



Oxygen Concentration during Oocyte Maturation in the Mouse

Kelly Michelle Banwell

1116320

Discipline of Obstetrics & Gynaecology,
The School of Paediatrics & Reproductive Health,
The University of Adelaide



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Abstract

Follicular antral oxygen tension is thought to influence subsequent oocyte developmental competence. Despite this, *in vitro* maturation (IVM) is routinely performed in either 5 or 20% oxygen and while low oxygen has been shown to be beneficial to embryo development in many species, the effects of altering oxygen concentration during IVM have not been adequately investigated. Here we investigated the effects of a range of oxygen concentrations (2, 5, 10 & 20% oxygen) during IVM of mouse oocytes on a range of oocyte and embryonic parameters as well as fetal/placental outcome measures and cumulus cell gene expression.

While common short term measures of oocyte developmental competence such as maturation, fertilisation, and embryonic development rates were not affected over the range of oxygen levels used, more in depth investigations found several striking differences. Following IVM at 5% oxygen, the oocyte mitochondria were found to have altered patterns of both membrane potential (a measure of mitochondrial activity) and distribution suggesting altered oocyte metabolism. Following IVF, the cellular make up of embryos was investigated. In blastocysts derived from low IVM oxygen (2%) there was found to be an increased number of trophoblast cells, an increased level of apoptosis (although this was not of sufficient magnitude to account for the cell number difference) and more cells positive for both Cdx2 and Oct4 (markers of trophoblast and inner cell mass cell types respectively) suggesting a less differentiated cell type. Furthermore, following embryo transfer, the ability of the embryos to implant or develop was not altered by IVM oxygen concentration; however, fetal and placental weights were reduced in the 5% oxygen group. Cumulus cell gene expression was also examined and was found to be altered both across IVM oxygen treatment groups and when compared to cells isolated from *in vivo* derived complexes. This change in gene expression elucidates some of the many ways in which oxygen concentration during IVM may be affecting the cumulus-oocyte complex (COC) and its future development. Together, this data highlights the importance of looking past common outcome measures when determining the effects of IVM culture

conditions. The results of this study also suggest that while IVM oxygen concentration contributes to the perturbing nature of current IVM systems, it is only one of many constituents that require proper investigation, understanding and optimisation.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Kelly M. Banwell

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Publications arising from these and related studies

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2. **Banwell, K. M.**, Thompson, J. G. (2008). *In vitro* maturation of mammalian oocytes: outcomes and consequences. *Semin. Reprod Med.* **26**(2): 162-74.

Abstracts arising from these and related studies

1. **Banwell, K. M.**, Lane, M., Russell, D. L., Kind, K. L., Thompson, J. G. (2005) "Blastocyst cell lineage is influenced by the oxygen concentration used during murine *in vitro* maturation" Proceedings of The Australian Society for Medical Research SA Conference, Adelaide, Australia.
2. **Banwell, K. M.**, Lane, M., Russell, D. L., Kind, K. L., Thompson, J. G. (2005) "Oxygen concentration during *in vitro* maturation of murine oocytes affects blastocyst cell lineage" Proceedings of The 36th Annual Conference of the Society for Reproductive Biology, Perth, Australia.
3. **Banwell, K. M.**, Lane, M., Russell, D. L., Kind, K. L., Thompson, J. G. (2005) "Effect of oxygen concentration during murine oocyte maturation on blastocyst cell lineage" Proceedings of The North Western Adelaide Health Service Research Day, Adelaide, Australia.
4. **Banwell, K. M.**, Lane, M., Russell, D. L., Kind, K. L., Thompson, J. G. (2006) "Oxygen concentration during murine oocyte maturation (IVM) influences oocyte and embryo parameters" Proceedings of The Australian Society for Medical Research SA Conference, Adelaide, Australia.
5. **Banwell, K. M.**, Lane, M., Russell, D. L., Kind, K. L., Thompson, J. G. (2006) "Fetal and placental outcomes are programmed by oxygen concentration during maturation of murine oocytes" Proceedings of the 37th Annual Conference of the Society for Reproductive Biology, Gold Coast, Australia.
6. **Banwell, K. M.**, Lane, M., Russell, D. L., Kind, K. L., Thompson, J. G. (2007) "Oxygen Concentration During *In vitro* Maturation of Murine Oocytes Influences Subsequent Fetal and Placental Outcomes" Proceedings of the Annual Conference of the International Embryo Transfer Society, Kyoto, Japan.
7. **Banwell, K. M.**, Lane, M., Russell, D. L., Kind, K. L., Thompson, J. G. (2007) "Mitochondrial consequences following *in vitro* maturation (IVM) of mouse oocytes in varying oxygen concentrations" Proceedings of The 38th Annual Conference of the Society for Reproductive Biology, Christchurch, New Zealand.

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