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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

there be any potential problems in the embryology laboratory, the use of atmospheric oxygen will exacerbate the issue, resulting in further compromised development. The concept that when stressors collide there are synergistic negative effects on the embryo is one that needs to be taken into consideration in order to optimize IVF cycle outcome (Awonuga et al., 2013).

Do we really need to offer blastocyst transfer?

In light of concerns raised by Maheshwari and colleagues, would we be better going back to day-3 transfers? What are the reasons/advantages for blastocyst transfer in the first place, and do they remain valid? In all mammalian species studied to date the asynchronous spatial transfer of cleavage-stage embryos to the uterus compromises resultant fetal development (Barnes, 2000; Walker et al., 2015), an observation not commonly acknowledged by the clinical IVF community. Hence, the development of more suitable and physiological culture systems that could support the development of viable human blastocysts was undertaken within the context of synchronicity, in order to increase implantation and live birth rates and to facilitate the move to single embryo transfer (Gardner and Lane, 2003). Further advantages of extended culture included the ability to identify the more viable embryos within a cohort (Gardner and Lane, 2003), the application of grading systems to quantitate both the inner cell mass and trophectoderm (TE; Gardner et al., 2000), the transfer of the embryo to the uterus at a time of decreased endometrial contractions (Fanchin et al., 2001) and the ability to undertake TE biopsy to facilitate preimplantation genetic screening (Fragouli et al., 2008). The last of these is important, as although the percentage of euploid embryos is higher on day 5 than at cleavage stages, several common aneuploidies are compatible with the formation of blastocysts of good morphology (Adler et al., 2014). Subsequent data confirm that blastocyst transfer gives rise to higher implantation and live birth rates. Further, under appropriate laboratory conditions, physiological oxygen being a prerequisite, there appear to be no detrimental effects on transfer outcome and the health of children born (Chambers et al., 2015; Oron et al., 2015).

Cumulative pregnancy rates and monozygotics

Maheshwari and colleagues also consider that overall there is no benefit to blastocyst transfer when one considers the cumulative pregnancy rate. However, one must consider that the majority of published studies concerning the transfer of cryopreserved blastocysts, the results of which are used to determine cumulative pregnancy rates, employed slow-freezing technologies rather than the significantly superior vitrification protocols that have now been adopted worldwide. Subsequently, it has been demonstrated that the move to blastocyst vitrification is associated with a significant increase in clinical pregnancy (50% increase) and live birth rates (40% increase) compared with those obtained with slow freezing (Li et al., 2014b). Consequently, cumulative pregnancy data for cleavage- and blastocyst-stage embryos must be re-examined and be based upon cycles where vitrified blastocysts were utilized. The latter have been reported to result in pregnancy rates and outcomes equivalent to, or even greater than, fresh transferred blastocysts (Li et al., 2014b; Roy et al., 2014).

A further concern of Maheshwari and colleagues is the potential for an increase in monozygotic twins; however, there are conflicting reports about this outcome (Papanikolaou et al., 2010), and in a more recent study, which controlled for embryo cohort quality, there was no reported increase in the incidence of monozygotic twins (Franasiak et al., 2015).

Conclusions

The delivery of healthy, normal-weight babies at term, resulting in healthy children and subsequently healthy adults, is the clear goal of assisted human conception. Discussions are now focused on which of the several procedures available to treat human infertility, while not necessarily being the most effective, are the safest. The questions and concerns about the health of children born following blastocyst transfer raised by Maheshwari and colleagues (Maheshwari et al., 2015) are not supported by more recent and more controlled analyses on birth outcomes. The reasons for this may lie not only in overall improvements in IVF laboratories in recent years, but plausibly through the utilization of 5% oxygen for extended culture; those countries and clinics utilizing physiological concentrations of oxygen reporting no adverse effects on the children born following extended culture (Chambers et al., 2015; Oron et al., 2015). Interestingly, in the meta-analysis considered by Maheshwari and colleagues, those studies reporting negative outcomes with extended culture appear to be from clinics/countries in which 20% oxygen is typically employed (Christianson et al., 2014). An extensive analysis of the literature concerning extended culture revealed that those clinics (or national databases) cited in the meta-analyses did indeed use atmospheric oxygen in the culture system.

Large prospective randomized trials on blastocyst transfer following culture in either 5% or atmospheric oxygen would validate, or disprove, the hypothesis presented here (although to many this would appear to be rather unethical given the numerous documented pathologies induced by 20% oxygen). Already one prospective randomized trial has been performed and determined that implantation and live-birth rates are significantly higher when blastocysts are cultured under reduced oxygen (5%) compared with atmospheric concentrations (Meintjes et al., 2009). Consequently, future prospective randomized trials on cleavage-stage versus blastocyst-stage transfers will ensure a focus on delivery outcomes. However, a plea for the use of 5% oxygen is recorded here, as is the request that all laboratory conditions be reported in future prospective studies if we are truly to understand the aetiology, or otherwise, of issues associated with extended culture. Based on extensive animal literature, it is proposed that the majority of so-called adverse outcomes of extended culture can be attributed to the use of atmospheric oxygen in the culture system. Furthermore, the use of vitrification for blastocyst cryopreservation will result in a significant increase in the overall efficacy of extended culture cycles.

Maheshwari et al. (2015) conclude; "The available data at present are weak and do not justify stopping extended culture but in the interest of long-term outcomes, it is perhaps time to rethink the current policy of blastocyst transfer". It would be more appropriate to conclude that given the overwhelming amount of data on the negative effects of atmospheric oxygen on the preimplantation mammalian embryo, and the ability of such high oxygen conditions to make the embryo more susceptible to other stressors present, it is time to rethink the current policy of all human embryo culture (cleavage stages and blastocyst) in the presence of atmospheric oxygen, and move to the routine use of 5% oxygen as advocated over 20 years ago (Gardner and Lane, 1993).

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