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COMMENTARY

The impact of physiological oxygen during culture, and vitrification for cryopreservation, on the outcome of extended culture in human IVF

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Abstract Extended culture has facilitated the move to single blastocyst transfer, resulting in significant increases in implantation and live birth rate, while concomitantly reducing fetal loss during pregnancy. However, concerns have been raised regarding subsequent neo-natal outcomes following extended culture. Analysis of the literature reveals differences in outcomes according to geographical region and between individual clinics. A common factor amongst reports of potentially adverse outcomes following blastocyst transfer appears to be that atmospheric (~20%) oxygen was typically employed for embryo culture. Clinics and countries utilizing physiological concentrations of oxygen (~5%) have not reported adverse perinatal outcomes with blastocyst transfer. Atmospheric oxygen imposes significant negative effects upon the embryo's molecular and cellular physiology, and further it increases the sensitivity of the preimplantation embryo to other stressors in the laboratory. With the recent adoption of vitrification for blastocyst cryopreservation, cumulative pregnancy rates per cycle with extended culture will increase significantly. Consequently, rather than perceiving extended culture as a potentially negative procedure, it is concluded that neo-natal data need to be interpreted in light of the conditions used to culture and cryopreserve blastocysts, and that furthermore a policy of embryo culture using 20% oxygen can no longer be justified.

KEYWORDS: blastocyst, culture, embryo transfer, implantation rate, pregnancy rate, pregnancy outcome

The communication by Maheshwari in this issue of *Reproductive BioMedicine Online* (Maheshwari et al., 2015) discusses possible concerns associated with the clinical outcomes following blastocyst transfer, focusing on the outcomes of two relatively recent meta-analyses based on observational data (Dar et al., 2014; Maheshwari et al., 2013). The conclusion of the two reports was that blastocyst transfer is associated with an increase in both preterm and very preterm delivery, and an increase in large-for-gestational-age babies compared with pregnancies resulting from the transfer of cleavage-stage embryos. Such meta-analyses have the advantage of relatively large numbers but lack the power to control for specific variables, many of which can have a direct effect on transfer outcome (Wale and Gardner, 2016). Hence, an issue facing any meta-analysis on human IVF is that not all publications list the precise conditions under which IVF and embryo culture were performed, making it extremely difficult to evaluate differences, and again highlights the need for clinical trials to list all conditions used.

Intriguingly, data from more recent studies, not considered by Maheshwari and colleagues, do not align with their conclusions (Chambers et al., 2015; Maxwell et al., 2015; Oron et al., 2015). The largest of these recent reports by Chambers and colleagues reports on over 50,000 infants born and

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did not describe any associations between blastocyst transfer and preterm births, low birth weights or small-for-gestational-age. So why do we see such contradictory reports in the literature? Here the possible reasons for such discrepancies are considered, and a common theme developed as to why certain centres and geographical areas have concerns with extended culture and perinatal outcomes.

Development of the human embryo to the blastocyst stage: all culture systems are not created equal

Until the middle of the 1990s attempts at blastocyst transfer were limited, and moderate success was achieved only through the use of co-culture systems. With the advent of physiologically based sequential media, it became possible to routinely culture the human embryo throughout the preimplantation period to the blastocyst. Initial studies, and the subsequent prospective randomized trial, showed significant increases in implantation rates (Gardner et al., 1998). Such studies were received with a healthy degree of scepticism, and over the ensuing years clinics around the world evaluated extended culture as a clinical procedure. What followed was extremely interesting; many laboratories were able to repeat the initial studies, others found no difference between blastocyst and cleavage-stage transfers and a minority actually reported that blastocyst culture gave inferior outcomes (Gardner and Balaban, 2006). Understanding the basis for this apparent conundrum led to the concept of considering the "embryo culture system" rather than simply embryo culture media, as it was evident that all aspects of the laboratory could have an impact on the effectiveness of the culture media and hence alter outcomes (Gardner and Lane, 2003). Variables evaluated included oxygen concentration, protein source, types of laboratory ware and embryo grouping versus single culture (to name but a few variables). In light of this "holistic approach" a request went out to the IVF community (Gardner and Lane, 2003) that all aspects of the culture system be reported in publications in an attempt to better understand and interpret the emerging data from different clinics, and to identify which factors were responsible for differences in IVF outcomes even when the same media were employed.

Subsequently meta-analyses of blastocyst versus cleavage-stage culture have been performed over the years, and have come out favourably towards blastocyst transfer. However, owing to the vast differences in culture systems between clinics such meta-analyses have really been comparing apples with oranges within the extended culture studies. One key variable, frequently not reported consistently between studies, is the concentration of oxygen used in the culture system. Here lies the heart of the problem: oxygen is one of the most powerful regulators of cell/embryo function (Wale and Gardner, 2016), but for many clinics, even countries, this does not appear to warrant concern.

The consensus (or otherwise) about oxygen concentrations in human IVF laboratories

A recent online survey, in which 265 clinics from 54 different countries participated, revealed that <25% of IVF human embryo culture is performed exclusively under physiological (~5%) oxygen (Christianson et al., 2014). Although this survey represents only a small fraction of the world's IVF clinics, what is notable from the Christianson paper, and from an extensive literature review of the past 10 years, is a clear geographic difference with regard to the use of 5% oxygen, with Australia, New Zealand and Japan representing the only countries to employ, almost exclusively, physiological oxygen for their human embryo culture. The widespread adoption of reduced oxygen in Australian IVF clinics can be readily attributed to several key studies dating back to 1969 from a number of Australian laboratories showing beneficial effects of reduced oxygen on the embryos of many different mammalian species (reviewed by Wale and Gardner, 2016). In the survey of Christianson and colleagues, 34% of clinics reported the use of 5% oxygen for some aspects of embryo culture while the majority of clinics did not use 5% oxygen at all. Given that even a transient exposure to oxygen has been shown to negatively affect development (Pabon et al., 1989; Wale and Gardner, 2010), it would appear that most human embryos worldwide experience oxidative stress in the IVF laboratory. So does oxygen concentration really matter and can it affect fetal development?

Does oxygen concentration during culture represent a key variable in determining embryo health?

Perhaps of all the things that affect embryo function and fetal development, oxygen is in the unique position of being amongst the most characterized and easily controlled, and yet ironically it remains the most ignored. It is evident that physiological concentrations of oxygen within the female tract are around 5% (Fischer and Bavister, 1993) whereas atmospheric oxygen is around 21% (depending upon altitude). There is an abundance of data on several mammalian species, including humans, showing that atmospheric oxygen negatively effects the preimplantation-stage embryo by: changes to the transcriptome (Gardner and Lane, 2005; Rinaudo et al., 2006), alterations to the proteome (Katz-Jaffe et al., 2005), compromising both carbohydrate and amino acid metabolism (Wale and Gardner, 2012), interfering with homeostasis (Wale and Gardner, 2013), differentially affecting male and female embryos (Gardner and Kelley, 2013), impacting the epigenome (Li et al., 2014a) and inducing premature X-chromosome inactivation (an epigenetic event; Lengner et al., 2010). This latter fact resonates with the concerns raised by Maheshwari et al. (2015) about the potential for increases in epigenetic changes associated with culture. Of note, none of the above negative effects change the appearance of the embryo itself; hence simply looking at the embryo (even through time-lapse microscopy) cannot determine the intracellular trauma being induced by atmospheric oxygen (Gardner et al., 2015).

Furthermore, and of immediate significance for this discussion, it has been documented that exposure of embryos to atmospheric oxygen predisposes them to greater susceptibility to other stressors in the IVF laboratory, for example ammonium accumulation in the surrounding medium (Wale and Gardner, 2013) or culture of embryos individually (Kelley and Gardner, 2015), to name just two. Consequently, should