The Distribution and Speciation of Trace Elements in Bovine Ovaries using Synchrotron Techniques

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Melanie Jasmine Ceko

25 August 2014

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I dedicate this thesis to the thousands of asylum seekers currently detained in offshore processing centres, particularly those in Nauru. There were times during my candidature when I felt overwhelmed, uninspired, and even frustrated by the tasks I was undertaking. Contemplating your lives almost instantly put my situation into perspective and for this I am both grateful and sorry. May you be granted the freedom you so deserve.

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Abstract

When studying the relationship of trace elements with human health, it becomes increasingly evident that keeping a balanced level of minerals in every cell of the human body may be crucial to optimising health and preventing disease. Dietary deficiencies of trace elements have been reported to alter various aspects of reproductive physiology, however, there is an alarming weighting towards studies focusing on their involvement in male reproductive function. Furthermore, those studies which are female focused have a tendency to investigate the role of trace elements in maintaining a healthy pregnancy, and the impact on offspring, as opposed to addressing preconception ovarian function.

This research predominantly used a combination of two synchrotron-based X-ray techniques, X-ray absorption spectroscopy (XAS) and X-ray fluorescence (XRF) imaging, to investigate the role that trace elements play in female reproductive function. More specifically, by probing the *in situ* bioaccumulation of trace elements in bovine ovaries, important findings were made regarding the distribution of iron (Fe), selenium (Se), and zinc (Zn) which were localised to specific structures. Additionally, widespread high levels of bromine (Br) were observed across all imaged tissues.

By classifying the ovarian follicles according to their health status, as well as their size (follicular diameter), and extracting average elemental concentrations for these follicles from the XRF images, it was possible to quantitatively analyse the cohort of 97 imaged follicles. Statistically significant differences were found for the elements Fe and Br (health status), and for Se (size). Se appeared to be the element which most greatly distinguished large (> 10 mm)

antral follicles from smaller counterparts and PCA scores plots supported this notion with the large follicles deviating from the rest of the cohort.

Owing to the statistically significant and very precise localisation of Se to the granulosa cells of large healthy follicles, a suite of additional biologically-based experiments were performed (quantitative real-time reverse transcription polymerase chain reactions (qRT-PCR), immunohistochemical staining, and Western immunoblotting) in order to identify the Se species as glutathione peroxidase 1 (GPx1). Taking into account what is already known about this selenoprotein, it was concluded that Se and selenoproteins may play a critical role as an antioxidant during late follicular development.

There has been much debate about the essentiality of Br to human and animal life. Owing to its high levels across all ovarian tissues imaged, subsequent experimentation was carried out to probe the chemical form of this element. Analyses of the X-ray absorption near-edge structure (XANES) spectra of a variety of mammalian tissues and fluids led to the conclusion that the form of Br in all samples, detected under normal physiological conditions, was bromide.

The application of synchrotron radiation to measure trace elemental distributions in bovine ovaries at such high resolutions has provided new insights into this organ. While this research was intended to form a baseline study for healthy ovaries, if extended to disease states in the future, our understanding of the biochemical mechanisms occurring in this complex organ could be significantly enhanced.

Linkage between Chapters

This thesis is comprised of eight chapters including five manuscripts which form the results chapters (Chapters 2 to 6). All the manuscripts were completed during candidature and Chapters 2 to 6 are intended to be published in peer-reviewed, international journals. Chapter 7 is a results chapter for which the decision was made not to publish the results owing to the lack of additional insight the analysis provided into the raw data.

Below is a list of the manuscripts that constitute Chapters 2 to 6 of this thesis: with Chapter 2 having already been published in Food Chemistry; Chapter 3 submitted to the Journal of Analytical Atomic Spectrometry; and Chapters 4, 5 and 6 ready to be submitted to the preferred journals. The broader research group is seeking more data to enhance the impact of Chapter 6, delaying submission of that manuscript and of those corresponding to Chapters 4 and 5 which refer to Chapter 6. Statements of Authorship which outline the contribution of the candidate and the co-authors to each chapter can be found at the beginning of Chapters 2 to 6. It should be noted that the methods presented in the manuscripts forming Chapters 3 to 6 are highly repetitious. Appropriate cross referencing of the materials and methods sections will be carried out dependent upon the order in which the papers are accepted for publication. Each chapter has been presented in the format which is appropriate for the journal it is intended to be published in.

Chapter 2: Ceko, M. J., Aitken, J. B., and Harris, H. H. (2014) Speciation of Cu in a range of food types by X-ray Absorption Spectroscopy. *Food Chemistry,* **164**, 50-54

Chapter 3: Ceko, M. J., Hummitzsch, K., Hatzirodos, N., Bonner, W., James, S. A., Spiers, K. M., Kirby, J. K., Rodgers, R. J., and Harris, H. H. (2014) Distribution and

speciation of bromine in mammalian tissue and fluids by X-ray fluorescence imaging and X-ray absorption spectroscopy. *Submitted to Journal of Analytical Atomic Spectrometry 02/08/2014*.

Chapter 4: Ceko, M. J., Hummitzsch, K., Bonner, W., Aitken, J. B., Rodgers, R. J., and Harris, H. H. (2014) Localization of the Trace Elements Iron, Zinc and Selenium in Relation to Anatomical Structures in Bovine Ovaries by X-ray Fluorescence Imaging. *To be submitted to Microscopy and Microanalysis*.

Chapter 5: Ceko, M. J., Hummitzsch, K., Hatzirodos, N., Rodgers, R. J., and Harris, H. H. (2014) Quantitative elemental analysis of bovine ovarian follicles using X-ray Fluorescence Micro-spectroscopy. *To be submitted to Metallomics*.

Chapter 6: Ceko, M. J., Hatzirodos, N., Hummitzsch, Bonner, W., Aitken, J. B., Rodgers, R. J., and Harris, H. H. (2014) X-ray Fluorescence Microscopy identifies a role for selenium in bovine female reproductive function. *Submitted, including additional data not presented in this thesis, to Metallomics.*

Chapter 1 is intended to serve as an introduction and provides a review of each of the five elements researched during candidature; namely, Br, Cu, Fe, Se and Zn. A general introduction into the biological relevance of each element is presented, in addition to a discussion surrounding its bioavailability, absorption *in vivo*, and anything that is known regarding its role in female reproductive function. The second half of the chapter is intended to introduce the key methodologies of this research and provides a theoretical introduction to XAS, XRF imaging, and ICP-MS, as well as discussing examples from the literature where XRF imaging techniques have been applied to animal systems.

In Chapter 2 the chemical form of Cu in a range of foods is analysed by fitting a suite of Cu model compound spectra to the XANES spectrum for each sample. ICP-MS is additionally employed to quantify the concentrations.

The aim of Chapter 3 was to distinguish the chemical form of Br across a wide range of predominantly mammalian tissues and fluids. ICP-MS analyses supported the high levels of Br that were initially observed in bovine ovarian tissue through XRF imaging, and these results are also presented.

Chapters 4 to 7 analyse the findings of two significant XRF imaging experiments, in which a number of subsections (n = 97 for the follicles alone) were imaged for Br, Fe, Se and Zn distributions. Chapters 4 and 5 may appear to have repetitious content but it should be pointed out that the focus of the former chapter is predominantly on qualitative observations and more specifically on the ability to differentiate between key structures in bovine ovarian tissues using elemental localisations alone. On a few occasions it was considered advantageous to quote approximate quantitative findings to more clearly articulate the differences in elemental levels between structures. In Chapter 5 not only does the focus become significantly more quantitative, but the content focuses purely on a single structure within the bovine ovaries (follicles) and explores the potential for differences in trace element levels, perhaps implying different protein expression, being used to distinguish between follicles at different stages of growth or atresia, or follicles of different sizes.

Chapter 6 builds on the key conclusions of Chapter 4 and 5; that being that Se is localised to the granulosa region of large healthy follicles. In this chapter a suite of biological

techniques are employed (qRT-PCR, immunohistochemical staining, and Western immunoblotting) to identify this protein.

Chapter 7 aims to address a common shortcoming of synchrotron-based XRF imaging; that being that it has a tendency to be purely observational in its nature. Having already extended beyond this in the quantitative analyses of Chapter 5, Chapter 7 explores the application of a multivariate statistical analysis technique to the sets of XRF generated elemental distribution maps collected for many of the ovarian sub sections.

Finally, Chapter 8 summarises the conclusions from this research and discusses potential future directions. Topics addressed include the significance of the work, its contribution to the discipline, problems encountered, and the necessity for further studies.