2007) and epigenome (Li et al., 2014). Despite this evidence, it is estimated that 20% oxygen is still used for human embryo culture in three- quarters of clinics worldwide (Christianson et al., 2014). Oxygen can change the embryo's response to other environmental stressors, demonstrated in the case of excess ammonium (Wale and Gardner, 2013), but it is not known if this applies to the stress of low embryo density. Evidence from the bovine suggests there may be an interaction between oxygen concentration and embryo density, but this has not yet been investigated in other species (Nagao et al., 2008).

To improve the development of singly cultured embryos, Vajta et al. (2000) developed the 'well-of-the-well' (WOW) culture system, in which embryos are cultured in a microwell to concentrate the embryo-secreted autocrine factors. In addition, it may be beneficial to reduce the contact between media and oil, particularly in small volumes or for individually cultured embryos (Hughes et al., 2010; Lane and Gardner, 1992). Embryo culture for time-lapse microscopy is typically carried out in commercially available microwell dishes, which may have multiple microwells under one drop of media (WOW), or each microwell may be under a separate drop of media (**Figure 1**). Culture of single bovine or porcine embryos in microwells increases cleavage rate and blastocyst rate compared with individual culture, equivalent to that observed in group culture (Hoelker et al., 2009; Kamiya et al., 2006; Pereira et al., 2005; Vajta et al., 2000, 2008). Single mouse embryos also benefit from culture in microwells, as blastocyst rates and cell numbers are reported to be equivalent to group culture (Chung et al., 2015; Pribenszky et al., 2010) or higher than standard single culture (Dai et al., 2012; Vajta et al., 2008), but these studies have all used 20% oxygen, which may influence outcome. Two trials of human embryos cultured in custom-made microwell dishes increased blastocyst rates (Hashimoto et al., 2012; Vajta et al., 2008), but most other human trials involving commercially available microwell dishes have not controlled for factors related to the incubator system. Although the use of microwells to improve single embryo culture is promising, further investigation is needed, in particular regarding the efficacy of commercially available dishes used in clinics.

An alternative to increasing embryo density or culture in microwells may be the development of synthetic embryo-conditioned media. For this to be achieved, the composition and effects of embryo-conditioned media must be better characterized. Several studies have shown that embryos condition the media immediately surrounding them and that neighbouring embryos (<160 µm away) benefit from this microenvironment (Gopichandran and Leese, 2006; Matoba et al., 2010; Somfai et al., 2010; Spindler and Wildt, 2002; Spindler et al., 2006; Stokes et al., 2005); only two studies have added embryo-conditioned media to single embryos. These indicate that embryo-conditioned media improves single mouse and bovine blastocyst development, but these experiments were conducted from the two-cell or four-cell stages in suboptimal conditions, so it is unknown if embryo-conditioned media will benefit single embryo development from the pronucleate stage in complex media and 5% oxygen (Fujita et al., 2006; Stoddart et al., 1996).

In the present study, we examine how culture conditions influence single mouse embryo development. First, we compare the development of mouse embryos in single culture for either the precompaction or postcompaction stages with group or single culture for the entire duration. Second, we investigate the combined effects of embryo density and oxygen on single embryo development. Third, we assess embryo development in 2 µl drops, Primo Vision WOW dishes, and EmbryoScope microwell dishes, using the same incubator. Finally, we investigate the effect of embryo-conditioned media supplementation on single embryo culture.

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