The role of reactive oxygen species and oxidative stress in post-ovulatory ageing and apoptosis of the mammalian oocyte

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B. Biotech (Hons Class I)

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Date: 15th July, 2015
Declaration

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

Thesis by Publication

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Signed…………………

Tessa Lord
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Publications and awards arising from work in this thesis

1. Publications

Chapter 1


Chapter 2


Chapter 3


Chapter 4

2. Statements of contribution

I attest that the Research Higher Degree candidate Tessa Lord has contributed upward of 50% towards data collection/analysis and manuscript preparation for all the publications included in this thesis for which I am a co-author.

L. Prof. John Aitken
Date: 8 July 2015

A. Prof. Brett Nixon
Date: 8 July 2015

Prof. Keith Jones
Date: 8 July 2015

Jacinta Martin
Date: 8 July 2015

Frances Martin (ADRT)
Date:
3. Conference Proceedings


**Lord, T.**, Aitken, R. J. Fertilization prevents entry of the MII oocyte into senescence by improving the capacity to cope with oxidative stress and subsequent damage. 44th annual conference of the Society for Reproductive Biology. Sydney, Australia. August 2013.


4. Awards

‘Best Presentation by a PhD Student’ prize - Australian Society for Medical Research Satellite Scientific Meeting, Newcastle, Australia (2014)

‘Faculty of Science and I.T. Conference Scholarship’ – University of Newcastle, Australia (2014)

‘Oozoa award’ for best student presentation - Society of Reproductive Biology, Sydney, Australia (2013)

Finalist for the Oozoa award for best student presentation, Society of Reproductive Biology, Gold Coast, Australia (2012)
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Abstract

Following ovulation, the MII stage oocyte awaits fertilization in the oviduct, or, in the case of assistive reproductive technologies (ART), in in vitro culture medium. In the absence of fertilization, however, the oocyte experiences a time-dependent deterioration in quality referred to as post-ovulatory ageing. Post-ovulatory ageing is associated with a decline in fertilization rate, as well as the production of poor quality embryos, an increased risk for post-implantation errors and production of offspring with compromised health. Although the consequences associated with post-ovulatory ageing are well defined, the molecular mechanisms which orchestrate this decline in oocyte quality, or conversely, act to prevent post-ovulatory ageing in the event that timely fertilization has occurred, are not well understood. In this thesis we decipher a critical role for reactive oxygen species (ROS) in the initiation of post-ovulatory ageing and apoptosis of the mammalian oocyte. Using a mouse model, we have characterised a time–dependent accumulation of intracellular ROS following retrieval of the ovulated oocyte. This elevation in ROS was found to instigate a self-perpetuating cycle of lipid peroxidation, electrophilic aldehyde production and mitochondrial damage; resulting in the initiation of an intrinsic apoptotic cascade. The elevation in levels of ROS and electrophilic aldehydes within the oocyte were directly associated with a decreased capacity to participate in fertilization and support embryo development.

Importantly, research within this thesis has demonstrated that timely fertilization of the oocyte is associated with an up-regulation of glutathione peroxidase activity, and accelerated DNA repair by the base excision repair (BER) pathway. These post-fertilization changes in oocyte biochemistry aide in circumventing the otherwise
inevitable initiation of post-ovulatory ageing by preventing the accumulation of ROS and oxidative DNA damage.

In identifying the critical role for ROS and electrophilic aldehydes in post-ovulatory ageing and apoptosis of the oocyte, it was possible to select antioxidant and aldehyde-reactive compounds to attenuate the onset of these processes. Specifically, melatonin was found to significantly improve fertilization rate, embryo formation rate and embryo quality in oocytes aged for 8 and 16 h \textit{in vitro}, as well as delay the initiation of apoptosis. Similarly, penicillamine was found to prevent the decline in fertilization rate and embryo formation rate associated with elevated levels of electrophilic aldehydes. The adaptation of these supplementation techniques for use in a human ART setting would be advantageous in lengthening the optimal window of time in which oocytes must be inseminated post-retrieval, as well as increasing the viability of re-insemination techniques such as rescue-ICSI; potentially minimising the likelihood that further ovarian stimulation cycles would be necessary following a failure to fertilize by IVF.

Collectively, these data provide a significant contribution to the field of knowledge surrounding degeneration and apoptosis of the mammalian oocyte, and provide novel methodologies for attenuating these events in an \textit{in vitro} setting.
CHAPTER 1 - Literature review

_Oxidative stress and ageing of the post-ovulatory oocyte_

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Chapter 1: Overview

The aim of the following literature review was to evaluate the current wealth of knowledge surrounding post-ovulatory ageing of the mammalian oocyte. Specifically, this review provided links between the established biochemical changes that occur within the oocyte in a time-dependent manner from ovulation and the clinical consequences associated with post-ovulatory ageing, in addition to speculating on the potential molecular mechanisms controlling these processes.

Upon acknowledgement of previous research that identifies an elevation in levels of reactive oxygen species within the oocyte with increasing time from ovulation, this chapter explored the possibility that oxidative stress is the initiator of the cascade of biochemical changes that result in the age-associated deterioration of oocyte quality. We have investigated the propensity for oxidative stress in the oocyte to influence a number of events associated with post-ovulatory ageing, including a decline in critical cell cycle factors such as MAPK, impaired calcium homeostasis, mitochondrial dysfunction, and a reduced capacity to participate in fertilization and support embryo development. This manuscript also explored strategies by which to prevent or delay post-ovulatory ageing in vitro, with a particular focus on the safety concerns associated with previously investigated compounds such as caffeine, as well as biochemical properties that are likely to determine the success of antioxidant compounds when supplementing in vitro culture medium.
Oxidative stress and ageing of the post-ovulatory oocyte

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Abstract

With extended periods of time following ovulation, the metaphase II stage oocyte experiences deterioration in quality referred to as post-ovulatory oocyte ageing. Post-ovulatory ageing occurs both in vivo and in vitro and has been associated with reduced fertilization rates, poor embryo quality, post-implantation errors and abnormalities in the offspring. Although the physiological consequences of post-ovulatory oocyte ageing have largely been established, the molecular mechanisms controlling this process are not well defined. This review analyses the relationships between biochemical changes exhibited by the ageing oocyte and the symptoms associated with the ageing phenotype. We also discuss molecular events that are potentially involved in orchestrating post-ovulatory ageing with a particular focus on the role of oxidative stress. We propose that oxidative stress may act as the initiator for a cascade of events that create the aged oocyte phenotype. Specifically, oxidative stress has the capacity to cause a decline in levels of critical cell cycle factors such as maturation-promoting factor, impair calcium homeostasis, induce mitochondrial dysfunction and directly damage multiple intracellular components of the oocyte such as lipids, proteins and DNA. Finally, this review addresses current strategies for delaying post-ovulatory oocyte ageing with a particular focus on the potential use of compounds such as caffeine or selected antioxidants in the development of more refined media for the preservation of oocyte integrity during IVF procedures.

Reproduction (2013) 146 R217–R227

Introduction

Upon release from the ovary, the prophase I oocyte undergoes a process of maturation involving resumption of meiosis, breakdown of the germinal vesicle and extrusion of the first polar body (reviewed by Sun et al. 2009). Following maturation, the oocyte once again enters meiotic arrest, now at metaphase II (MII). The MII stage oocyte awaits fertilization by the spermatozoon either in the oviduct of the female reproductive tract or, in an assisted reproduction setting, in in vitro culture media. An optimal window exists in which fertilization of this MII stage oocyte should occur; in mammalian species, this is generally within 10 h of ovulation; however, successful fertilization can still occur in the mouse for up to 15 h (Marston & Chang 1964, Tarin et al. 1999). In the event that no fertilization occurs within this time frame, oocytes experience a process of degradation referred to as ‘post-ovulatory ageing’. It should be noted that post-ovulatory ageing is a process distinct from that of ‘ovarian ageing’ (reviewed by Bentov & Casper 2013), which occurs within the ovary of females towards the end of reproductive life, as they approach the climacteric. All references to oocyte ageing within this review refer to the deterioration of MII oocytes that occurs following their release from the ovarian follicle both in vivo and in vitro.


The cellular deterioration associated with post-ovulatory ageing can strongly influence the outcome of fertilization in vivo and in vitro. This is not surprising when considering that the oocyte provides factors to the embryo that orchestrate the early events of embryogenesis (Schultz & Heyner 1992, Minami et al. 2007), remodel the genome (Torres-Padilla et al. 2006) and repair DNA damage in both male and female nuclei.
that fertilization could involve an oocyte. This, in turn, results in an increased likelihood synchrony between intercourse and release of the spermatozoa to the female reproductive tract is relatively a result of such mechanisms is that the delivery of oestrus, or the act of mating itself triggers ovulation. The ovulation, as determined by the onset of behavioural post-ovulatory ageing is not uncommon. In the majority of mammalian species, mating occurs only at the time of ovulation, (Genesca et al. 1992). Unfortunately, the occurrence of post-ovulatory ageing is not uncommon. In the majority of mammalian species, mating occurs only at the time of ovulation, as determined by the onset of behavioural oestrus, or the act of mating itself triggers ovulation. The result of such mechanisms is that the delivery of spermatozoa to the female reproductive tract is relatively synchronized with oocyte release. In humans and certain primates, however, there are no visual signs of ovulation and, as a result, no mechanisms in place to ensure synchrony between intercourse and release of the oocyte. This, in turn, results in an increased likelihood that fertilization could involve an in vivo aged oocyte and freshly ejaculated spermatozoa. In an in vitro setting, oocytes employed in assisted reproduction technologies (ART) are often unavoidably subjected to extended periods of culture prior to fertilization. One circumstance in which this may occur is when ‘rescue ICSI’ is performed on failed-to-fertilize oocytes. In light of such considerations, it would clearly be beneficial to establish some control over the process of post-ovulatory oocyte ageing, particularly in an in vitro setting, where the demand for ART continues to increase exponentially (Wang et al. 2011).

This review explores the negative consequences associated with post-ovulatory oocyte ageing, discusses the poorly understood mechanisms that control this process and considers the current means by which post-ovulatory ageing can be prevented or delayed. Specifically, in light of recent research, we revisit the proposal that oxidative stress may act as the ‘trigger’ for a cascade of other events associated with oocyte ageing and hence might be an attractive approach for deterring this degenerative process in post-ovulatory oocytes.

### Clinical implications of oocyte ageing

The clinical implications of oocyte ageing include a decreased capacity for fertilization both in vivo (Marston & Chang 1964) and in vitro (Ben-Rafael et al. 1986, Badenas et al. 1989), production of poor-quality embryos (Yanagida et al. 1998, Lord et al. 2013), an increased likelihood of early pregnancy loss (Wilcox et al. 1998) and abnormalities in offspring (Tarin et al. 1999). The attributes of aged oocytes responsible for these adverse outcomes are discussed below.

### Decreased fertilization rate

A decrease in fertilization rate has been associated with aged oocytes in many species including the humans (Lash & Whittaker 1974), mice (Marston & Chang 1964, Liu et al. 2009b), cows (Chian et al. 1992) and pigs (Kikuchi et al. 2000). This phenomenon can be attributed to multiple biochemical and functional alterations to the oocyte that accumulate with post-ovulatory age. First, aged murine oocytes are known to experience premature cortical granule exocytosis (Szollosi 1971, Dodson et al. 1989, Ducibella et al. 1990, Xu et al. 1997) and zona hardening (Longo 1981, Dodson et al. 1989, Xu et al. 1997); changes that would obviously impose limitations on the ability of the zona pellucida to interact with a spermatozoon and initiate the acrosome reaction. Additionally, as levels of lipid peroxidation are known to be elevated within post-ovulatory aged oocytes (Takahashi et al. 2003; Table 1), the fluidity of the oolemma may be decreased, reducing the likelihood of sperm–oolemma fusion and thereby fertilization.

In the event that sperm–zona interaction and sperm–oolemma fusion progress unimpaired, the abilities of the

### Table 1 Aberrations to oocyte biology associated with mammalian post-ovulatory ageing.

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Consequences associated with ageing</th>
<th>In vivo/in vitro</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zona pellucida</td>
<td>Zona hardening</td>
<td>Both</td>
<td>Longo (1981), Dodson et al. (1989) and Xu et al. (1997)</td>
</tr>
<tr>
<td>Cortical granules</td>
<td>Partial exocytosis</td>
<td>Both</td>
<td>Szollosi (1971), Dodson et al. (1989), Ducibella et al. (1990), Xu et al. (1997) and Liu et al. (2009a, 2009b)</td>
</tr>
<tr>
<td>Spindle</td>
<td>Elongation, dispersal or disruption</td>
<td>In vitro</td>
<td>Takahashi et al. (2003)</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>Lipid peroxidation</td>
<td>Both</td>
<td>Takahashi et al. (2003)</td>
</tr>
<tr>
<td>ER</td>
<td>Depletion of Ca^{2+} stores</td>
<td>Both</td>
<td>Takahashi et al. (2000)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Increased generation of ROS</td>
<td>Both</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>DNA</td>
<td>Loss of membrane potential</td>
<td>In vitro</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>Decreased ATP production</td>
<td>In vitro</td>
<td>Chi et al. (1988)</td>
</tr>
<tr>
<td></td>
<td>Epigenetic changes</td>
<td>Both</td>
<td>Liang et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>Misaligned chromosomes</td>
<td>Both</td>
<td>Wakayama et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>Premature centromere separation</td>
<td>Both</td>
<td>Spielmann et al. (1985) and Mailhes et al. (1998)</td>
</tr>
</tbody>
</table>

IP$_3$, inositol 1,4,5-trisphosphate; ROS, reactive oxygen species.
post-ovulatory aged oocyte to exhibit normal activation and initiate embryo development following fertilization are nonetheless compromised. Upon fertilization, inositol 1,4,5-trisphosphate (IP$_3$) receptors located on the oocyte’s endoplasmic reticulum (ER) initiate calcium (Ca$^{2+}$) oscillations in response to IP$_3$ produced by the sperm-derived factor PLCζ1 (Yoon et al. 2008, Kuroda 2010). However, post-ovulatory aged oocytes experience a decline in IP$_3$ receptor/channel functionality (Zhang et al. 2011), as well as suppressed ER Ca$^{2+}$-ATPase activity, and exhibit a consequential depletion of Ca$^{2+}$ stores that reside within the ER (Takahashi et al. 2000). As a result of this impaired Ca$^{2+}$ homoeostasis, aged oocytes exhibit abnormal Ca$^{2+}$ oscillations at fertilization that have significantly higher frequency and lower amplitude than those of fertilized fresh oocytes (Igarashi et al. 1997, Takahashi et al. 2003). These abnormal Ca$^{2+}$ oscillations are purportedly associated with the onset of apoptosis following fertilization, rather than entry into a developmental pathway (Gordo et al. 2000, 2002).

**Poor embryo quality**

While the fertilization potential of post-ovulatory aged oocytes is obviously compromised, circumstances exist in which fertilization does still occur, particularly in clinical practice when ICSI is used to bypass the physiological mechanisms that would normally prevent the association of defective gametes. Not surprisingly, embryos originating from post-ovulatory aged oocytes are generally of poor quality and exhibit delayed or impaired development during subsequent embryogenesis (Yanagida et al. 1998, Lord et al. 2013).

The poor quality of these embryos is highlighted when we assess their capacity to implant in the endometrium and carry a pregnancy to term. With extensive periods of post-ovulatory ageing (>24 h in vitro), ICSI-generated embryos reportedly lose their capability for uterine implantation following embryo transfer (Chen et al. 1995). Despite this, oocytes that are subjected to shorter periods of post-ovulatory ageing can still produce embryos capable of implantation, but are prone to post-implantation errors. A clinical study assessing the effects of in vivo post-ovulatory oocyte ageing found a significant correlation between early pregnancy loss and the likelihood that delayed fertilization had occurred (Wilcox et al. 1998). These findings have also been reported in other mammalian species such as the mouse (Marston & Chang 1964).

The decline in embryo quality associated with post-ovulatory aged oocytes is presumably attributed to multiple factors. First, cytoplasmic ageing is likely to greatly hinder the capacity of the oocyte to support and orchestrate embryo development, particularly when changes in both the protein and mRNA composition of the oocyte are demonstrable with prolonged ovulation to fertilization latency (Tarin et al. 2000, Hamatani et al. 2004). Additionally, aged oocytes are likely to display a corresponding increase in the proportion of non-viable embryos following fertilization as a result of chromosomal abnormalities. Post-ovulatory aged oocytes purportedly exhibit significantly higher instances of chromosome misalignment, potentially as a consequence of meiotic spindles that are elongated, dispersed or disrupted (Wakayama et al. 2004). These characteristics increase the likelihood for erroneous chromosome separation and hence aneuploidy (Mailhes et al. 1998). Further to this, in certain species such as the bovine, advancing oocyte age is also associated with a significantly increased susceptibility to polyspermy (Chian et al. 1992), which again results in the generation of embryos that are essentially non-viable.

**Abnormalities in offspring**

Despite the obstacles associated with fertilization of post-ovulatory aged oocytes and subsequent embryo development and implantation, birth of live offspring originating from these gametes does still occur. It was originally thought that aged oocytes that manage to support embryo development to the blastocyst stage were no less capable of producing healthy offspring than their fresh oocyte counterparts (Wilcox et al. 1998). However, more recent research has demonstrated a significant decline in live birth rates and an elevated instance of abnormalities in offspring (Tarin et al. 1999).

Tarin et al. (1999, 2002) demonstrated that offspring originating from in vivo aged oocytes exhibited growth retardation, delayed development, heightened emotional state, decreased reproductive fitness and importantly decreased longevity. Although the specific factors within the post-ovulatory aged oocyte that so prominently affect offspring integrity have not been determined, it has been hypothesized that these abnormalities may stem from the transference of a subpopulation of dysfunctional mitochondria (Tarin et al. 2002). Impaired mitochondrial function has indeed been linked with diseases such as schizophrenia (Whatley et al. 1996), Alzheimer’s disease (Hutchin & Cortopassi 1995, Budd & Nicholls 1998) and a decreased lifespan (Sont & Vandenbroucke 1993).

An alternative explanation for the abnormalities observed in the offspring of in vivo and in vitro aged oocytes may lay with an altered epigenetic profile, which would essentially affect gene expression in the embryo. Both the male and female genomes experience significant demethylation events following fertilization (Mayer et al. 2000) and post-ovulatory oocyte ageing has been shown to significantly alter the methylation pattern of imprinted genes in both the oocyte and the developing placenta (Liang et al. 2008, 2011).
Factors affecting oocyte ageing and apoptosis

Post-ovulatory oocyte ageing is clearly a prominent issue affecting fecundity; however, the molecular mechanisms that control this process are not well elucidated. It is currently undetermined as to whether a single event acts to trigger a cascade of other factors, or if several biochemical and functional changes occur separately to create an ‘aged’, degenerating MII oocyte. Below we assess factors that are known to influence the onset of post-ovulatory ageing and specifically highlight the potential role of oxidative stress as the ‘trigger’ for ageing and apoptosis of the oocyte both in vitro and in vivo.

Cumulus cells

In the presence of cumulus cells, MII stage oocytes purportedly ‘age’ more rapidly. In vivo aged oocytes and in vitro aged oocytes enclosed within the cumulus cell complex experience increased rates of spontaneous activation and fragmentation (Miao et al. 2005, Wu et al. 2011), accelerated decline of MPF/MAPK (Miao et al. 2005) and decreased levels of blastocyst formation (Wu et al. 2011) when compared with denuded oocytes aged in vitro. Qiao et al. (2007) reported that the addition of cumulus cells to culture medium containing denuded oocytes caused an acceleration of post-ovulatory ageing on par with that of oocytes enclosed in a cumulus–oocyte complex, while other research groups have demonstrated that blocking gap junctions within the cumulus–oocyte complex does not prevent accelerated ageing (Wu et al. 2011). Following these observations, it has been hypothesized that cumulus cells secrete soluble/paracrine factor(s) that promote post-ovulatory ageing, potentially an event that coincides with the entry of the cumulus cells into apoptosis (Wu et al. 2011). Additionally, the accelerated depletion of the oocyte metabolite pyruvate from culture medium by cumulus cells (Downs et al. 2002) appears to directly affect oocyte metabolism and hence post-ovulatory ageing, potentially by causing downstream inhibition of protein synthesis within the oocyte and upsetting redox equilibrium (Liu et al. 2009b, Li et al. 2011).

An alternative explanation for cumulus cell-associated acceleration of post-ovulatory ageing has been detailed in publications by Perez et al. (2005) and Kujjo & Perez (2012). These researchers propose that the bioactive sphingolipid, ceramide, generated by the cumulus cells may be responsible for mitochondrial dysfunction and subsequent ageing and apoptosis in post-ovulatory oocytes. This is attributed to an increased sensitivity of aged oocytes to cytosolic ceramide spikes and the ability of ceramide to act as a pro-apoptotic factor upstream of Bax.

Although the presence of cumulus cells clearly exacerbates degeneration in ageing post-ovulatory oocytes, additional mechanisms must be at play in controlling this process, as oocytes that have been denuded of their cumulus cells also experience ageing and apoptosis (Miao et al. 2005, Lord et al. 2013).

MPF and MAPK

The critical cell cycle factors MPF and MAPK control meiotic resumption in germinal vesicle stage oocytes and then act to maintain cell cycle arrest once oocytes have reached the MII phase. A gradual decline in the concentration of these factors within the MII oocyte has been detected with post-ovulatory age and has been associated with increased levels of parthenogenetic activation and fragmentation (Kikuchi et al. 2000). The mechanism by which MPF specifically becomes degraded with post-ovulatory age has been studied extensively – particularly as a potential target for reversing the ageing process (to be discussed later in this review). MPF comprises two molecules: the catalytic subunit p34cdc2 and the regulatory subunit cyclin B. In the active form of MPF, these two subunits are coupled and T-161 of the catalytic subunit is phosphorylated. Following fertilization, MPF is inactivated by the dephosphorylation of T-161 and degradation of the decoupled cyclin B. Interestingly, inactivation of MPF occurs via an alternate pathway in the ageing post-ovulatory oocyte. Under these circumstances, the inactive compound ‘pre-MPF’ is formed, in which the two subunits are still bound but phosphorylation of T-14 and/or T-15 of p34cdc2 occurs (Kikuchi et al. 2002). The accumulation of pre-MPF is clearly a factor controlling at least some aspects of post-ovulatory ageing as acceleration of pre-MPF formation using the tyrosine phosphatase inhibitor, vanadate, has been shown to increase susceptibility to parthenogenetic activation and fragmentation, while maintenance of active MPF levels within the oocyte using caffeine decreases levels of parthenogenesis and fragmentation (Kikuchi et al. 2002), and extends the window for fertilization (Ono et al. 2011).

Mitochondrial dysfunction

Mitochondrial dysfunction appears to be another potent mediator of the post-ovulatory oocyte ageing process. Operational mitochondria are crucial to normal oocyte function, with these organelles representing the primary source of ATP production within both oocytes and early embryos (Dumollard et al. 2007). The functionality of the mitochondria does, however, become compromised with extended periods of time following ovulation, a factor that is thought to heavily influence oocyte quality. Diminished mitochondrial integrity in the in vitro aged oocyte has been demonstrated by a loss of mitochondrial membrane potential (Zhang et al. 2011) and a decline in levels of ATP production (Chi et al. 1988).

As damage to the mitochondria is known to cause an increased production of reactive oxygen species (ROS)
and the release of pro-apoptotic factors such as cytochrome c (Liu et al. 2009a), which recruit members of apoptotic machinery such as caspases (Takai et al. 2007) and endonucleases (Fujino et al. 1996), it is likely that mitochondrial dysfunction is the link between post-ovulatory oocyte ageing and apoptosis. Additionally, loss of mitochondrial integrity can result in aberrant protein synthesis and inactivation or loss of mitochondrial DNA (mtDNA; reviewed by Shigenaga et al. (1994)).

The pronounced control that mitochondrial function has over post-ovulatory ageing has been demonstrated in studies utilizing microinjection of isolated mitochondria into in vitro aged oocytes. During a 24-h culture period, MII oocytes whose mitochondrial pool was increased by 5% via microinjection showed a significant decline in levels of programmed cell death when compared with control oocytes (Perez et al. 2000).

**Oxidative stress: the initiator of post-ovulatory ageing?**

It has been demonstrated by several research groups that ROS – particularly hydrogen peroxide (H2O2), superoxide anion (O2·−) and peroxynitrite (ONOO−) – accumulate in oocytes with increasing amounts of time following ovulation both in vitro and in vivo (Takahashi et al. 2003, Tatone et al. 2011, Lord et al. 2013). ROS production within the MII oocyte likely occurs as a by-product of oxidative phosphorylation; however, environmental factors that oocytes are subjected to in vitro (exposure to light, lack of antioxidant rich follicular and tubal fluids and increased oxygen tension) are also thought to facilitate ROS production (Mastroianni & Jones 1965, Mass et al. 1976, Goto et al. 1993, Guerin et al. 2001). While the oocyte does offer some intracellular defence against oxidative attack in the form of the antioxidant glutathione, these resources are depleted with post-ovulatory age (Boerjan & de Boer 1990, Yoshida et al. 1993). As a consequence of the progressive increase in ROS production and the concomitant depletion of antioxidant protection, the post-ovulatory aged oocyte experiences a state of oxidative stress. We hypothesize that this oxidative stress may act as the ‘trigger’ for a cascade of other factors that orchestrate post-ovulatory ageing, as well as directly affect multiple aspects of oocyte biochemistry and functionality. The idea that oxidative stress may be the initiator of ageing in the MII oocyte is supported by recent findings indicating that the onset of oxidative stress is a relatively early event in in vitro culture (Lord et al. 2013). Indeed, links can be established between oxidative stress in the oocyte and the aforementioned decline in critical cell cycle factors, mitochondrial dysfunction, apoptosis, impaired Ca2+ homoeostasis, decreased fertilization rate, poor embryo quality and abnormalities in offspring. These associations will be discussed below.

We propose that the decline in the critical cell cycle factor, MPF, which reportedly orchestrates some facets of post-ovulatory ageing, may be a symptom of oxidative stress in the MII oocyte. As mentioned previously, the accumulation of ‘pre-MPF’ occurs with increasing periods following ovulation, with phosphorylation at multiple sites on the catalytic subunit of MPF effectively inactivating this compound (Kikuchi et al. 2000, 2002). The enzymes that purportedly control these phosphorylation events are cdc25 (a tyrosine phosphatase) and Wee1/Myt1 (tyrosine kinases) (Kikuchi et al. 2002). Interestingly, ROS have previously been shown to have a capacity for both inhibition of tyrosine phosphatases (Monteiro et al. 1991, Sullivan et al. 1994), including cdc25 specifically (Brisson et al. 2007), and stimulation of tyrosine kinases (Chan et al. 1986). As a result, the induction of oxidative stress in the post-ovulatory oocyte may directly affect levels of MPF within the cell, resulting in the onset of associated downstream symptoms of oocyte ageing such as parthenogenesis and fragmentation (Kikuchi et al. 2002).

Oxidative stress that occurs with post-ovulatory age also has the potential to directly affect mitochondrial function. The DNA, proteins and lipids within the mitochondria are particularly susceptible to oxidative attack, not only because of their close proximity to the source of ROS production (the electron transport chain (ETC)) but also in the case of mtDNA, because of the absence of protective histones and mechanisms for DNA repair (reviewed by Shigenaga et al. (1994)). Oxidative stress has been linked with mtDNA damage and deletions (Sohal & Dubey 1994), loss of mitochondrial membrane potential (Liu et al. 2000), increased ROS generation by the ETC (Liu et al. 2009a) and a decline in ATP production (Melov et al. 1999). Importantly, factors such as increased ROS generation and a decline of ATP production are also known to be associated with post-ovulatory ageing (Chi et al. 1988, Lord et al. 2013), suggesting that a link between oxidative stress and age-related mitochondrial dysfunction is certainly plausible. Notably, oxidative damage to mtDNA in the oocyte could potentially be the basis for the aforementioned abnormalities associated with impaired mitochondrial function in offspring originating from aged oocytes (Tarin et al. 2002).

Oxidative stress also has the capacity to directly influence the onset of apoptosis in post-ovulatory aged oocytes. H2O2 treatment of MII oocytes in vitro has been shown to cause a decline in levels of the anti-apoptotic molecule Bcl-2 (Takahashi et al. 2009), induce expression of pro-apoptotic Bax and caspase-3, and precipitate both DNA fragmentation (Chaubé et al. 2007) and cytochrome c release (Liu et al. 2000). Importantly, these pathologies are common to both oxidative stress and oocyte ageing, and relief from oxidative stress by way of antioxidant treatment has been shown to delay the onset of fragmentation and caspase activation associated with post-ovulatory ageing (Lord et al. 2013).
In addition to potentially being a precursor to MPF/MAPK depletion, mitochondrial dysfunction and subsequent apoptosis, the damaging nature of oxidative stress itself is likely to be directly involved in generation of the ‘aged oocyte’ phenotype. First, oxidative stress may be accountable for a perturbation of Ca\(^{2+}\) homoeostasis in aged oocytes. Previous research has demonstrated that these two events are implicitly linked, as treatment of fresh oocytes with H\(_2\)O\(_2\) results in abnormal Ca\(^{2+}\) oscillations following fertilization, which exhibit similar frequency and amplitude to those produced by post-ovulatory aged oocytes (Igarashi et al. 1997, Takahashi et al. 2003). The basis for this interaction lies with the capacity for ROS to directly affect the Ca\(^{2+}\) channels and Ca\(^{2+}\)-ATPases of the ER (Rohn et al. 1993, Doan et al. 1994, Wesson & Elliot 1995), as well as the Ca\(^{2+}\) signalling molecule calmodulin (Gao et al. 2001). Further facets of oocyte biochemistry have also been shown to be vulnerable to oxidative attack; specifically zona pellucida dissolution time and cortical granule loss; which are significantly altered following treatment with H\(_2\)O\(_2\) and O\(_2\).

The mechanisms behind these pathologies remain elusive, although it is plausible that oxidative stress influences processes under the control of cytoskeletal fibres, such as cortical granule exocytosis and polar body extrusion, by generating damage to the mitochondria that results in decreased ATP production (Chi et al. 1988). With decreased ATP availability, the dynamics of actin and tubulin assembly–disassembly would likely be impeded (Bershadsky et al. 1980).

Finally, oxidative stress is also capable of generating global damage to lipid, protein and DNA constituents of the cell (reviewed by Guerin et al. (2001)). As previously mentioned, peroxidation to lipids in the plasma membrane may decrease the oocyte’s potential for sperm–oocyte fusion. Additionally, such global acquisition of oxidative damage in aged oocytes is presumably related to poor embryo quality following fertilization, as relief from oxidative stress in the aged MII oocyte has been linked with improved developmental potential (Lord et al. 2013).

In conclusion, we propose that the development of oxidative stress is a relatively early event following ovulation that may initiate a decline in MPF levels, generate mitochondrial damage resulting in apoptosis, perturb Ca\(^{2+}\) homoeostasis and instigate damage to the lipid, protein and DNA components of the cell, as well as to the cytoskeleton and zona pellucida. The acquisition of such oxidative damage post-ovulation may culminate in the observed reduction in fertilization rate, decline in embryo quality and increased likelihood for abnormalities in offspring associated with the aged oocyte (Fig. 1). If oxidative stress is indeed the instigator for post-ovulatory degeneration of the MII oocyte, it would be interesting to establish whether the biochemical changes that occur within the oocyte following timely fertilization (i.e. prior to the onset of ‘ageing’) involve increased expression of antioxidant enzymes or enzymes involved in repair of oxidative damage. Certainly, recent research within our laboratory suggests an increased capacity to cope with oxidative insult in oocytes post-fertilization (T Lord and R J Aitken 2013, unpublished observations).

**Mechanisms for preventing/delaying oocyte ageing**

The final portion of this review looks into current strategies for delaying or preventing post-ovulatory oocyte ageing and assesses their effectiveness and potential for adaptation to clinical use in the future.

**Culture conditions and media composition**

Alterations to the concentration of metabolites within oocyte culture media has been shown to prominently affect post-ovulatory ageing in vitro. Specifically, increasing the concentration of pyruvate within culture media has been shown to delay post-ovulatory ageing by maintaining cortical granule integrity, decreasing susceptibility to activating stimuli, preventing an age-associated decline in levels of MPF and glutathione, improving blastocyst formation rates following fertilization and delaying the onset of apoptosis (Liu et al. 2009b, Li et al. 2012). As the oocyte and early embryo preferentially utilize pyruvate over glucose to drive metabolism (Leese & Barton 1984), it has been proposed that pyruvate supplementation delays oocyte ageing by fuelling prolonged ATP production within oocytes and also by maintaining intracellular redox potential (Liu et al. 2009b, Li et al. 2011). Increased pyruvate concentration is particularly effective in preventing accelerated in vitro ageing of the cumulus-enclosed MII oocyte, a result likely attributed to the more rapid depletion of pyruvate from culture media in the presence of cumulus cells (Downs et al. 2002).

Alteration of culture temperature has also been shown to delay the onset of post-ovulatory ageing in vitro. Porcine oocytes supplemented with pyruvate and stored at 15 °C were able to be successfully fertilized and maintain developmental potential after 42 h in vitro, whereas oocytes exposed to the same conditions at 37 °C could only be successfully fertilized after 6 h of culture (Li et al. 2012). The mechanism behind delayed ageing at low temperatures is likely related to a down-regulation of oocyte metabolism (Chip et al. 2011), resulting in reduced levels of ROS production and, subsequently, a decreased likelihood for oxidative damage and ROS-induced apoptosis (Li et al. 2012).

**Maintenance of MPF levels**

Currently, one of the most thoroughly investigated methods for preventing/delaying post-ovulatory oocyte
ageing in vitro is by maintaining levels of critical cell cycle factors within the cell. Caffeine is one such compound that has been utilized in this regard, as it acts to inhibit phosphorylation of MPF to pre-MPF by Wee1/Myt1. Supplementation of oocyte culture media with caffeine has indeed been shown to maintain levels of MPF post-ovulation and to subsequently decrease levels of parthenogenesis and fragmentation (Kikuchi et al. 2002). Additionally, recent studies have established that the addition of caffeine or MG132 (a proteasome inhibitor) to oocyte culture can increase fertilization rates by ICSI and decrease fragmentation in embryos (Ono et al. 2011). Further modes of action for caffeine in relation to post-ovulatory ageing include a delay in the deterioration of Ca^{2+} release mechanisms, although the underlying pharmacology is not yet clear (Zhang et al. 2011).

While live offspring have been obtained using oocytes that were exposed to caffeine treatment (Ono et al. 2011), previously published research has implicated caffeine as an inhibitor of DNA repair mechanisms (Selby & Sancar 1990). This impairment of DNA repair has been demonstrated to directly affect oocytes, as caffeine-treated golden hamster eggs lost their capacity to repair aberrations in both chromatids and chromosomes following fertilization by spermatozoa possessing DNA damage (Genesca et al. 1992). This clearly creates concern as to the safety of utilizing caffeine as an agent for preventing post-ovulatory oocyte ageing in vitro, as un-repaired DNA damage within the embryo may result in abnormalities/birth defects in offspring.

In a similar fashion to caffeine, MG132 has also been demonstrated to maintain high MPF levels in oocytes in vitro, by preventing degradation of cyclin B. Oocytes treated with MG132 have been shown to produce embryos with improved developmental potential; however, the capacity to produce live offspring is reduced (Yu et al. 2005), potentially as a result of inhibition of necessary protein degradation (Gao et al. 2005). Again,
MG132 is clearly not a suitable compound for utilization in an ART setting.

In addition to the aforementioned impacts of caffeine or MG132 on cultured oocytes, recent research has suggested that not all facets of post-ovulatory ageing can be controlled by manipulation of cell cycle factors. It has been demonstrated that caffeine-supplemented oocytes do not experience any delay in the onset of age-associated apoptosis, nor any relief from accumulation of oxidative stress (Lord et al. 2013). If accumulation of oxidative stress is indeed an upstream event that is intricately linked with age-associated declines in critical cell cycle factors, then relief from oxidative stress by way of antioxidant supplementation should maintain MPF/MAPK levels in vitro.

Antioxidants

If oxidative stress acts as a ‘trigger’ for ageing in post-ovulatory oocytes, then it would be expected that antioxidant treatment would effectively delay this process, either via supplementation of oocyte culture medium to delay ageing in vitro or by oral administration to prevent ageing of oocytes in vivo.

In vitro studies have indeed demonstrated that antioxidant supplementation can attenuate the process of post-ovulatory oocyte ageing; however, this phenomenon is reliant on the type of antioxidant utilized. Early research by Tarin et al. (1998) demonstrated that L-ascorbic acid (vitamin C) and 6-methoxy-2,5,7,8-tetramethylchloromane-2-carboxylic acid (trollox/vitamin E) could not significantly improve fertilization rate nor prevent fragmentation associated with post-ovulatory ageing, while β-mercaptoethanol and L-cysteine in fact decreased the likelihood of aged oocytes reaching the blastocyst stage following fertilization. However, this research also established that the addition of the reducing agent diithiothreitol (DTT) to oocyte culture medium resulted in increased fertilization and blastocyst formation rates, potentially by protecting free thiol groups within these ‘ageing’ oocytes from becoming oxidized. Despite the reported benefits associated with DTT, the application of this method for delaying post-ovulatory ageing in a clinical setting is limited as DTT has been associated with DNA damage within cells (Held et al. 1996, Oikawa et al. 2002).

Treatment of oocytes with the nitric oxide (NO•) donor S-nitroso-N-acetylpenicillamine has also been shown to attenuate certain signs associated with post-ovulatory ageing such as cortical granule exocytosis, zona hardening and poor embryo quality (Goud et al. 2008b). NO• has been identified in multiple studies as a free radical crucial to oocyte and embryo function (Gouge et al. 1998, Kuo et al. 2000). NO• does exhibit antioxidant characteristics – specifically a capacity to scavenge O2•− (Robak & Grygleweski 1993); however, the exact mechanism by which NO• elicits its age-attenuating effects remains elusive.

More recently, a study conducted by our laboratory (Lord et al. 2013) has identified that the potent antioxidant melatonin provides protection from post-ovulatory ageing when used to supplement in vitro culture medium. Oocytes treated with melatonin experienced a delayed onset of apoptosis, decreased levels of fragmentation, an increased optimal window for fertilization and improved embryo quality when compared with control aged oocyte counterparts. These effects are presumably a direct result of relief from oxidative stress, as melatonin-treated aged oocytes exhibited significantly reduced levels of ROS (Lord et al. 2013). Further to this, previous studies have highlighted the ability of melatonin to reverse the deleterious effects of H2O2 treatment on MI oocytes (Tamura et al. 2008) and prevent hypochlorous acid-induced abnormalities in chromosomes and microtubules (Banerjee et al. 2012). Importantly, melatonin has a lack of demonstrable toxicity (Jahnke et al. 1999), making it a primary candidate for utilization in an assisted reproduction setting.

Although the ability of antioxidants to influence post-ovulatory ageing is well characterized in vitro, the capacity for these compounds to delay in vivo ageing is poorly defined. Oral administration of antioxidants such as co-enzyme Q10, melatonin and N-acetyl-L-cysteine has been linked with improved oocyte quality, as well as increased fertilization rates and litter sizes in clinical and laboratory studies (Tamura et al. 2008, Burstein et al. 2009, Liu et al. 2012); however, these reports focus primarily on the relationship between antioxidants and ovarian ageing. To the authors’ knowledge, no in vivo studies have been conducted to establish the effects of orally administered antioxidants on post-ovulatory ageing specifically.

Although the beneficial effects of antioxidants on post-ovulatory oocyte ageing are dependent on the antioxidant utilized, these compounds may be at the forefront in terms of delaying in vitro ageing prior to IVF or ICSI, particularly if oxidative stress does act as the ‘trigger’ for downstream pathological changes. The variation in therapeutic potential between antioxidants trialled to date may be attributed to the different reactive species scavenged by each compound, their stability in in vitro culture media, their capacity to infiltrate multiple organelles within the cell and also their potency at concentrations that are considered safe for clinical use. Following the success of antioxidant supplementation in vitro, it would be beneficial to establish the capability of these compounds to delay the onset of senescence in MI oocytes in vivo.

Concluding remarks

Ageing of post-ovulatory oocytes clearly has a major impact on fertilization potential, the quality of resulting
embryos, the likelihood of carrying a pregnancy to term and the health and well-being of offspring. Although factors such as MPF/MAPK decline and mitochondrial dysfunction are known to be involved in orchestrating post-ovulatory ageing, the exact molecular mechanisms that control these processes remain elusive. This review has proposed that oxidative stress may act as the ‘initiator’ of a chain of events that create the aged oocyte phenotype. Oxidative stress can be directly linked not only with MPF decline and mitochondrial dysfunction but also with the acquisition of many abnormalities in oocyte biology such as aberrant Ca\(^{2+}\) homeostasis and damage to proteins, lipids and DNA. We have proposed that relieving oxidative stress using antioxidant supplementation may provide a safe and effective avenue for delaying post-ovulatory ageing, specifically in an assisted reproduction setting where extended periods of time between ovulation and fertilization may be unavoidable.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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References

Dodson MG, Minhas BS, Curtis SK, Palmer TV & Robertson JL 1989 Spontaneous zona reaction in the mouse as a limiting factor for the time in which an oocyte may be fertilized. Journal of In Vitro Fertilization and Embryo Transfer 6 101–106. (doi:10.1007/BF01130735)


Ono T, Mizutani E, Li C, Yamagata K & Wakahaya T 2011 Offspring from intracytoplasmic sperm injection of aged mouse oocytes treated with caffeine or MgCl2. Genesis 49 460–471. (doi:10.1002/dvg.20756)


Oxidative stress and the ageing oocyte


Yoon SY, Jellerette T, Salicioni AM, Lee HC, Yoo MS, Coward K, Patterson J, Grow D, Cibelli JB, Visconti PE et al. 2008 Human sperm iodovod of PLC, zeta 1 fails to induce Ca2+ release and are unable to initiate the first step of embryo development. Journal of Clinical Investigation 118 3671–3681. (doi:10.1172/JCI36942)


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CHAPTER 2

*Melatonin prevents postovulatory oocyte ageing in the mouse and extends the window for optimal fertilization in vitro*¹

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Chapter 2: Overview

The aim of the following manuscript was to establish a direct link between the accumulation of reactive oxygen species that occurs in the post-ovulatory oocyte and the degeneration and eventual apoptosis of this cell. For this study we utilised a mouse model and focused specifically on post-ovulatory ageing of the oocyte in vitro.

In addition to establishing a timeline for the elevation in ROS levels in mouse oocytes and the appearance of apoptotic markers in relation to hCG injection and in vitro culture time, this study demonstrated a direct association between the spike in intracellular ROS that occurs post-ovulation and the onset of apoptosis, as well as a decline in oocyte functionality. Oocytes treated with the antioxidant melatonin maintained low levels of ROS throughout extended periods of in vitro culture and associatively exhibited a delayed onset of apoptosis, saw no decline in fertilization rate after up to 16 h in vitro culture time, and produced embryos which exhibited no detriment in quality when compared to embryos derived from freshly retrieved oocytes.

The data provided within this manuscript strongly suggests that the accumulation of ROS that occurs within hours of ovulation of the mammalian oocyte may indeed be the trigger for the deterioration in quality referred to as post-ovulatory ageing. Additionally, these findings provide a basis for the development of in vitro culture mediums containing antioxidant agents to preserve oocyte quality prior to assisted reproduction procedures.
Melatonin Prevents Postovulatory Oocyte Aging in the Mouse and Extends the Window for Optimal Fertilization In Vitro

Tessa Lord, Brett Nixon, Keith T. Jones, and R. John Aitken

INTRODUCTION

The health and integrity of the oocyte can greatly influence the success of fertilization as well as the developmental competence of the embryo [1]. This dependence on oocyte quality is of little surprise considering that this cell contains factors responsible not only for remodeling the maternal and paternal genomes [2, 3] but also for orchestrating the early stages of embryogenesis [4, 5]. Furthermore, the oocyte is the sole source of mitochondria in the developing embryo, with these organelles not only being responsible for ATP production but also conducting key cellular processes such as apoptosis in response to appropriate developmental cues [6].

Following ovulation, the prophase I oocyte resumes meiosis and undergoes a maturational process involving germinal vesicle breakdown, migration of the metaphase spindle, and extrusion of the first polar body. Following these events, meiosis is once again arrested—now at metaphase II (MII)—and remains in this state until fertilization occurs [7, 8]. Unfortunately, with increasing time following ovulation, the MII oocyte undergoes a process of deterioration in vivo and in vitro, referred to as oocyte aging. Oocyte aging is associated with many deleterious effects (for review, see [9, 10]); the aging oocyte experiences partial cortical granule exocytosis [11–14] and zona hardening [12, 14, 15], making it less receptive to fertilization [16–18]. A decrease in critical cyclin-dependent kinase factors, particularly maturation-promoting factor (MPF) and mitogen-activating protein kinase, also becomes evident [19]. Additionally, these oocytes commonly exhibit spindle abnormalities and losses of chromosomal integrity [20] and are increasingly susceptible to polyspermy [21, 22]. As a result, fertilization of aged oocytes is associated with poor developmental potential of embryos [22], elevated risk for early pregnancy loss [23], and abnormal/retarded development in offspring [24, 25]. The “end point” of this oocyte aging process is cell death via an apoptotic pathway characterized by phosphatidylserine externalization [26], caspase activation [27], accumulation of the apoptotic signaling protein Bax (Bcl-2-associated X) and suppression of Bcl-xL (B-cell lymphoma-extra large) [28, 29], and DNA fragmentation [30].

The intracellular signals controlling postovulatory oocyte aging have not been well defined; however, because oxidative stress is purportedly a prominent mediator of aging and disease in many cell and tissue types [31], reactive oxygen species (ROS) are certainly potential orchestrators of this process. Levels of ROS (particularly hydrogen peroxide) and lipid peroxidation have been shown to significantly increase with oocyte age [32], whilst oocytes under oxidative stress exhibit a decreased fertilization rate [32] and are more likely to enter into apoptosis [33]. Despite these data suggesting involvement of oxidative stress in the postovulatory oocyte aging process, little success has been achieved in delaying aging with antioxidant supplementation [34]. Although some nonantioxidant compounds such as caffeine have been shown to extend the window for fertilization using intracytoplasmic sperm injection (ICSI) [35] by preventing inactivation of MPF within the cell [19] and alleviating the deterioration of calcium release mechanisms [36], the clinical significance of such observations is questionable. For example, caffeine supplementation of in vitro culture medium has been shown to suppress DNA repair mechanisms in somatic cells [37] and has been demonstrated to reduce the ability of hamster oocytes to repair extensive DNA damage in human spermatozoa [38]. This is clearly a
worrisome factor when considering the assisted reproductive technology (ART) patient population, because many male patients exhibit significantly elevated levels of DNA damage in their spermatozoa compared to donor populations [39].

In the present study, we demonstrate that oxidative stress does indeed play an integral role in curtailing the structural and functional integrity of the postovulatory oocyte. We also show that the potent antioxidant melatonin can effectively relieve aging mouse oocytes of oxidative stress in vitro, delaying the onset of apoptosis and preventing fragmentation. Importantly, we also demonstrate that melatonin-supplemented oocytes experience an extended window for optimal fertilization and produce embryos of improved quality compared with their control aged oocyte counterparts. Finally, we showed that compounds targeted at maintenance of MPF levels (e.g., caffeine) are inadequate to delay all facets of oocyte aging; specifically, they do not prevent accumulation of oxidative stress in these cells. We propose that melatonin may be a compound that can be safely utilized to prevent oocyte aging in a clinical setting (e.g., by reducing the undesirable consequences associated with next-day rescue ISCI).

MATERIALS AND METHODS

Oocyte Collection

Three- to five-week-old C57BL6/CBA F1 female mice were administered intraperitoneal injections of equine chorionic gonadotropin (Intervet) followed 48 h later by human chorionic gonadotropin (hCG; Intervet) to induce superovulation. Mice were culled 14–15 h after hCG injection using CO2 asphyxiation. Ovaries were removed immediately and placed in PBS at 37°C before cumulus mass retrieval from the ampullae. Oocytes were denuded from cumulus cells using a 5-min incubation in hyaluronidase (300 μg/ml; Sigma-Aldrich) at 37°C. Oocytes were then washed from three to five times in M2 medium (Sigma-Aldrich) to completely remove the cumulus cells. The use of animals in this project was approved by the University of Newcastle Animal Care and Ethics Committee, and all animals were obtained from breeding programs run in the University of Newcastle Central Animal House.

Aging of Oocytes In Vitro

To establish the effects of melatonin supplementation on oocyte aging, collected oocytes were immediately placed in a 20-μl droplet of either M2 medium, M2 medium containing 1 mM melatonin (Sigma-Aldrich), or M2 medium plus ethanol (vehicle control) under mineral oil (Sigma-Aldrich) at 37°C. Oocytes were aged in these droplets in groups of 8–10 at 37°C before cumulus mass retrieval from the ampullae. Oocytes were denuded from cumulus cells using a 5-min incubation in hyaluronidase (300 μg/ml; Sigma-Aldrich) at 37°C. Oocytes were washed three to five times in M2 medium (Sigma-Aldrich) to completely remove the cumulus cells. The use of animals in this project was approved by the University of Newcastle Animal Care and Ethics Committee, and all animals were obtained from breeding programs run in the University of Newcastle Central Animal House.

To establish the effects of melatonin supplementation on oocyte aging, collected oocytes were immediately placed in a 20-μl droplet of either M2 medium, M2 medium containing 1 mM melatonin (Sigma-Aldrich), or M2 medium plus ethanol (vehicle control) under mineral oil (Sigma-Aldrich). Oocytes were “aged” in these droplets in groups of 8–10 at 37°C under gas (5% O2, 6% CO2 in N2) for 1, 8, 24, or 48 h from the time of oocyte retrieval. In experiments that compared the ability of melatonin and caffeine to delay oocyte aging, the above-described protocol was followed; however, a portion of the oocytes was also aged in M2 medium containing 5 mM caffeine (Sigma-Aldrich) as well as in M2 medium containing a combination of both 1 mM melatonin and 5 mM caffeine. The optimal concentration of 1 mM melatonin was predetermined via a dose-response study (Supplemental Fig. S1; available online at www.biolreprod.org), whereas the optimal concentration of 5 mM caffeine was obtained from previously published studies on postovulatory oocyte aging [19, 35, 36, 40].

Carboxy-DFFDA

To identify oxidative stress/ROS levels in aged oocytes, 5′-carboxy-2′,7′-dihydrodihydrofluorescein diacetate (carboxy-DFFDA; Molecular Probes), a fluorescent probe capable of detecting powerful oxidants such as H2O2 and peroxynitrite, was utilized. Oocytes were incubated in a 10 μM solution of carboxy-DFFDA in M2 medium for 15 min at 37°C under gas. Oocytes were then washed three times in M2 medium before mounting on a glass slide for microscopy. To compare ROS levels between different treatment groups, images were generated using an Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging GmbH), and a pixel intensity value was calculated for each oocyte using ImageJ software (National Institutes of Health).

Caspase Assay

A FAM-FLICA Poly Caspase Assay Kit (ImmunoChemistry Technologies) was used to establish levels of caspase activation in aged oocytes. Oocytes were incubated in working solution (created as per the manufacturer’s instructions) for 30 min at 37°C under gas. Following incubation, oocytes were washed three times in M2 medium before mounting for fluorescence microscopy. Pixel intensity values were again used to compare activated caspase levels between treatment groups.

Annexin-V Assay

An Annexin-V conjugate (Molecular Probes) was used to identify phosphatidylserine exteriorization in apoptotic cells. Oocytes were incubated in Annexin-V for 15 min before being transferred to a 0.25 mg/ml solution of propidium iodide (Sigma-Aldrich) to allow recognition of necrotic cells. Oocytes were washed three times in M2 medium before mounting and analysis via fluorescence microscopy.

In Vitro Fertilization

Fertilization studies were conducted on oocytes aged for 8, 16, and 24 h in vitro with or without 1 mM melatonin supplemented into the culture medium. The in vitro fertilization (IVF) procedure followed in the present study was modified from that used by the MRC Harwell (Oxfordshire, U.K.), which is based on a series of publications by Takeo and colleagues [41–43]. Spermatozoa were retrieved from the cauda epididymides from adult Swiss mice and capacitated in Biggers, Whitten, and Whittingham medium [44] supplemented with 1 mg/ml of polyvinyl alcohol and 1 mg/ml of methyl-beta-cyclodextrin for 1 h at 37°C and 6% CO2 under mineral oil. Immediately before completion of capacitation, aged oocytes were washed four times in human tubal fluid (HTF) [41–43] and then placed in fertilization medium (HTF containing 1 mM reduced glutathione [GSH]). Denuded, freshly retrieved oocytes were also fertilized and used as a control. Aliquots (3–5 μl) of capacitated sperm suspension were added to the fertilization dishes before a 4-h period of incubation at 37°C under gas. Following fertilization, oocytes were washed four times in HTF and incubated overnight to the 2-cell stage. The 2-cell embryos were transferred to G-1 PLUS medium (Vitrolife) on the morning of Day 2, then transferred to G-2 PLUS medium (Vitrolife) on the morning of Day 4. The percentage of oocytes that fertilized and reached the blastocyst stage was calculated on the morning of Day 5.

TUNEL Assay

A TUNEL assay (Roche Diagnostics) was conducted on Day 5 blastocysts to identify DNA fragmentation related to apoptosis in nuclei. The TUNEL assay was conducted as described previously [45], and blastocysts were analyzed using confocal microscopy (Zeiss LSM 510; Carl Zeiss MicroImaging GmbH).

Statistical Analyses

All experiments were conducted at least three times on independent samples, and results were analyzed by ANOVA using JMP version 9.0.0 (SAS Institute Inc.). A post hoc comparison of group means was conducted using a Fisher protected least significant difference test. Analysis of paired samples was conducted using a paired Student t-test. A value of P < 0.05 was considered to be statistically significant.

RESULTS

Oxidative Stress Precedes the Appearance of Markers of Aging and Apoptosis in Oocytes In Vitro

To establish a relationship between the onset of oxidative stress and the onset of apoptosis in C57BL6/CBA F1 aging mouse oocytes, levels of ROS and of apoptotic markers were assessed at a series of time points over the course of 48 h in vitro. MII oocytes showed a significant time-dependent increase in ROS levels (P < 0.001) as detected by the carboxy-DFFDA fluorescent probe (Fig. 1A). ROS levels were significantly elevated after only 8 h in culture and continued to rise exponentially up to 48 h. Oocytes therefore appear to experience oxidative stress from as early as 8 h following...
FIG. 1. Changes associated with postovulatory oocyte aging. A) From 8 h of in vitro culture onward, oocytes experienced a significant increase in ROS levels as detected using the carboxy-DFFDA fluorescent probe ($P < 0.001$); ordinate axis represents percentage of the control value at 1 h. By 48 h of culture, levels of oxidative stress were increased by more than 200% above the 1-h fresh oocyte control ($n = 18$). Images below the histogram depict a carboxy-DFFDA negative oocyte (1 h of culture) and an oxidatively stressed oocyte showing high levels of carboxy-DFFDA fluorescence at 48 h of culture. B) The percentage of Annexin-V-positive, propidium iodide-negative oocytes increased in a time-dependent manner from oocyte retrieval. Less than 5% of oocytes were Annexin-V positive at 1 h after retrieval; however, this was significantly increased to 19.5% by 8 h and to more than 50% by 48 h ($P < 0.001$) ($n = 5$). Images below the histogram depict staining patterns seen in Annexin-V-negative (1 h of culture) and -positive oocytes (48 h of aging). C) A significant increase in levels of caspase activation was detected in aging oocytes from 24 h ($P < 0.05$) and 48 h ($P < 0.001$) ex vivo; ordinate axis represents percentage of the control value at 1 h ($n = 13$). Images below the histogram demonstrate low levels of caspase activity at 1 h of culture and a 48-h aged oocyte showing high levels of fluorescence related to caspase activation. D) The percentage of oocytes that exhibited abnormal morphology (examples demonstrated in accompanying images) related to aging was elevated significantly from 1 h to 24 and 48 h of culture ($P < 0.001$) ($n = 12$). Values are plotted as the mean ± SEM. Independent replicates were conducted with a minimum of 40 oocytes/replicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Bar = 50 μm.
retrieval. Our results further suggested that oxidative stress is a factor associated with the onset of apoptosis in oocytes as they age in vitro. Thus, a significant increase in the percentage of Annexin-V-positive cells was observed simultaneously with the aforementioned early increase in ROS levels, with the Annexin-V conjugate identifying a significant increase in the exteriorization of phosphatidylserine after 8 h ($P < 0.05$), 24 h ($P < 0.05$), and 48 h ($P < 0.001$) in culture (Fig. 1B). Furthermore, a significant rise in levels of caspase activation (a late apoptotic marker) was observed; this rise achieved statistical significance after 24 h ($P < 0.05$) and 48 h ($P < 0.001$) in culture (Fig. 1C). The onset of oxidative stress in these aging oocytes was also demonstrated to precede morphological features of cellular stress (fragmentation related to apoptosis or spontaneous activation), which were significantly elevated after 24 h ($P < 0.001$) and 48 h ($P < 0.001$) of culture, respectively (Fig. 1D).

**Melatonin Relieves Oxidative Stress in Aging Oocytes and Delays the Onset of Apoptosis**

If oxidative stress is indeed a prominent mediator of oocyte aging, then supplementation with a potent antioxidant compound that would be stable under the culture conditions employed in the present study should stop or delay the aging process. By supplementing the culture medium with 1 mM melatonin, we did indeed detect a 41% decrease ($P < 0.05$) in ROS levels following 48 h of in vitro culture (Fig. 2A). This prominent reduction in ROS levels was accompanied by a delay in the onset of apoptosis, with a 52% decline in levels of caspase activation ($P < 0.05$) (Fig. 2B) as well as a 32% decrease in morphological abnormalities ($P < 0.001$) (Fig. 2, C–E). It was concluded from these results that oxidative stress certainly influences at least some facets of oocyte aging and that a degree of attenuation of this process could be enforced using the antioxidant melatonin.

**Melatonin Extends the Optimal Window for Fertilization and Improves Embryo Quality**

In preventing the accumulation of ROS during the aging process, melatonin effectively extended the optimal window for fertilization of the oocyte. Specifically, 72% ± 8.4% (values are ± SEM throughout) of oocytes aged for 8 h in the presence of melatonin reached the 2-cell stage following insemination, compared to only 49% ± 10.8% of oocytes aged in control media ($P < 0.05$) (Fig. 3A). Similarly, oocytes inseminated after 16 h of in vitro culture with melatonin had a rate of 2-cell embryo formation 17% greater that of the control ($P < 0.05$) (Fig. 3B), whereas after 24 h of culture, the rate of 2-cell embryo formation was increased by 26% in the presence of melatonin ($P < 0.05$) (Fig. 3C). The developmental progression of embryos generated from oocytes aged in the presence of melatonin was also superior to the progression of those established from control oocytes. Thus, 54% ± 10.0% of oocytes aged for 8 h in the presence of melatonin reached the blastocyst stage, whereas fertilization of control aged oocytes resulted in a 29% ± 13.8% blastocyst formation rate ($P < 0.01$) (Fig. 3A). Melatonin also increased the rate of blastocyst formation in oocytes inseminated following 16 h of aging in vitro by 16.8% ($P < 0.01$) (Fig. 3B). Despite an increased 2-cell embryo formation rate in oocytes exposed to melatonin for 24 h relative to aged controls, very few of these embryos progressed to produce viable blastocysts, indicating a dramatic loss of developmental potential when oocytes are aged for such prolonged periods of time (Fig. 3C).

Blastocysts formed from control oocytes aged for 8 and 16 h before insemination were of poor quality, displaying elevated levels of apoptosis ($P < 0.05$), with 18% ± 4.2% and 16% ± 4.6% of blastomeres, respectively, being TUNEL positive (Fig. 3, D–H) compared to under 7% of blastomeres in embryos formed from fresh oocytes (Fig. 3D). Significantly, embryos originating from oocytes aged for the same periods of time in the presence of melatonin did not experience elevated levels of apoptosis; the percentage of TUNEL-positive blastomeres was not significantly different in these melatonin-supplemented cultures compared with the fresh oocyte controls (Fig. 3, D and E).

**Comparison Between the Effects of Melatonin and Caffeine on Oocyte Aging**

In the interest of comparing mechanisms by which aging is delayed in oocytes supplemented with melatonin versus oocytes supplemented with caffeine, a comparison of the oxidative and apoptotic status of oocytes exposed to these reagents was conducted. Interestingly, despite its ability to extend the window in which murine oocytes can be fertilized [35], caffeine supplementation provided no relief from oxidative stress following 48 h of in vitro culture; with ROS levels decreasing below that of control aged oocytes only in the presence of melatonin ($P < 0.05$) (Fig. 4A). Additionally, caffeine-treated aged oocytes did not experience any relief from the onset of apoptosis. Again, levels of caspase activation were only significantly reduced with melatonin supplementation or when caffeine and melatonin were combined in culture medium ($P < 0.05$) (Fig. 4B).

**DISCUSSION**

With the demand for ART increasing exponentially in recent years, the drive to refine these technologies has become paramount, particularly considering that less than 20% of initiated IVF cycles result in the production of live offspring [46] and that these offspring have a significantly increased risk of possessing birth defects [47]. The present study has focused specifically on the contribution of postovulatory oocyte aging in vitro to fertilization efficiency and embryonic developmental potential. We have identified oxidative stress as a major contributor to the postovulatory oocyte aging process and have found that supplementation of culture medium with the potent antioxidant melatonin can delay multiple facets of oocyte aging and apoptosis in vitro, thereby extending the optimal window for fertilization and preventing an age-associated decline in embryo quality.

This research provides confirmation that oxidative stress is indeed a key mediator of oocyte aging in vitro and appears to act as a trigger for induction of the intrinsic apoptotic pathway, just as has been observed for spermatozoa [48]. Our results demonstrate that the onset of oxidative stress may be a relatively early event associated with in vitro culture, because a significant elevation in ROS levels is evident from the 8-h time point. The onset of oxidative stress early in the oocyte aging process is not particularly surprising when taking into consideration the reported decline in GSH levels within the oocyte with time postovulation as well as the absence of antioxidant-rich follicular fluid to provide protection [49]. Additionally, the in vitro environment itself is a potential promoter of oxidative stress in oocytes due to increases in oxygen tension [50, 51] and exposure to light [52]. The free-radical theory of aging proposes that ROS produced by the mitochondria as by-products of oxidative phosphorylation may
result in the progressive accumulation of toxic oxidative metabolites in the cell with age, inflicting damage on these organelles and initiating further ROS production in a damaging redox cycle [53]. Such a chain of cause-and-effect has recently been demonstrated in human spermatozoa, with mitochondrial ROS generation triggering an increase in cellular 4-hydroxy-ynonenal and the latter then forming adducts with enzymes in the mitochondrial electron-transport chain, stimulating yet more ROS generation in a self-perpetuating manner [54]. As a further consequence of this process, the intrinsic apoptotic cascade ensues, characterized by mitochondrial pore formation, cytochrome c release, and subsequent cellular degradation by

FIG. 2. A reduction in oocyte aging is achieved with supplementation of 1 mM melatonin to culture medium. A) Melatonin-supplemented oocytes that had been aged for 48 h in vitro showed a 40% reduction in ROS levels compared to control aged oocytes (P < 0.05); ordinate axis represents percentage of the control value at 1 h (n = 8). B) Melatonin significantly decreased levels of caspase activation in oocytes aged for 48 h, suggesting that the onset of apoptosis had been delayed (P < 0.05) (n = 10). C) The percentage of oocytes that acquired abnormal morphology over time was significantly reduced below the control in the presence of melatonin after both 24 and 48 h of culture (P < 0.001) (n = 15). D) and E) Heavy cytoplasmic fragmentation could be visualized in control oocytes following extended periods of aging (>48 h); D), whereas oocytes supplemented with melatonin during this time retained relatively normal morphology (E). Values are plotted as the mean ± SEM. Independent replicates were conducted with a minimum of 50 oocytes/replicate. *P < 0.05, **P < 0.001. Bar = 50 μm.
caspases and endonucleases [55]. The onset of oxidative stress observed following the culture of oocytes for 8 h in vitro is consistent with the notion that ROS represent the instigator of apoptosis in these cells, with early markers for apoptosis (phosphatidylserine externalization) appearing simultaneously with oxidative stress whereas caspases, involved in the subsequent processing of cellular proteins [55], materialize later.

In alignment with our research, previous studies have also identified oxidative stress as a factor that likely is involved in the aging of postovulatory oocytes [32, 56]. However, until now, little success had been achieved in delaying the onset of this phenomenon using antioxidant supplementation. Tarin et al. [34] showed that supplementation of oocyte culture medium with l-ascorbic acid and vitamin E could not improve fertilization rates or embryo development in aged oocytes in vitro, whereas treatment with l-cysteine caused a decreased developmental rate to the blastocyst stage [34]. In contrast to previous research, we found in the present study that the antioxidant melatonin was able to successfully delay aging and apoptosis in oocytes retrieved from the hybrid mouse strain C57BL6/CBA F1, thereby increasing both embryo formation rate and embryo quality after in vitro incubation periods of up to 16 h (the comparable effect of melatonin on oocytes retrieved from other mouse strains has not yet been assessed).

The superior ability of melatonin to influence oocyte quality in the present study may relate to several key features of this compound’s chemistry. First, melatonin is extremely stable in aqueous solution [57], meaning that no rapid inactivation of its ROS scavenging capabilities occurs in oocyte culture medium. Importantly, unlike many other antioxidant agents, melatonin does not produce pro-oxidant by-products from its interaction with ROS. In fact, all known intermediates generated through the reaction between melatonin and ROS are free-radical scavengers themselves, meaning that even at low concentrations, melatonin induces a powerful “free radical scavenging
appears to be directly linked with oxidative stress levels in the blastomeres produced from aged oocytes in the present study of poor-quality embryos. A high degree of apoptotic nuclei in oxidative stress in aging oocytes is linked with the production of developmental potential, we have found that the onset of reach the S phase [63]).

will not pass through the G 1 checkpoint of the cell cycle to pathway than to continue with embryonic development (i.e., clearly are more likely to default to the intrinsic apoptotic and mitochondrial DNA. Oocytes with excessive DNA damage also act as a protector from oxidative attack for many other intracellular components such as proteins as well as nuclear and mitochondrial DNA. Oocytes with excessive DNA damage clearly are more likely to default to the intrinsic apoptotic pathway than to continue with embryonic development (i.e., will not pass through the G 1 checkpoint of the cell cycle to reach the S phase [63]).

Further to its effect on embryo formation rate and developmental potential, we have found that the onset of oxidative stress in aging oocytes is linked with the production of poor-quality embryos. A high degree of apoptotic nuclei in blastomeres produced from aged oocytes in the present study appears to be directly linked with oxidative stress levels in the oocyte before insemination, because relieving this stress using melatonin decreased the incidence of TUNEL-positive blastomeres. Not surprisingly, high percentages of apoptotic blastomeres within the embryo have been associated with poor embryo quality [64] and with a tendency for early embryos to undergo resorption [65, 66]. Therefore, oxidative stress in the oocyte before insemination may affect not only the preimplantation embryo but also postimplantation events and, hence, the likelihood of producing healthy, live offspring.

The final component of the present study highlighted potential downsfalls associated with utilizing compounds such as caffeine, which influence MPF levels within the oocyte, to inhibit aging. Although the temporal window for fertilization is purportedly extended with the prevention of MPF phosphorylation [35], the present study has demonstrated that other facets of oocyte aging such as the accumulation of oxidative stress and the onset of apoptosis are not controlled by this reagent. As previously discussed, the consequences of fertilizing an oxidatively stressed oocyte may include a higher rate of apoptotic nuclei in blastocysts and a subsequent elevated risk for embryo resorption. We propose that melatonin is likely to be a more effective and safer compound for potential utilization in ART, as, not only can this antioxidant positively affect embryo formation rate and blastocyst quality, but is also reported to have a lack of demonstrable toxicity [67].

It is worth noting that although compounds such as melatonin and caffeine are capable of delaying several aspects of postovulatory oocyte aging, a threshold window for development still exists beyond which a successful outcome is unlikely. This was demonstrated by our fertilization studies, which showed that although 24-h aged oocytes experienced increased levels of 2-cell embryo formation following melatonin supplementation, these embryos had mostly lost the capability to reach the 4-cell and blastocyst stages of development. The transient capacity for maintenance of high-quality oocytes with media supplementation is not surprising, particularly because any single compound is unlikely to hold the potential for prevention of all consequences associated with oocyte aging. For instance, although the relief from oxidative stress provided by melatonin delays the acquisition of morphological abnormalities and apoptosis, it may not exert influence on other facets of aging such as loss of cytoskeletal integrity and spindle organization. With future gains in understanding of the complex mechanisms controlling oocyte aging, it may be possible to create oocyte culture media containing a combination of active compounds, including an antioxidant agent such as melatonin, to target an extensive array of age-related changes and allow prolonged incubation periods in vitro before IVF/ICSI.

In conclusion, our research has demonstrated, to our knowledge for the first time, that by preventing oxidative stress using melatonin supplementation, aspects of the aging process that mouse oocytes undergo postovulation in vitro can be delayed. Oocytes aged in the presence of melatonin experienced a delay in the onset of apoptosis, an increased optimal window for fertilization, and improved embryo quality compared to their untreated aged counterparts. The present study also demonstrated not only that oxidative stress is a prominent mediator of the oocyte aging process but also that oxidative stress in aged oocytes is directly responsible for a decline in embryo formation rate and embryo quality. We have also provided evidence to suggest that melatonin may be more effective and safer than caffeine for inhibiting the oocyte aging process in a clinical setting.
REFERENCES

20. Takeo T, Nakagata N. Combination medium of cryoprotective agents containing l-glutamine and methyl-beta-cyclodextrin in a preincubation medium yields a high fertilization rate for cryopreserved C57BL/6 mouse sperm. Lab Anim 2010; 44:132–137.
Chapter 2: Supplementary Figures

Supplementary Fig. 1: A dose response study was conducted to establish the most effective concentration (1 mM) of melatonin for delaying post-ovulatory oocyte aging. (A) Following 48 h culture time in vitro, oocytes treated with 0 - 0.1 mM melatonin displayed no differences in the acquisition of morphological abnormalities. Contrastingly, populations of oocytes treated with 1 mM melatonin showed significantly decreased percentages of morphological abnormalities when compared to that of the control ($P<0.001$) (n=3). (B) Concentrations of 0.01, 0.05 and 0.1 mM melatonin had no significant effect on levels of caspase activation in 48 h aged oocytes, however oocytes treated with 1 mM melatonin displayed decreased levels of caspase activation when compared to the untreated control ($P<0.01$) (n=3). Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 50 oocytes per replicate.
CHAPTER 3

Accumulation of electrophilic aldehydes during post-ovulatory ageing of mouse oocytes causes reduced fertility, oxidative stress and apoptosis


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Chapter 3: Overview

Following the determination that reactive oxygen species play an integral role in the initiation of post-ovulatory ageing of the mammalian oocyte (Chapter 2), the following manuscript aimed to further elucidate the molecular events that culminate from this early onslaught of oxidative stress, and thence orchestrate the degeneration of the female germ cell. Again, experiments were conducted using mouse oocytes in an in vitro setting, using equivalent in vitro ‘ageing’ periods to those established in Chapter 2.

Data within this chapter has demonstrated that an increase in lipid peroxidation coincides with elevated ROS levels in the ageing oocyte. As a consequence, the oocyte experiences an accumulation of cytotoxic electrophilic aldehydes such as 4-hydroxynonenal, and acrolein. These cytotoxic aldehydes were found to covalently modify an array of proteins within the ageing oocyte, including succinate dehydrogenase – an integral component of complex II of the electron transport chain (ETC). Evidence within this manuscript strongly suggests that aldehyde adduction of succinate dehydrogenase impairs ETC function, resulting in further mitochondrial ROS production, mitochondrial membrane collapse, and the initiation of an intrinsic apoptotic cascade.

In further characterising the biochemical events that ensue during post-ovulatory oocyte ageing it was possible to identify additional compounds that are likely to preserve oocyte integrity if included in culture medium prior to assisted reproduction procedures. In this circumstance, the thiol-reactive compound penicillamine was found to reverse/prevent the decline in fertilization rate associated with elevated levels of electrophilic aldehydes in the oocyte, and allowed for a significantly improved embryo formation rate.
Accumulation of Electrophilic Aldehydes During Postovulatory Aging of Mouse Oocytes Causes Reduced Fertility, Oxidative Stress, and Apoptosis

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ABSTRACT

With increasing periods of time following ovulation, the metaphase II (MII)-stage oocyte experiences overproduction of reactive oxygen species and elevated levels of lipid peroxidation that are implicitly linked with functional deficiencies acquired during postovulatory oocyte aging. We have demonstrated that the electrophilic aldehydes 4-hydroxynonenal (4HNE), malondialdehyde, and acrolein are by-products of nonenzymatic lipid peroxidation in the murine MII-stage oocyte, adding to multiple proteins within the cell. The covalent modification of oocyte proteins by these aldehydes increased with extended periods of time postovulation; the mitochondrial protein succinate dehydrogenase (SDHA) was identified as a primary target for 4HNE adduction. Time- and dose-dependent studies revealed that exposure to elevated levels of electrophilic aldehydes causes mitochondrial reactive oxygen species production, lipid peroxidation, loss of mitochondrial membrane potential, and eventual apoptosis within the MII oocyte, presumably as a consequence of electron transport chain collapse following 4HNE adduction. Additionally, we have determined that short-term exposure to low doses of 4HNE dramatically impairs the oocyte’s ability to participate in fertilization and support embryonic development; however, this loss of functionality can be prevented by supplementation with the antioxidant penicillamine. In conclusion, this study has revealed that the accumulation of electrophilic aldehydes is linked to postovulatory oocyte aging, causing reduced fertility, oxidative stress, and apoptosis of this highly specialized cell. These data highlight the importance of timely fertilization of the mammalian oocyte postovulation and emphasize the potential advantages associated with antioxidant supplementation of oocyte culture medium in circumstances where reinsemination of oocytes may be desirable (i.e., rescue intracytoplasmic sperm injection), or where in vitro fertilization may be delayed.

electrophilic aldehydes, oxidative stress, postovulatory oocyte aging

INTRODUCTION

In mammalian species, timely fertilization of the oocyte following ovulation is crucial for a successful reproductive outcome. In the majority of mammals, biology assures synchrony between the release and interaction of gametes, either as a result of induced ovulation at the time of mating as in rabbits, cats, and camels [1] or receptivity to mating only at the time of ovulation (behavioral estrus), for example, cows [2]. In the case of humans and certain primates, no biological mechanisms are in place to ensure synchrony between intercourse and ovulation, elevating the likelihood that the metaphase II (MII) oocyte will experience degeneration as a consequence of postovulatory aging before meeting with the fertilizing spermatozoon; however, biochemical alterations to both the zona pellucida and oolemma during oocyte degeneration often prevent the occurrence of such in vivo fertilization (reviewed by Lord and Aitken [3]). Unfortunately, the utilization of assisted reproductive technologies (ART) can encourage postovulatory aging of the oocyte in vitro culture as well as enable fertilization of these degenerating cells by overriding natural selection mechanisms that would normally prevent the union of defective gametes. In vitro aging of oocytes is particularly problematic in situations where extended periods of in vitro maturation are required (generally in livestock species) prior to in vitro fertilization (IVF). In these situations, a delicate balance exists in that the culture time must be sufficient for nuclear maturation to occur but not so extensive as to instigate in vitro aging [4]. This delicate equilibrium is further complicated by the fact that bovine sperm, for example, take several hours from the initiation of IVF to actually penetrate the oocyte. In a human ART setting, the rapid degeneration of oocytes in vitro culture means that if fertilization by IVF fails due to a male factor (i.e., lack of zona binding by the spermatozoon), then these oocytes cannot be reinseminated using rescue intracytoplasmic sperm injection (ICSI); thus, the patient must be subjected to a subsequent round of expensive and invasive fertility treatment.

As previously mentioned, postovulatory oocyte aging has a detrimental effect on fertilization rate; however, it also negatively influences embryo quality as well as the health of the resulting offspring [5–7]. At a molecular level, oocytes exhibit numerous symptoms of aging with extended periods of time following ovulation, including a decline in cell cycle factors required to maintain meiotic arrest [8] and an associated increased susceptibility to spontaneous activation [9], partial cortical granule exocytosis and zona pellucida hardening [9–13], loss of chromosomal and spindle integrity [14–17], and also mitochondrial dysfunction [18–21]. This degenerative process of postovulatory oocyte aging culminates in cell death and exhibits many of the features of apoptosis [7, 22].

Although the molecular mechanisms controlling postovulatory oocyte aging and apoptosis are not thoroughly understood (reviewed by Lord and Aitken [3]), oxidative stress has been found to be a key mediator of this process. An elevation in levels of reactive oxygen species (ROS) within the oocyte can be identified following as little as 8 h in vitro culture time and has been directly linked to a decline in fertilization rate and embryo quality [7], impaired calcium homeostasis [19, 23], abnormalities in chromosomes and microtubules [24], and the appearance of apoptotic markers such as fragmentation and activated caspases [7, 25]. The accumulation of ROS within the oocyte is thought to be exacerbated by in vitro culture conditions, for example,
increased oxygen tension, exposure to light, and an absence of antioxidant-rich follicular and tubal fluids [26–29]. In conjunction with the overproduction of ROS in the aging MII oocyte, an elevation of lipid peroxidation is also observable with increasing periods of time following ovulation [19]. Electrophilic aldehydes such as 4-hydroxynonenal (4HNE), malondialdehyde (MDA), and acrolein are well-known by-products of the nonenzymatic peroxidation of polyunsaturated fatty acids, generally arachidonic and linoleic acids [30]. These aldehydes are highly reactive with histidine, cysteine, and lysine residues on proteins [31, 32], forming Michael adducts with a hemiacetal structure [33], which can result in impairment of enzymatic activity and interfere with signaling pathways [32]. Significantly, 4HNE is known to play a key role in inducing subfertility in the male germ cell [34], and 4HNE has been found to be directly responsible for induction of both mitochondrial ROS generation and apoptosis in the spermatozoon by adducting to the flavoprotein component of the mitochondrial protein succinate dehydrogenase (SDHA), thereby stimulating electron leakage and superoxide generation from the electron transport chain (ETC) at Complex II. Because oxidative stress and lipid peroxidation are relatively early events in the oocyte aging process in vitro, we hypothesize that the production of electrophilic aldehydes may ensue, initiating an escalating cycle of oxidative stress and apoptotic damage involving the oocyte’s mitochondria in a similar fashion to oxidatively stressed spermatozoa [34]. This study therefore aimed to establish the role of electrophilic aldehydes in postovulatory aging of murine oocytes in vitro. Specifically, we have utilized immunocytochemistry and immunoblotting techniques to establish the presence of 4HNE, MDA, and acrolein adducts in the aging oocyte and immunoprecipitation to identify adduction targets. To ascertain the effects of physiological concentrations of electrophilic aldehydes (0–200 μM) on oocyte integrity [35–38], MII-stage oocytes have been subjected to 4HNE and acrolein exposure in a time- and dose-dependent manner, and their capacity for fertilization and embryo development, levels of oxidative stress, and apoptotic status assessed.

MATERIALS AND METHODS

Chemicals and Materials

All the chemicals used in this study were purchased from Sigma Aldrich unless otherwise stated. The anti-4HNE antibody was from Jomar Diagnostics (STA-035), the acrolein antibody from Novus Biologicals (NB200-556), and the MDA antibody from Abcam (ab6463). All the fluorescent probes used in this study were obtained from Molecular Probes unless otherwise stated.

Oocyte Retrieval

Three- to four-week-old C57BL6 x CBA F1 female mice were administered intraperitoneal injections of equine chorionic gonadotropin (eCG) (Intervet), followed 48 h later by human chorionic gonadotropin (hCG) (Intervet) to induce superovulation. MII-stage oocytes were collected at 14 h post-hCG injection and denuded as described previously [7]. The use of animals in this project was approved by the University of Newcastle Animal Care and Ethics Committee, and all the animals were obtained from breeding programs run in the University of Newcastle Central Animal House.

Immunocytochemistry with 4HNE, Acrolein, and MDA Antibodies

Following retrieval, oocytes were washed three times in phosphate-buffered saline (PBS) containing 3 mg/ml polyvinylpyrrolidone prior to fixation in 3.7% paraformaldehyde for 1 h at room temperature (RT). Fixed oocytes were again washed in PBS/polyvinylpyrrolidone before permeabilization with 0.25% Triton-X in PBS for 10 min at RT. Following permeabilization, oocytes were placed in a blocking solution of 3% bovine serum albumin (BSA) in PBS where they remained overnight at 4°C. The following day, oocytes were washed in 1% BSA/PBS before a 1 h incubation in anti-4HNE primary antibody (1:200 in 1% BSA/PBS), anti-MDA antibody (1:100 dilution in 1% BSA/PBS), or anti-acrolein primary antibody (1:100 in 1% BSA/PBS) at 37°C. This incubation step was followed by a 1 h incubation in Alexa Fluor 488 goat-antirabbit secondary antibody (1:1000 in 1% BSA/PBS; Invitrogen) at 37°C. Finally, oocytes were mounted on microscope slides in polyvinyl alcohol (mowiol) and fluorescence was assessed against a secondary antibody-only control using a FV1000 Confocal Microscope (Olympus). A subset of oocytes was also incubated in Mitotracker Red (Molecular Probes) prior to fixation for immunocytochemistry (as per the manufacturer’s instructions) to establish any colocalization between these electrophilic aldehydes and the mitochondria.

SDS-PAGE and Western Blot Analysis

Extraction of protein lysates from mouse oocytes was achieved via a 5 min incubation in SDS extraction buffer (2% [w/v] SDS and 10% [w/v] sucrose in 0.1875 M Tris, pH 6.8) containing a protease inhibitor cocktail (Roche) at 100°C to achieve denaturation of secondary and tertiary protein structures. Protein lysates were stored at −20°C for no more than 48 h prior to use. Protein extracted from approximately 100 oocytes per treatment was loaded onto a 4%–20% gradient SDS polyacrylamide gel (NuSep) and separated by electrophoresis. Proteins were then transferred onto nitrocellulose membrane using standard Western blot transfer techniques. Nitrocellulose membranes were blocked in 5% skim milk in Tris–0.02 M NaCl (0.15 M) TBS containing 0.1% Tween (TBST) at RT for 1 h prior to incubation with anti-4HNE primary antibody (1:500), anti-acrolein antibody (1:1000), or anti-MDA antibody (1:2000) in 1% skim milk in TBST. Immunooblots remained in primary antibody solution overnight at 4°C and were washed three times in TBST before a 1 h incubation in goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G secondary antibody (1:1000 dilution in 1% skim milk in TBST). Nitrocellulose membranes were developed using an enhanced chemiluminescence kit (GE Healthcare) as per the manufacturer’s instructions. Densitometric analysis was conducted using the public sector image-processing program Image J (version 1.45S; National Institutes of Health), and values were normalized to the loading control (tubulin).

Immunoprecipitation of 4HNE

Immunoprecipitation was utilized to identify proteins that were covalently modified by 4HNE within the oocyte. Ten micrograms of 4HNE antibody was cross-linked with Protein G Dynabeads (Life technologies) using 2 mM 3,3-dithiobis-sulfosuccinimidyl propionate as per the manufacturer’s instructions. The 4HNE-bound Dynabeads were then resuspended in 2 μg oocyte protein lysate that had been precleared of any nonspecific binding partners via a 1 h incubation with unbound Protein G Dynabeads (4°C). Extraction of oocyte protein (while still maintaining protein–protein interactions) was achieved via a 2 h incubation in CHAPS lysis buffer (10% glycerine, 10 mM CHAPS, 10 mM HEPES in distilled water) at 4°C with constant rotation. Coinubulation of 4HNE-bound Dynabeads and precleared oocyte lysate was conducted overnight with constant rotation. Following this, antibody-antigen-bound beads were washed three times in PBS before being resuspended in SDS loading buffer—0.2% (w/v) SDS, 50% (v/v) 0.375 M Tris, 10% (v/v) sucrose, 4% (v/v) β-mercaptoethanol, and 0.001% bromphenol blue—and incubated at 100°C for 5 min to elute proteins. The eluted protein was loaded onto a 4%–20% gradient gel along with the following controls: 1 μg 4HNE antibody, elution from Dynabeads used for the preclearing step, Dynabeads-only control, and a wash control containing lysis buffer that was used to wash the Dynabeads following cross-linking with 4HNE antibody (see Supplemental Fig. S1, available online at www.biolreprod.org). Gel electrophoresis, Western blot transfer, and probing of the nitrocellulose membrane were conducted as described above.

Oocyte Aging and Treatment with Electrophilic Aldehydes

Oocytes undergoing in vitro aging without 4HNE/acrolein treatment remained in 20 μl droplets of M2 medium under mineral oil for 1, 3, or 24 h at 37°C under gas (5% O2 and 6% CO2 in N2). Subsets of oocytes were also aged for 8 or 24 h in vitro (equates to 22 and 38 h post-hCG, respectively) in the presence of 4HNE (Cayman Chemicals) or acrolein at physiological concentrations of 0, 50, 100, or 200 μM in M2 medium [35–38]. At each time point, oocytes were subjected to a series of fluorometric assays (described below) to ascertain their oxidative and apoptotic status.

Identifying Oxidative Stress and Apoptosis related to 4HNE and Acrolein Exposure

MitoSox Red (MSR) was used to detect mitochondrial ROS production within the oocyte, while 5′-carboxy-2′,7′-dichlorofluorescein diacetate
(DFF-DA) was used to detect H₂O₂ and peroxynitrite. Levels of lipid peroxidation were determined using the BODIPY C₁₁ probe. The onset of apoptosis was identified using a FAM FLICA Poly Caspases Assay Kit (Immunochemistry Technologies) to identify caspase activation and a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche) to identify DNA fragmentation resulting from apoptosis. An associated loss of mitochondrial membrane potential was determined using the JC-1 probe by monitoring the red/green fluorescence ratio. At each analyzed time point, oocytes were incubated with the desired fluorometric probe for 30 min and washed three times in M₂ medium before mounting for analysis using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging Inc.). Fluorescence levels exhibited by oocytes were quantified by pixel intensity analysis using Image J, and values were normalized to the untreated control for graphical representation and statistical analyses.

**IVF**

In order to assess the effects of 4HNE accumulation within the oocyte on fertilization potential and subsequent embryo development, oocytes were exposed to 0, 50, 100, or 200 µM 4HNE in M₂ medium for 1.5 h prior to IVF, which was conducted as described previously [7]. Additionally, oocytes were exposed to 100 µM 4HNE (the lowest concentration that was found to significantly decrease both fertilization rate and embryo formation rate within 1.5 h) in the presence of penicillamine (250 µM, 500 µM, or 1 mM) prior to IVF to assess any counteraction of the negative effects of 4HNE by this antioxidant. In order to confirm that the beneficial effects of penicillamine were due to intracellular reaction with cytotoxic aldehydes rather direct adduction of the added 4HNE, this experiment was repeated using a transient 4HNE exposure protocol. Following selection of 1 mM as the optimal concentration of penicillamine, this compound was incorporated into oocyte culture medium following a limited exposure to 4HNE. Oocytes were exposed to 100 µM 4HNE for 1.5 h, washed three times in M₂ medium, and then placed in M₂ medium or M₂ medium containing 1 mM penicillamine for 3 h prior to IVF. An untreated control and penicillamine-only control were also included (penicillamine alone had no effect on fertilization rate or embryo formation rate when compared to untreated controls).

Oocytes were assessed at 4 h postinsemination for signs of fertilization, namely formation of pronuclei and extrusion of the second polar body. Additionally, 24 h following IVF, the percentage of 2-cell embryos formed within each treatment category was assessed. On the morning of Days 3 and 5, the percentage of 4-cell and blastocyst formation, respectively, was also assessed in penicillamine/4HNE experiments. Values are represented normalized to the untreated control (control value set at 100%).

**Statistical Analyses**

All the experiments were conducted at least three times on independent samples, and the results were analyzed by ANOVA using JMP version 9.0.0. A post hoc comparison of group means was conducted using a Fisher protected least significant difference test. Analysis of paired samples was conducted using a paired Student t-test. A value of $P < 0.05$ was considered to be statistically significant.

**RESULTS**

**Aging Oocytes Experience an Accumulation of Electrophilic Aldehydes**

In order to establish whether electrophilic aldehydes are produced in the MII-stage oocyte as a result of lipid peroxidation and subsequently whether these electrophiles play a role in postovulatory aging, immunocytochemistry and immunoblotting techniques were utilized. Analysis by immunocytochemistry identified 4HNE, acrolein, and MDA within the MII-stage oocyte at 15 h post-hCG (1 h in vitro culture) with fluorescence associated with these antibodies distributed throughout the ooplasm (representative images provided). B) Western blot analysis of oocyte lysates revealed that 4HNE, acrolein, and MDA covalently modify multiple proteins within the oocyte. The primary target for adduction by 4HNE, acrolein, and MDA was a 70 kDa protein(s), whilst acrolein and MDA adduction was also prominent in a high molecular weight protein(s) of >170 kDa. Tubulin shown as control. Independent replicates were conducted with a minimum of 100 oocytes per replicate. Bar = 50 µm.
throughout the ooplasm at levels visibly higher than the secondary antibody-only control (Fig. 1A). Western blot analysis confirmed adduction of 4HNE, acrolein, and MDA to multiple proteins within lysates retrieved from these oocytes (Fig. 1B). The primary target for covalent modification by all three aldehydes was a protein(s) of 70 kDa, whilst acrolein and MDA were also found to adduct to a high molecular weight protein(s) of >170 kDa in size. Importantly, covalent modification by 4HNE, acrolein, and MDA appeared to become more prevalent in protein lysates from oocytes that had been aged for 24 h in vitro (Fig. 2, A–C, respectively). A visible increase in the intensity of the 70 kDa band was detected in aged oocyte lysates above fresh oocyte lysates and was confirmed using densitometric analysis normalized to the tubulin loading control (4HNE, \( P < 0.001 \); acrolein, \( P < 0.01 \); MDA, \( P < 0.001 \)). Furthermore, the global adduction of proteins, including to the >170 kDa protein(s), by 4HNE, acrolein, and MDA visibly increased in intensity with postovulatory age.

**Electrophilic Aldehyde Exposure Causes Oxidative Stress and Apoptosis in the MII Oocyte**

Because the extended culture of MII oocytes in vitro is associated with oxidative stress, lipid peroxidation, and the onset of apoptosis [7, 19], we next determined whether these pathological changes might be precipitated by the time-dependent accumulation of electrophilic aldehydes demonstrated within the oocyte. Both 4HNE and acrolein were utilized to assess the time- and dose-dependent effects of electrophilic aldehyde exposure, using doses of 0, 50, 100, and 200 \( \mu \text{M} \) at 8 and 24 h (as well as a 1 h untreated control). Importantly, the selected doses coincide with the predicted physiological ranges of these compounds: 10 \( \mu \text{M} \)–5 mM for
4HNE [35] and 30–180 μM for acrolein [36–38]. Additionally, the selected time points correspond with a previous study conducted by our research group [7] that detected an elevation in ROS levels following only 8 h in vitro culture in untreated C57Bl6 x CBA oocytes that was exacerbated and accompanied by indicators of apoptosis at 24 h.

In conjunction with previous studies that depict lipid peroxidation as an identifiable characteristic of in vitro aging, levels of fluorescence associated with the BODIPY C11 probe were elevated at both 8 h (P < 0.001) and 24 h (P < 0.001) in untreated MI-stage oocytes (Fig. 3A). This lipid peroxidation was exacerbated in a dose-dependent manner by the presence of both 4HNE and acrolein. BODIPY C11 fluorescence was significantly elevated above control values following 8 h of 100 and 200 μM 4HNE treatment (both P < 0.01) and 200 μM acrolein treatment (P < 0.05) (Fig. 3B). Following 24 h of exposure, a significant elevation in lipid peroxidation above the untreated control could again be identified within both 100 and 200 μM 4HNE treatments (both P < 0.01), whilst a prominent time-dependent elevation in BODIPY C11 fluorescence could be identified in acrolein-treated oocytes, found to be significantly elevated from the control at all concentrations tested (P < 0.05) (Fig. 3C and D). Similarly, levels of mitochondrial ROS production were found to be increased in untreated oocytes following both 8 h (P < 0.01) and 24 h (P < 0.01) of in vitro culture as a consequence of postovulatory aging (Fig. 3E). This mitochondrial ROS production was further exacerbated in the presence of the highest dose of both 4HNE (P < 0.01) and acrolein (P < 0.05) for 8 h (Fig. 3F) and by 100 μM (P < 0.05) and 200 μM (P < 0.001) 4HNE and all doses of acrolein (P < 0.01) for 24 h (Fig. 3G and H). Pixel intensity values associated with MSR fluorescence again demonstrated a visible time-dependent increase within oocytes exposed to elevated levels of electrophilic aldehydes. Finally, the DFF-DA fluorescent probe further confirmed a significant time-dependent increase in ROS levels within untreated oocytes undergoing postovulatory aging at both 8 h (P < 0.05) and 24 h (P < 0.01) (Fig. 3I). Again, ROS production was found to be elevated above controls following 8 h 4HNE (100 μM, P < 0.05; 200 μM, P < 0.01) and acrolein (200 μM, P < 0.05) exposure (Fig. 3J) and further exacerbated following 24 h exposure to both compounds (100 μM 4HNE, P < 0.01 and 200 μM 4HNE, P < 0.001; all doses acrolein, P < 0.01, P < 0.01, and P < 0.001 respectively) (Fig. 3K and L).

In addition to stimulating oxidative stress; exposure of oocytes to the electrophilic aldehydes 4HNE and acrolein resulted in an accelerated onset of apoptosis and an associated loss of mitochondrial membrane potential. In accordance with previously published literature [7], levels of caspase activation within untreated oocytes increased in a time-dependent manner as a result of apoptosis associated with postovulatory aging (P < 0.01) (Fig. 4A). Apoptosis was accelerated in these oocytes in a time- and dose-dependent fashion in response to electrophilic aldehyde treatments. Oocytes treated with 200 μM 4HNE/acrolein for 8 h (P < 0.05 and P < 0.01 respectively) (Fig. 4B) and higher doses (100 and 200 μM) of both 4HNE (P < 0.001) and acrolein (P < 0.05 and P < 0.01, respectively) for 24 h exhibited significantly elevated levels of fluorescence relating to caspase activation when compared to untreated controls (Fig. 4C and D). Analysis of DNA fragmentation relating to apoptosis using the TUNEL assay revealed a similar trend. In untreated oocytes, fluorescence associated with DNA fragmentation was significantly elevated at 24 h due to apoptosis associated with postovulatory age (P < 0.01) (Fig. 4E). Exposure to 200 μM of both 4HNE and acrolein as well as 100 μM 4HNE significantly elevated levels of TUNEL fluorescence in oocytes above control values following 24 h of culture (P < 0.001, P < 0.05, and P < 0.05, respectively) (Fig. 4, F–H). Finally, a loss of mitochondrial membrane potential within oocyte populations also accompanied at both 8 h (P < 0.01) and 24 h (P < 0.01) of in vitro culture (Fig. 4I) and was exacerbated by 8 and 24 h exposure to 100 μM (P < 0.001) and 200 μM of 4HNE (P < 0.01, P < 0.05) and acrolein (P < 0.001) (Fig. 4, J–L). Further confirmation that these electrophilic aldehydes were inducing apoptosis (rather than necrosis) within the oocyte was achieved via morphological analysis, which revealed shrinking of the cytoplasm in aldehyde-treated oocytes when compared to untreated controls as well as apoptotic body formation (Fig. 4M).

As we have demonstrated, postovulatory oocyte aging is associated with elevated levels of lipid peroxidation and ROS production followed by the onset of apoptosis characterized by caspase activation, DNA fragmentation, and a loss of mitochondrial membrane potential. The time-dependent increase in levels of protein modification by endogenous electrophilic aldehydes in the in vitro cultured oocyte (depicted in Fig. 2) may be directly linked with these biochemical changes because exposure to elevated levels of both 4HNE and acrolein exacerbated oxidative stress within MII oocytes and accelerated entry into apoptosis.

4HNE Stimulates Mitochondrial ROS Production in the Oocyte by Adducting the Mitochondrial Protein SDHA

We next aimed to ascertain whether electrophilic aldehydes may be covalently binding to the mitochondrial protein SDHA within the oocyte, stimulating the observed ROS production and the subsequent onset of postovulatory aging and apoptosis via dysfunction of the ETC as has been described in the spermatozoon [34]. Western blot analysis revealed that the 70 kDa band corresponding to SDHA colocalizes with the primary target for 4HNE, acrolein, and MDA adduction in oocyte lysates (Fig. 5A). In order to confirm a covalent modification of SDHA, immunoprecipitation experiments were performed using anti-4HNE antibody. The 4HNE-modified proteins were pulled down from oocyte lysates using 4HNE antibody-bound beads, and the presence of SDHA within this subset of proteins was confirmed via Western blot analysis, clearly displaying a 70 kDa band corresponding to SDHA in the elution (Fig. 5B). Further to these findings, immunocytochemistry analyses on untreated oocytes subjected to 8 h in vitro culture demonstrated a distinct colocalization between 4HNE-associated fluorescence and the mitochondria as detected by Mitotracker red (Fig. 5C). Together these results suggest that increasing levels of covalent modification of the mitochondrial flavoprotein SDHA by 4HNE during in vitro aging of the oocyte may cause dysfunction of the ETC, resulting in the observed increase in ROS production/lipid peroxidation and eventual apoptosis that occur throughout postovulatory aging.

Low-Dose 4HNE Exposure Impedes Fertilization and Embryo Competency

Finally, we explored the effects of short-term exposure to the cytotoxic aldehyde 4HNE on the functionality of the oocyte in terms of its capacity to participate in fertilization and generate a viable embryo. Oocytes exposed to the higher doses of 4HNE (100–200 μM) for only 1.5 h prior to insemination exhibited a significant reduction in fertilization rate when compared to untreated controls (100 μM, P < 0.01; 200 μM, P < 0.05) (Fig. 6A). Additionally, exposure to all doses of 4HNE...
FIG. 3. Exposure of MII-stage oocytes to elevated levels of 4HNE and acrolein instigates oxidative stress in a time- and dose-dependent manner. A–D) Analysis of levels of lipid peroxidation using the fluorescent probe BODIPY C11 revealed a time-dependent elevation in fluorescence in untreated MII-stage oocytes and a time- and dose-dependent elevation in fluorescence resulting from both 4HNE and acrolein exposure that achieved statistical significance at higher doses (100 and 200 μM) of 4HNE and all doses of acrolein. E–H) Similarly, mitochondrial ROS production was found to be elevated in untreated oocytes with increasing periods of in vitro culture time, whilst 4HNE and acrolein treatment significantly increased levels of mitochondrial ROS.

I–L) Further, 4HNE and acrolein treatment significantly increased levels of DFF-DA (a marker of mitochondrial depolarization).
postovulatory aging has also been associated with aging in other tissue types [41]. The accumulation of aldehydes with postovulatory age observed in this study complements previously published reports of elevated levels of ROS [7] and lipid peroxidation [19] in the oocyte with extended periods of in vitro culture time. Indeed, these phenomena were again observed within the confines of the current study, with levels of MSR and BODIPY C11 increasing in a time-dependent manner in untreated in vitro cultured oocytes. Although the antioxidant glutathione (GSH) is abundant within the ooplasm, and is known to provide an effective defense against compounds such as 4HNE within the cell by binding of the electrophile to the cysteine residue (reviewed by Perluigi et al. [40]), the MII-stage oocyte becomes depleted of GSH with increasing amounts of time following ovulation [42, 43], thus explaining the observable accumulation of both 4HNE and ROS within this cell.

Time- and dose-dependent exposure of oocytes to both 4HNE and acrolein revealed that an accumulation of electrophilic aldehydes with postovulatory age could certainly be responsible for numerous pathologies previously identified to be associated with the aged phenotype. It is important to emphasize that the doses of 4HNE and acrolein used in these experiments are within the range of concentrations expected to be achieved during a state of oxidative stress. While the resting concentration of these electrophilic aldehydes is thought to be 5–10 μM for 4HNE [35] and 30–50 μM for acrolein [36, 38], at times of oxidative stress, these concentrations are thought to increase to as high as 5 mM [35] and 180 μM [36], respectively. We have observed that exposure of the oocyte to elevated levels of exogenous 4HNE (100–200 μM) and acrolein (50–200 μM) causes a propagation of ROS production and lipid peroxidation, eventually leading in a loss of mitochondrial membrane potential, activation of caspases, and DNA fragmentation related to apoptosis. The ability of acrolein to propagate ROS production and lipid peroxidation at a lower concentration than that seen with 4HNE is likely attributed to the fact that acrolein is the strongest electrophile of the \( \alpha, \beta \)-unsaturated aldehydes and demonstrates the highest reactivity with nucleophiles such as proteins [44]. Following from this, the proposed mechanism by which these electrophilic aldehydes elicit the aforementioned effects on oocyte biochemistry is thought to be via covalent modification of the mitochondrial flavoprotein SDHA, as demonstrated by colocalization and immunoprecipitation analyses. Michael addition reactions of 4HNE with other mitochondrial proteins, such as cytochrome c oxidase [45] and mitochondrial ATPase [46], has been demonstrated to stimulate ROS production in other cell types, while addition to SDHA has been shown to be responsible for increased ROS production in the spermatozoon [34]. Covalent modification of SDHA within the oocyte’s mitochondria likely results in autooxidation and subsequent transference of electrons to oxygen rather than to coenzyme Q [47]. This stimulation of ROS production would initiate an escalating cycle of lipid peroxidation, electrophilic aldehyde formation, and free radical generation, resulting in oxidative stress (diagrammatically represented in Fig. 7), which would,
FIG. 4. Exposure to 4HNE and acrolein initiates a time- and dose-dependent appearance of apoptotic markers and loss of mitochondrial membrane potential within the oocyte. A–D) Levels of caspase activation were significantly elevated with increasing periods of in vitro culture time in untreated oocytes as a consequence of postovulatory aging. Following both 8 and 24 h exposure to 4-HNE and acrolein, levels of caspase activation were found to be significantly elevated above control values. E–H) Similarly, DNA fragmentation relating to apoptosis (TUNEL assay) was detected in untreated oocytes following 24 h in vitro culture as a result of postovulatory aging. However, this DNA fragmentation was exacerbated in response to electrophilic aldehyde.
in turn, precipitate the onset of apoptosis in the MII-stage oocyte [7, 25].

In addition to its role in directing the oocyte into an apoptotic pathway during postovulatory aging, we have also established a direct link between electrophilic aldehyde accumulation and a decline in functionality of the oocyte in terms of its capacity to participate in fertilization and support embryo development. Short-term exposure (1.5 h) to only low levels of 4HNE was found to severely impair the functionality of the oocyte long before the appearance of apoptotic markers. The observed reduction in fertilization rate associated with 4HNE exposure is likely to be directly linked to an associated elevation in lipid peroxidation within the oolemma; increasing membrane rigidity [48] and interfering with sperm-oolemma fusion. Impairment of the oocyte’s capacity to support embryo development, as represented by a significant decline in the percentage of 2-cell embryo formation associated with short-term exposure to low levels of 4HNE (50 μM), and a prominent loss of developmental potential associated with exposure to higher levels of 4HNE (100 and 200 μM) is likely attributed to interference with multiple intracellular components by accumulated aldehydes. Firstly, actin is known to be a particularly vulnerable target for covalent modification by 4HNE [49], with any impairment in actin functionality having the capacity to interrupt several crucial postfertilization events such as spindle rotation, polar body formation, and pronuclear

exposure, with levels of TUNEL fluorescence being significantly elevated above untreated controls following 24 h exposure to 4HNE and acrolein. 

Finally, a time-dependent loss of mitochondrial membrane potential, as determined by the JC-1 assay, was identified in populations of untreated oocytes subjected to in vitro aging. Loss of mitochondrial membrane potential was further exacerbated by exposure to elevated levels of electrophilic aldehydes for 8 and 24 h. Following 24 h electrophilic aldehyde treatment, oocytes exhibited morphological characteristics of apoptosis, such as shrinking of the cytoplasm and apoptotic body formation. Images accompanying each histogram depict a representation of untreated oocytes at 1 h and oocytes treated with 200 μM 4HNE or acrolein for 24 h for each fluorescent probe utilized. Bar = 50 μm. Mean ± SEM values are plotted in the histograms. Independent replicates were conducted with a minimum of 100 oocytes per replicate; *P < 0.05, **P < 0.01, ***P < 0.001.
FIG. 6. Short-term exposure of the oocyte to 4HNE prior to insemination significantly impairs the ability of the cell to support fertilization and embryo development but can be reversed by the antioxidant penicillamine. 

A) Oocytes exposed to higher doses of 4HNE (100–200 μM) for 1.5 h experienced a significant decline in fertilization rate at 3 h postinsemination. 

B) Additionally, short-term exposure to all doses of 4HNE resulted in a dose-dependent reduction in the formation of 2-cell embryos at 24 h postinsemination when compared to untreated controls. 

C, D) Coincubation with the antioxidant penicillamine (250 μM–1 mM) throughout the 100 μM 4HNE treatment reversed the negative effects of this electrophile on fertilization rate and improved 

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Furthermore, 4HNE has the capacity to reduce levels of intracellular calcium [23] and disrupt calcium homeostasis [44]. Aberrant calcium oscillations at the time of fertilization are known to trigger apoptosis rather than initiate a developmental pathway, as has been demonstrated in both aged and oxidatively stressed oocytes, which exhibit calcium oscillations with reduced amplitude and increased frequency compared with freshly ovulated oocytes, culminating in embryo fragmentation [19, 23]. Finally, electrophilic aldehydes such as 4HNE and acrolein also have the ability to irreversibly damage DNA within the cell [51, 52], with elevated levels of DNA damage in the zygote potentially preventing continuation of the cell cycle past the G₁ checkpoint, resulting in developmental arrest at the 1-cell stage of development and likely entry into apoptosis [53]. Importantly, these data suggest that the demonstrated accumulation of electrophilic aldehydes during in vitro aging may be directly linked to multiple insufficiencies acquired with increasing time postovulation, cumulatively resulting in a reduced capacity to produce viable embryos.

At this juncture, it is important to consider that electrophilic aldehydes, particularly acrolein, are toxicants that can exist within the environment (e.g., herbicides or compounds formed during combustion of organic materials) [54], and that are present at high levels within cigarette smoke [37]. Cigarette smokers in particular are predicted to have elevated levels of systemic acrolein [37, 55], presenting a potential risk for oocyte damage within the ovary prior to ovulation. Certainly, toxicants within cigarette smoke are known to cause follicle depletion and oocyte apoptosis in antral follicles, whilst surviving oocytes exhibit elevated levels of mitochondrial ROS production and lipid peroxidation, along with a reduced capacity to participate in fertilization [56]. The commonality between these pathologies and those demonstrated in our acrolein dose- and time-dependent studies in vitro (Figs. 3 and 4), as well as the demonstrated presence of acrolein adducts in

FIG. 7. Proposed role of electrophilic aldehydes in postovulatory oocyte aging. With increasing amounts of time from ovulation (>10 h), the MII-stage oocyte experiences a depletion of antioxidant protection by way of GSH stores and an accumulation of ROS, resulting in oxidative stress. As a result of increased levels of ROS, lipid peroxidation occurs, generating harmful electrophilic aldehydes such as 4HNE. These electrophilic aldehydes covalently modify the mitochondrial flavoprotein SDHA, affecting the functionality of the ETC and causing further ROS production. This self-perpetuating cycle directly affects the functionality of the oocyte, impairing its capacity to support fertilization and embryo development and culminates in oxidative stress-driven initiation of an apoptotic cascade.
freshly ovulated oocytes (Fig. 1), suggest that exposure to elevated levels of acrolein through cigarette smoke may be directly linked with compromises in female fertility.

Finally, when considering the irreversible nature of damage inflicted by endogenously produced electrophilic aldehydes within the oocyte during postovulatory aging in vitro, it would clearly be beneficial to prevent the accumulation of these compounds, particularly in an assisted reproduction setting where oocytes may be exposed to extended periods of in vitro culture time before fertilization by IVF or ICSI. Previous research has indicated that various antioxidants (GSH reductase, peroxiredoxin, catalase) are capable of reducing levels of lipid peroxidation products, such as 4HNE, when supplemented into culture medium [57, 58]. Our study has shown that the reactive thiol, penicillamine [39], can counteract the negative effects of 4HNE exposure on oocyte functionality, improving fertilization rate and embryo formation rate following 4HNE exposure, making this antioxidant a promising candidate for preventing degeneration of oocytes as a result of endogenously produced aldehydes when supplemented into culture medium in an in vitro setting. Certainly, supplementation of oocyte culture medium with melatonin, an antioxidant compound known to counteract lipid peroxidation [59], has been previously demonstrated to delay the onset of fragmentation and caspase activation during postovulatory aging in vitro as well as extend the optimal window for fertilization and improve quality of embryos resulting from oocytes that have been exposed to extended periods (8–16 h) of in vitro culture time [7]. It is possible that a combination of antioxidant agents capable of minimizing lipid peroxidation as well as neutralizing electrophilic aldehydes may provide a methodology for greatly prolonging the window for vitality of MII-stage oocytes awaiting insemination.

In conclusion, this study has demonstrated that in vitro aging of the MII-stage oocyte postovulation is associated with an accumulation of cytotoxic electrophilic aldehydes. We propose that this accumulation of aldehydes is a component of an escalating cycle involving ROS production, lipid peroxidation, and subsequent aldehyde formation that results in a state of oxidative stress in the aging oocyte, eventually triggering an intrinsic apoptotic cascade. We have found that electrophilic aldehydes such as 4HNE can covalently modify the mitochondrial protein SDHA within the oocyte, providing one potential mechanistic link between the accumulation of 4HNE and mitochondrial ROS production observed with postovulatory aging. Additionally, we have demonstrated that the decreased capacity for fertilization and embryo formation observed in aging oocytes may be directly linked with electrophilic aldehyde accumulation. This research emphasizes the importance of timely fertilization of the oocyte postovulation and, furthermore, highlights the beneficial nature of antioxidant supplementation of oocyte culture medium in circumstances where IVF may be delayed (e.g., livestock ART) or where reinsemination of oocytes may be advantageous following a failure to fertilize by IVF (i.e., rescue ICSI).

REFERENCES
Chapter 3: Supplementary Figures

Supplementary Fig. 1: 4HNE-immunoprecipitation including control lanes. Protein eluted from 4HNE bound Dynabeads was subjected to SDS-PAGE and western transfer along with the following controls; 1 µg 4HNE antibody (Ab control), unbound Protein G Dynabeads (bead only), elution from Dynabeads used to ‘pre-clear’ oocyte lysates (pre-clear), and lysis buffer used to wash the Protein G Dynabeads post-crosslinking with 4HNE antibody (wash control). A 70 kDa band corresponding to SDHA could be identified in the elution lane (and not control lanes) following incubation with α-SDHA antibody, confirming that 4HNE covalently modifies this mitochondrial flavoprotein. The nitrocellulose membrane was also probed with α-4HNE antibody, verifying successful immunoprecipitation and depicting several adduction targets within the elution lane.
CHAPTER 4

Fertilization increases 8-hydroxy-2’-deoxyguanosine repair and antioxidant protection to prevent mutagenesis in the embryo

Under review: Developmental Biology

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Chapter 4: Overview

The aim of this manuscript was to elucidate the molecular and biochemical changes that occur within the oocyte at the time of fertilization that prevent otherwise inevitable entry into post-ovulatory ageing and apoptosis. In light of our previous findings which depict the intracellular accumulation of ROS as the initiator of oocyte degeneration, we investigated post-fertilization alterations to the antioxidant capacity of the oocyte, as well as its propensity for repair of oxidative damage.

Data within this manuscript demonstrated that the unfertilized oocyte is inherently more sensitive to oxidative insult than its fertilized counterpart – exhibiting an increased propensity for vitality loss and accumulation of oxidative DNA base adducts in response to hydrogen peroxide treatment. The increased tolerance of the fertilized oocyte to such oxidants was found to be attributed to an improved capacity for ROS breakdown by way of increased glutathione peroxidase activity, as well as upregulated activity of enzymes involved in oxidative DNA damage repair.

This manuscript provides characterisation of two previously unbeknownst mechanisms controlling the ‘molecular switch’ that redirects the fate of the oocyte from a degenerative pathway to a developmental pathway. These findings also provide unique insight into the biochemical defences possessed by the zygote to prevent mutagenesis upon the instigation of embryogenesis. This manuscript is currently under review in Developmental Biology (revised manuscript resubmitted as of 05/07/15).
Fertilization stimulates 8-hydroxy-2’deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo

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ABSTRACT

Oxidative DNA damage harboured by both spermatozoa and oocytes at the time of fertilization must be repaired prior to S-phase of the first mitotic division to reduce the risk of transversion mutations occurring in the zygote and subverting the normal patterns of cell differentiation and development. Of the characterised oxidative DNA lesions, 8-hydroxy-2’-deoxyguanosine (8OHdG) is particularly mutagenic. The current study reveals for the first time a marked acceleration of 8OHdG repair in the mouse oocyte/zygote by the base excision repair (BER) pathway following fertilization. Specifically, fertilization initiates post-translational modification to BER enzymes such as OGG1 and XRCC1, causing nuclear localisation and accelerated 8OHdG excision. Additionally, both the nuclear and mitochondrial genomes appear to benefit from increased protection against further 8OHdG formation by a fertilization-associated increase in glutathione peroxidase activity. The major limitation of the characterised 8OHdG repair system is the relatively low level of OGG1 expression in the oocyte, in contrast to the male germ line where it is the only constituent of the BER pathway. The male and female germ lines therefore collaborate in the repair of oxidative DNA damage, and oocytes are vulnerable to high levels of 8OHdG being carried into the zygote by the fertilizing spermatozoon.

INTRODUCTION

Repair of oxidative DNA damage within the one-cell zygote, prior to the initiation of S-phase of mitosis, is a critical step in the creation of viable embryos and healthy offspring. In the absence of successful repair of oxidative lesions such as 8-hydroxy-2’-deoxyguanosine (8OHdG), G-C to T-A transversion mutations (Wood et al.,
1992) can occur during DNA replication that not only alter the genetic profile of the zygote itself but also every cell generated by the rapid mitotic divisions that characterize embryogenesis. Thus, transversion mutations within the zygote have the propensity to irreversibly alter gene expression profiles and thence the fidelity of normal embryonic development (Bruner et al., 2000; Wu et al., 2013; Ohno et al., 2014). Despite the importance of repairing oxidative DNA damage at this early stage of development, gametes harbouring high levels of 8OHdG at the time of fertilization are known to undergo inadequate DNA repair in the zygote, resulting in detrimental effects on the pre-implantation development of the embryo (Takahashi, 2012; Lane et al., 2014) and on foetal growth and development (Chabory et al., 2009; Lane et al., 2014), as well as defects in offspring, including cancer and a significant reduction in lifespan (Ronen and Glickman, 2001; Vinson and Hales, 2002; Aitken et al., 2009).

Unfortunately, both spermatozoa and oocytes are known to harbour oxidative DNA lesions that can be contributed to the zygote following fertilization. The spermatozoon is particularly vulnerable to oxidative attack as a consequence of its propensity to produce reactive oxygen species (ROS) during the promotion of sperm capacitation (Aitken et al., 1995; Rivlin et al., 2004; O’Flaherty et al., 2006), its lack of antioxidant protection as a result of the restricted distribution and minimal volume of sperm cytoplasm, and a paucity of DNA repair mechanisms within a cell that possesses very little capacity for transcription or translation [reviewed by Aitken and De Iuliis (2010)]. Further to this, the increased utilization of assisted reproductive technologies (ART) such as ICSI to treat sub-fertile patient populations that are known to possess significantly elevated levels of 8OHdG in their spermatozoa (De Iuliis et al., 2009; Aitken et al., 2010) increases the likelihood that a spermatozoon harbouring mutagenic lesions will achieve fertilization by bypassing a number of natural selection strategies that would normally be operating in vivo.

As a consequence of the spermatozoon’s purported deficiency in DNA repair capacity, the responsibility for resolving the oxidative lesions contributed to the zygote by both male and female gametes is traditionally thought to lie entirely with the oocyte (Shimura et al., 2002). Single nucleotide repair of 8OHdG lesions in eukaryotic cells is primarily conducted by the base excision repair (BER) enzymes: oxoguanine glycosylase (OGG1), apurinic/apyrimidinic endonuclease (APE1) and X-ray repair cross complementing protein (XRCC1). Although repair of oxidatively damaged DNA can also be conducted by the enzymes of the nucleotide excision repair (NER) pathway,
this complex, multistep repair process is generally reserved for lesions that are causing structural distortion of the DNA (Brierley and Martin, 2013). Within the BER pathway, recognition of the 8OHdG adduct and subsequent base excision is performed by the DNA glycosylase OGG1. The residual abasic site is then hydrolyzed by the endonuclease APE1, allowing for replacement of the purine or pyrimidine base by polymerase β. Ligation of the strand nick is then performed by DNA ligase in association with the scaffolding protein XRCC1 [reviewed by David et al. (2007)]. Indeed, the oocyte is known to accumulate an abundance of mRNA’s and proteins involved in DNA repair within its vast cytoplasm during oogenesis (Zheng et al., 2005; Menezo et al., 2007), as the opportunity to transcribe new DNA repair genes is not available to the embryo until the 2-cell stage in the mouse (Flach et al., 1982), and the 4-cell stage in humans (Braude et al., 1988).

In reality however, repair of oxidative DNA damage potentially involves a measure of co-operation between male and female gametes prior to the initiation of embryo development. The first enzyme of the BER pathway, OGG1, has been clearly identified in the chromatin of human spermatozoa (Smith et al., 2013b). This sperm-derived OGG1 was not only found to be present at both the mRNA and protein level, but was also capable of cleaving 8OHdG adducts from sperm nuclear DNA to create the corresponding abasic sites. Despite the presence of OGG1 in sperm chromatin, the subsequent enzymes of the BER pathway, APE1 and XRCC1, could not be identified within human spermatozoa suggesting that this repair pathway can only be driven to completion by the oocyte, post-fertilization (Smith et al., 2013).

The current study utilizes a mouse model to characterize BER in the zygote, prior to the initiation of S-phase, with a particular focus on the potential for collaboration between sperm- and oocyte- derived BER enzymes. The results reveal a previously unappreciated upregulation of 8OHdG repair following fertilization as result of maternally driven post-translational modification to selected BER enzymes. Additionally, a fertilization-associated increase in antioxidant activity that decreases vulnerability of the zygote to oxidant-induced DNA damage was characterized. Cumulatively, these molecular mechanisms are likely to be critically important for protecting the genetic integrity of the zygote to allow for unimpeded transition through embryogenesis. However, low levels of OGG1 expression detected within the murine oocyte in this study highlight the vulnerability of the zygote to mitotic progression in
the absence of absolute DNA repair, as well as the necessity of OGG1 activity in the sperm cell prior to fertilization to lower the burden of 8OHdG repair on the oocyte.

MATERIALS AND METHODS

Chemicals and Materials

All chemicals were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise stated. Anti-XRCC1 antibody was purchased from Epitomics (Littleton, CO, USA) (catalog number 3631-1), while antibodies against OGG1, APE1, phosphorylated XRCC1 (phospho S518, T519 and T523) and PMP70 were purchased from Abcam (Cambridge, UK) (ab91421, ab194, ab84417 and ab3421 respectively). Anti-phospho-serine (P5747) and –phosphor-threonine (P6623) antibodies were both from Sigma Aldrich.

Oocyte collection

Three to four week old C57BL6/CBA F1 female mice were subjected to a superovulation regime and oocytes were collected and denuded as described previously (Lord et al., 2013). The use of animals in this project was approved by the University of Newcastle Animal Care and Ethics Committee, and all animals were obtained from breeding programs run in the University of Newcastle Central Animal House.

In Vitro Fertilization (IVF)

IVF was performed as described previously (Lord et al., 2013) using spermatozoa from 8 week old Swiss mice, or from the OGG1-deficient senescence-accelerated mouse prone 8 (SAMP8) (Choi et al., 1999) or senescence-resistant control strain of mouse, SAMR1. At 4 h post-insemination successful fertilization was determined via identification of the second polar body and/or formation of pronuclei.

Reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR) of OGG1, APE1 and XRCC1

RNA extraction and reverse transcription were conducted as described previously (Sobinoff et al., 2013). Briefly, 2 µg of total RNA was extracted from a pooled population of oocytes, DNase treated to remove genomic DNA, and reverse transcribed with oligo(dT) primer (Promega, Madison, WI, USA) and M-MLV Reverse Transcriptase (Promega). Reverse transcription was verified by RT-PCR using cDNA
amplified with GoTaq Flexi (Promega). All primers utilized have been provided in a supplementary data Table (Supplementary Table S1). These primers were screened for specificity via a nucleotide BLAST search (NCBI) prior to use. Quantitative PCR was conducted using SYBR Green GoTaq qPCR master mix (Promega) over 40 amplification cycles on a LightCycler 96 SW 1.0 (Roche Diagnostics, Mannheim, Germany). LightCycler Analysis Software (Roche) was used to quantify SYBR Green fluorescence after each amplification cycle. The optimal annealing temperature for OGG1, APE1 and XRCC1 was 57°C, as determined via a calibration curve, with all primer efficiencies lying between 1.8 and 2. Quantification of transcript abundance within oocytes was calculated relative to two housekeeping control genes, beta-2 microglobin (B2M) and beta-glucuronidase (Gusβ), which display similar primer efficiencies at 57°C. A negative control in which reverse transcriptase had been omitted was also performed for each qPCR replicate.

**Immunocytochemistry**

Oocytes/zygotes were washed 4 × in PBS containing 3 mg/ml polyvinylpyrrolidone (PVP) prior to fixation in 3.7% paraformaldehyde for 1 h at room temperature (RT). Following fixation, oocytes were again washed in PBS/PVP before permeabilization in 0.25% Triton-X for 10 min at RT. Oocytes were then washed in PBS/PVP and blocked in 3% BSA/PBS for 1 h at 37 °C. Following this blocking step, oocytes were incubated in the desired primary antibody (all used at a concentration of 1/100 in 1% BSA/PBS) overnight at 4 °C before washing with 1% BSA/PBS and incubating in anti-rabbit Alexa Fluor 488 (1/1000) for 1 h at 37 °C. Immunofluorescence was observed using confocal microscopy (Olympus FV1000 confocal microscope; Notting Hill, VIC, Australia). Quantification of levels of fluorescence within oocytes was achieved using the public sector program ImageJ (US National Institutes of Health).

Immunocytochemistry was carried out on spermatozoa as described by Smith et al. (2013b), using hydrogen peroxide (H2O2) and dithiothreitol to de-condense the highly compacted chromatin, and using primary antibodies within the same conditions described for oocytes.

**SDS-PAGE and Western Blotting**
Protein extraction was achieved by adding sodium dodecyl sulphate (SDS) extraction buffer (2% w/v SDS, 10% w/v sucrose in 0.1875 M Tris, pH 6.8) to cells and incubating at 100°C for 5 min. The samples were then centrifuged (500 g for 5 min) and the supernatant stored at -20°C prior to use. Extracted proteins were loaded onto an SDS polyacrylamide gel (10 µg sperm protein per lane, or protein lysate from 100 oocytes per lane) and separated via electrophoresis. 1 µg of recombinant OGG1 (rOGG1), recombinant APE1 (rAPE1) (both from New England Biolabs, Ipswich, MA, USA), and Jurkat whole cell lysate (Novus Biologicals, Littleton, CO, USA) was loaded onto the polyacrylamide gels as a positive control for OGG1, APE1 and XRCC1 antibodies respectively. Following electrophoresis, proteins were transferred to a nitrocellulose membrane using standard Western transfer techniques. The nitrocellulose membrane was blocked with 5% skim milk for 1 h and washed 3 x in TBS containing 0.1% Tween (TBST). Blots were then incubated overnight at 4°C with the appropriate primary antibody diluted in 1% skim milk in TBST. Primary antibodies were used at the following concentrations; OGG1 - 1/500, APE1 - 1/2000, XRCC1 - 1/5000. Blots were washed free from primary antibody in TBST, and incubated in goat anti-rabbit IgG-HRP (Sigma Aldrich) secondary antibody (1/1000 in 1% skim milk/TBST) for 1 h at RT. Membranes were developed using an enhanced chemiluminescence kit (GE Healthcare, Castle Hill, Australia) according to the manufacturer’s instructions.

Detection of extracellular 8OHdG – ELISA assay

A DNA oxidative damage ELISA kit (Cayman chemical, Ann Arbor, MI, USA) that is capable of detecting 8OHdG released into the extracellular space following excision by the cell’s DNA repair machinery was utilized to assess levels of 8OHdG repair in the oocyte pre- and post-fertilization. Following the retrieval and denuding of oocytes, half were subjected to IVF, while half of the oocytes remained unfertilized. Following a 4 h incubation, oocytes were treated with 1 mM H₂O₂ for 1 h to induce 8OHdG formation. Upon removal of oocytes/zygotes from their respective media and assessment of fertilization rate, the culture media was collected and stored at -80°C until required. The ELISA assay was carried out as per the manufacturer’s instructions, and absorbance was read using a Fluostar Optima spectrophotometer (BMG LabTechnologies, Durham, NC, USA). Values of 8OHdG release into the extracellular space are reported as pg/ml, normalized to the number of oocytes in each droplet. Fertilization rate was factored into calculations when formulating a per-oocyte value in
‘fertilized’ treatments, and replicates were only included where fertilization rate was over 80%.

Detection of intracellular 8OHdG in the oocyte and zygote and vitality assessment

In order to assess levels of intracellular 8OHdG in oocytes and zygotes, an OxyDNA test was conducted (Argutus Medical, Dublin, Ireland). Following the 4h incubation to allow for fertilization and the 1 h incubation in 1 mM H₂O₂, cells were either subjected to fixation for the OxyDNA test or cultured for an additional 3 h ‘recovery’ period in M2 medium before being assessed for vitality using 50 µg/ml propidium iodide.

Fixation for the OxyDNA test was carried out in 3.7% paraformaldehyde overnight at 4°C. The following day, oocytes and zygotes were permeabilized in 0.25% Triton-X in PBS for 10 min at RT. Oocytes/zygotes were then incubated for 1 h at 37°C in OxyDNA reagent (1:100 dilution; as per the manufacturer’s instructions). Finally, cells were counterstained for 5 min with propidium iodide before mounting for analysis by fluorescence microscopy. Pixel intensity values reflecting 8OHdG levels in oocytes and zygotes were generated using ImageJ in order to create a comparison between treatments.

Pharmacological inhibitor studies

Inhibitors of both the BER pathway [“BER inhibitor” (Merck, Darmstadt, Germany], and NER pathway [X80 (Sigma Aldrich)] were utilized at doses which have been previously recognized to be effective. Oocytes were exposed to either 10 µM of the BER inhibitor (Madhusudan et al., 2005), 100 µM X80 (Neher et al., 2010), or the equivalent concentration of DMSO, throughout the 4 h incubation which allowed for fertilization, as well as throughout the 1 h H₂O₂ treatment. Following this culture time, culture media was collected for analysis via the 8OHdG ELISA assay and zygotes were fixed and subjected to the OxyDNA test, followed by pixel intensity analysis using ImageJ.

Inhibition of CK2 in order to impair phosphorylation of XRCC1 (Parsons et al., 2010) was also conducted as described above. Oocytes were exposed to 4,5,6,7-Tetrabromo-2-azabenzimidazole (TBB) throughout the culture period initially in a dose–dependent manner (0 – 1000 µM) and finally at an effective but non-lethal concentration of 100 µM. Following incubation, culture media were again collected for
ELISA analysis and oocytes were subjected to immunocytochemistry (see above) using the phospho-XRCC1 antibody.

Finally, 20 μg/ml cycloheximide was used as an inhibitor of translation/protein synthesis (Schneider-Poetsch et al., 2010) in the manner described above in order to further elucidate the mechanisms of increased 8O HdG excision post-fertilization.

**Duolink – Proximity ligation assay (PLA)**

A Duolink in situ fluorescence PLA (Sigma Aldrich) was used to identify phosphorylation of the BER enzymes at specific residues in the absence of commercially available phospho-antibodies. Oocytes and zygotes were fixed and permeabilized as detailed above (see Immunocytochemistry) before conducting the Duolink PLA preparation, detection, and analysis procedures as per the manufacturer’s instructions. In the circumstance that two antibodies, such as those against OGG1 and phosphoserine, localize to the same target within the oocyte (<30 nm distance), a punctate region of red fluorescence is emitted. In order to confirm that fluorescence detected within these experiments was not a result of non-specific binding or background signals, a series of technical and biological controls were carried out. These included the omission of one antibody, and conducting the assay between our antibodies of interest and an unrelated antibody with which they are not expected to interact, in this case, α-tubulin.

**Assessing levels of ROS/antioxidant activity within the MII stage oocyte and zygote**

In order to assess levels of ROS, and to make inference as to the antioxidant capacities of oocytes and zygotes following H2O2 treatment, a 5’-carboxy-2’,7’-difluorodihydrofluorescein diacetate (DFF DA probe) (Molecular Probes, Eugene, OR, USA) was utilized. Untreated and 1 mM H2O2 treated MII stage oocytes and zygotes were incubated in a 10 μM solution of DFF DA in M2 media for 30 min at 37°C. Oocytes were then washed 3 × before mounting for analysis by fluorescence microscopy using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging Inc, Sydney, Australia). Quantification of average fluorescence levels between treatments was conducted using ImageJ pixel intensity analysis.

An antibody against the peroxisomal membrane marker PMP70 was used to identify any alterations to peroxisomal abundance – and thus catalase activity – within the oocyte pre- and post-fertilization (see Immunocytochemistry). Additionally, a
glutathione peroxidase (GPx) assay kit (Cayman chemical) was used to assess GPx activity in populations of MII oocytes and zygotes (50 oocytes in each population). Lysates were prepared as per the manufacturer’s instructions and absorbance was read using a Fluostar Optima spectrophotometer.

**Statistical analyses**

All experiments were conducted at least 3 × on independent samples and results were analysed by ANOVA using JMP version 9.0.0. A post hoc comparison of group means was conducted using a Fisher’s Protected Least Significant Difference test. Analysis of paired samples was conducted using a paired Student’s t-test. A value of $P < 0.05$ was considered to be statistically significant.

**RESULTS**

*OGG1 is under-represented in the murine oocyte, potentially affecting functionality of the BER pathway*

In order to investigate the functionality of the BER enzymes in the mammalian oocyte and zygote, qualitative and quantitative analyses were conducted on the key enzymes within this pathway: OGG1, APE1 and XRCC1. As reported by microarray analyses conducted on MII stage oocytes from the rhesus monkey (Zheng et al., 2005), our study has demonstrated an under-representation at the transcript level of the first enzyme in the BER pathway (OGG1) within C57BL6/CBA mouse oocytes. Quantitative PCR analysis revealed low levels of both OGG1 and APE1 mRNA within MII stage oocytes, relative to the selected housekeeping genes B2M and Gus $\beta$, while XRCC1 was expressed at significantly higher levels ($P < 0.05$) (Fig. 1A). Importantly, analysis at the protein level revealed a similar trend in terms of OGG1 expression. Western blotting techniques demonstrated the presence of both APE1 and XRCC1 within oocyte lysates (100 oocytes per lane, approximately 1 µg protein) at 35 kDa and 85 kDa respectively; whilst no distinct band associated with OGG1 expression could be detected at 37 kDa at this protein concentration (Fig. 1B). Expression of each BER enzyme in oocyte lysates was compared to a positive control (1 µg): recombinant OGG1 (rOGG1), recombinant APE1 (rAPE1) or jurkat cell lysate for the XRCC1 antibody. In a similar fashion to our Western blotting analyses, immunocytochemistry revealed high levels of fluorescence associated with APE1 and XRCC1 dispersed throughout the ooplasm, while the fluorescence associated with OGG1 was low (Fig. 1C). Despite this,
Figure 1: OGG1 is under-represented in MII-stage mouse oocytes. (A) Quantitative PCR analysis revealed a low abundance of OGG1 and APE1 mRNA within the murine oocyte (relative to B2M and Gusβ), whilst XRCC1 transcripts were present at significantly higher levels. (B) Immunoblotting analysis of the protein composition of these oocytes identified bands corresponding to APE1 and XRCC1 at 35 kDa and 85 kDa respectively; whilst a 37 kDa band corresponding to OGG1 could not be detected at this concentration of protein (100 oocytes per lane, equivalent to ~1 µg protein). Positive controls; rOGG1, rAPE1 and Jurkat cell lysate respectively (~1 µg). (C, D) In support of Western blotting data, immunocytochemistry identified fluorescence associated with APE1 and XRCC1 throughout the ooplasm. Fluorescence relating to OGG1 was low, however upon pixel intensity analysis was still found to be significantly greater than the ‘secondary only’ control (“C”). Scale bar = 50 µm. Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 40 oocytes per replicate. *P < 0.05, **P < 0.01, ***P < 0.001.

pixel intensity analysis revealed that levels of fluorescence associated with the OGG1 antibody were significantly elevated above that of the secondary only control (P < 0.001) (Fig. 1D), suggesting that this enzyme is still present within the oocyte, albeit at low levels. It should be noted that in addition to OGG1 being under-represented in comparison to APE1 and XRCC1 within the mammalian oocyte itself, a comparison of
oocyte protein lysates to equivalent concentrations of protein from other tissue types (spleen and whole ovary protein lysates) revealed that OGG1 concentrations are up to 50% lower in oocyte lysates than that of the alternative tissue types observed (Supplementary Figure 1).

Following this investigation of the BER enzymes, an analysis of the oocyte’s capacity for 8OHdG repair was conducted. Despite the under-representation of OGG1 within MII stage oocytes, ELISA analysis did detect the release of 8OHdG into the extracellular space (oocyte culture media), suggesting that some capacity for OGG1-mediated 8OHdG excision does exist (Fig. 2A). Interestingly however, one-cell stage zygotes exhibited a superior capacity for 8OHdG excision compared with unfertilized MII stage oocytes; excising and releasing significantly higher levels of 8OHdG into the extracellular space (P < 0.001) following a 5 h incubation period that allowed for in vitro fertilization and included terminal exposure to 1 mM H2O2 for 1 h. (Fig. 2A). This highly significant increase in 8OHdG release was not an artefact created by the presence of spermatozoa within the ‘fertilized’ oocyte culture medium, as levels of 8OHdG release into the extracellular space within the ‘sperm only’ control were significantly lower than the increase observed between the MII and zygotic stages (Fig. 2A). To complement these ELISA data describing 8OHdG levels in the extracellular space, analysis of intracellular 8OHdG levels using an oxidative DNA damage assay revealed distinct variations between pre- and post- fertilization oocytes. The OxyDNA assay revealed significantly lower levels of 8OHdG within H2O2 treated zygotes compared with MII stage oocytes (P < 0.05) (Fig. 2B, C), with 8OHdG being identified within both the nuclear DNA (metaphase plate) (Fig. 2D) and the mitochondrial DNA (Fig. 2E); as demonstrated by co-localisation between the 8OHdG probe and the nuclear stain DAPI for the former, and Mitotracker red for the latter.

These data suggested that while repair of oxidative DNA damage is minimal in the MII oocyte due to low levels of OGG1, fertilization is associated with an accelerated rate of 8OHdG excision and extracellular release, suggesting upregulation of the BER pathway. Additionally, these data could also reflect a change in antioxidant activity within the oocyte post-fertilization.
Figure 2: Repair of oxidative DNA damage is accelerated within the oocyte post-fertilization. (A) Following insemination and a 1 h exposure to H$_2$O$_2$, fertilized oocytes exhibited significantly higher levels of 8OHdG excision and release into the extracellular space than unfertilized oocytes. (B, C) Additionally, MII oocytes experienced a prominent increase in levels of intracellular 8OHdG (indicated by FITC staining in representative images, red counterstain - propidium iodide) following H$_2$O$_2$ treatment, whilst fertilized oocytes exhibited little change in levels of oxidative DNA damage. Upon analysis of pixel intensity values, MII oocytes were found to contain significantly elevated levels of intracellular 8OHdG above fertilized oocytes following H$_2$O$_2$ treatment. (D) Representative image of 8OHdG fluorescence co-localizing with the metaphase plate (DAPI) following H$_2$O$_2$ treatment. (E) Fluorescence associated with 8OHdG was also found to co-localize with the mitochondrial DNA, as depicted by the Mitotracker red fluorescent probe. Scale bar = 50 µm. Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 40 oocytes per replicate. *$P < 0.05$, ***$P < 0.001$.

**Fertilization-associated increases in 8OHdG excision are orchestrated by the BER pathway, however unlikely to be reliant on sperm-derived OGG1**

The investigation next focused on the characterization of mechanisms orchestrating the acceleration of 8OHdG repair in the zygote prior to the initiation of S-phase. Firstly, the observed elevation in 8OHdG excision was confirmed to be a result of BER enzyme activity. Impairment of the BER pathway was achieved via utilization
of a commercially available pharmacological inhibitor (‘BER inhibitor’). Exposure to the BER inhibitor (10 μM) throughout the 5 h incubation period prevented the fertilization-associated elevation in 8OHdG excision \((P < 0.05)\) (Fig. 3A). Additionally, the OxyDNA assay also revealed an elevation in levels of intracellular 8OHdG when oocytes were treated with the BER inhibitor in conjunction with \(\mathrm{H}_2\mathrm{O}_2\) \((P < 0.01)\) (Fig. 3B). Contrastingly, oocytes which were exposed to an inhibitor of the NER pathway, X80 (Neher et al., 2010), continued to exhibit an acceleration of 8OHdG excision post-fertilization (Fig. 3C) and experienced no change from control oocytes in intracellular 8OHdG levels in response to \(\mathrm{H}_2\mathrm{O}_2\) treatment (Fig. 3D). It should be noted that both the inhibitors utilized were observed to have no demonstrable effect on fertilization rate or viability of oocytes.

Given the apparent acceleration of BER observed in the early zygote, we next endeavoured to determine whether this upregulation was a consequence of the OGG1 contributed to the zygote by the fertilizing spermatozoon. Firstly, immunocytochemistry/immunoblotting techniques were utilized to verify that, as in human spermatozoa, murine spermatozoa possessed a truncated BER pathway characterized by the presence of the OGG1 enzyme but lacking APE1 and XRCC1. As predicted, immunocytochemistry identified the presence of OGG1 within both the nucleus (sperm head) and midpiece (mitochondria), whilst APE1 was identified only within the mitochondria, and XRCC1 primarily within the mitochondria with some punctate staining within sperm nuclei (Fig. 4A). Similarly, both OGG1 and XRCC1 were identified by Western blotting analysis in sperm protein lysates; however APE1 could not be detected (Fig. 4B) as a consequence of very low levels of expression, solely in association with the mitochondria.

In order to determine whether the OGG1 present in sperm chromatin is responsible for the increased 8OHdG excision observed, we utilized spermatozoa from the SAMP8 mouse which possesses severely defective OGG1 \([10-40\% \text{ of the activity exhibited by the senescence-resistant (SAMR1) control mouse}]\) as a consequence of an arginine to tryptophan mutation at codon 304 (Choi et al., 1999). Importantly, this impairment in OGG1 enzyme activity has been confirmed to exist in the spermatozoa of SAMP8 mice (Smith et al., 2013a). Despite this, fertilization of wild type oocytes with these OGG1-deficient SAMP8 spermatozoa did not influence the accelerated 8OHdG excision associated with fertilization, as detected by the ELISA assay (Fig. 4C). Similarly, levels of intracellular 8OHdG were not observed to be significantly elevated
Figure 3: The BER enzymes are responsible for the accelerated excision of 8OHdG post-fertilization. (A) Pharmacological inhibition of the BER pathway using a commercially available compound ‘BER inhibitor’ dampened the accelerated 8OHdG excision associated with fertilization, as determined by the ELISA assay. (B) Similarly, exposure to the BER inhibitor in conjunction with H₂O₂ resulted in a significant increase in levels of intracellular 8OHdG in zygotes. (C) In contrast, inhibition of the NER pathway using X80 did not affect the fertilization-associated up-regulation of 8OHdG excision, nor levels of intracellular 8OHdG (D). Intracellular 8OHdG values are normalized to the untreated control. Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 60 oocytes per replicate. *P < 0.05, **P < 0.01.

in zygotes derived from SAMP8 sperm (Fig. 4D), and developmental progression to 2-cell stage was not hindered (Fig. 4E). Although it is possible that very low levels of functional OGG1 contributed to the oocyte by the fertilizing spermatozoon (such as those contributed by SAMP8 sperm) are sufficient to drive the observed rise in 8OHdG repair, with wild type sperm providing an ‘excess’ of protein that is not necessarily
required, it is more plausible that maternally-derived factors are orchestrating this acceleration of BER in response to fertilization-associated cues.

Figure 4: OGG1 is present in mouse spermatozoa however is unlikely to be responsible for the acceleration of 8O HdG excision associated with fertilization. (A) Immunocytochemistry revealed OGG1 within the nucleus of the mouse spermatozoon, as previously reported in human sperm. APE1 could only be identified in the midpiece (mitochondria) of the sperm cell, whilst XRCC1 was identified within the midpiece, with some punctate staining within the nucleus. (B) Western blot analyses using sperm protein lysates (swiss, SAMR1 and SAMP8 mice) revealed that whilst OGG1 and XRCC1 are present, the pathway is truncated due to the absence of the endonuclease enzyme APE1. (C) Pronuclear stage zygotes produced via fertilization of wild type oocytes with sperm from either SAMR1 (control) mice, or OGG1-deficient SAMP8 mice, did not exhibit any changes in 8O HdG excision, suggesting that sperm-derived OGG1 is unlikely to orchestrate the burst of 8O HdG repair observed post-fertilization. (D) Similarly, zygotes derived from SAMP8 sperm did not exhibit elevated levels of intracellular 8O HdG above SAMR1-derived zygotes following H2O2 treatment. (E) Additionally, wild type oocytes fertilized with OGG1-deficient SAMP8 sperm did not display any impairment in developmental progression to the 2-cell stage. Scale bar = 20 µM. Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 50 oocytes per replicate.
Post-translational modifications instigate increased BER activity post-fertilization

Following the discovery that a significant contribution of sperm-derived OGG1 to 8OHdG excision post-fertilization is unlikely, further investigation was carried out to characterize the events driving accelerated 8OHdG repair in the zygote. Although increased transcription of OGG1, APE1 or XRCC1 genes post-fertilization would explain the observed elevation in 8OHdG repair, no changes in levels of mRNA or protein relating to any of the BER enzymes could be detected between the MII and zygotic stages (Fig. 5A, B). Further confirmation that the post-fertilization increase in 8OHdG excision was not a consequence of changes in the expression of DNA repair proteins was achieved via exposure to the translation/protein synthesis inhibitor cycloheximide (20 µg/ml) throughout the 5 h incubation. Inhibition of translation throughout this period had no demonstrable effect on levels of 8OHdG excision by the zygote, as detected by the ELISA assay (Fig. 5C).

In light of these data, we next examined the possibility that post-translational modifications, known to strengthen the association between key BER enzymes and accelerate rates of 8OHdG excision (Hu et al., 2005; Almeida and Sobol, 2007), may be controlling this post-fertilization event. This analysis revealed distinct global changes in protein phosphorylation between the MII stage oocyte and the zygote as depicted in Fig. 6. An abundance of phosphorylated cytoplasmic proteins could be identified throughout the ooplasm of both the MII oocyte and zygote by both anti-phospho-serine (Fig. 6A) and anti-phospho-threonine (Fig. 6B) antibodies, with threonine phosphorylation levels being significantly elevated in the zygote ($P < 0.001$) (Fig. 6C). Significantly, these cytoplasmic signals were augmented by a prominent nuclear phosphoprotein signal associated with male and female pronuclei within the zygote (Fig. 6).

This fertilization-triggered association of phosphorylated proteins with the pronuclei was found to extend specifically to enzymes involved in the BER pathway. A Duolink in situ fluorescence proximity ligation assay (PLA) depicted localization of OGG1 phosphorylated at serine and threonine residues to the nuclei of the zygote; while no distinct association with the metaphase plate could be identified in MII oocytes (Fig. 7A, B). Control images have been included that depict low levels of punctate red fluorescence where one antibody has been omitted, and where an antibody not predicted to interact with the antibodies-of-interest (in this case α-tubulin) has been utilized (Fig. 7). In adopting an antibody targeting phospho-XRCC1 (S518, T519, T523) we also
Figure 5: Accelerated 8OHdG repair post-fertilization is not attributed to up-regulated expression of the BER enzymes. (A) Quantitative PCR analysis revealed no significant elevation in OGG1, APE1 or XRCC1 transcript levels (relative to B2M and Gusβ) following fertilization. (B) Similarly, no visible changes in fluorescence could be detected via immunocytochemistry when comparing OGG1, APE1 and XRCC1 in MII stage oocytes and zygotes. (C) Exposure to the translation/protein synthesis inhibitor cycloheximide (CH) throughout fertilization did not inhibit the fertilization-associated elevation in 8OHdG excision, thereby; increased expression of the BER enzymes could not be responsible for the acceleration of 8OHdG excision observed following fertilization. Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 60 oocytes per replicate. Blue staining = DAPI. Scale bar = 50 µm.

identified a uniform cytoplasmic distribution of fluorescence prior to fertilization that became localized to the pronuclei (Fig. 7C) following fertilization. In contrast, no phopho-XRCC1 was found in the vicinity of the metaphase plate in unfertilized MII oocytes (Fig. 7C).

In order to determine the mechanism responsible for relocating these BER enzymes to the pronuclei of freshly fertilized oocytes, the latter were exposed to TBB, an inhibitor known to impair casein kinase 2 (CK2) -directed phosphorylation of XRCC1 (Kubota et al., 2009). Zygotes exposed to TBB experienced a dose-dependent loss of vitality (Fig. 7D); presumably an indication of the vital role that CK2 plays at this stage of development. A dose of 100 µM TBB was finally selected for experiments on CK2 inhibition, as no significant effects on either vitality or fertilization rate were detected at this concentration (Fig. 7D). As hypothesized, zygotes exposed to 100 µM TBB throughout the 5 h incubation period experienced diminished nuclear localization of phospho-XRCC1 (Fig. 7C). Importantly, this decline in phosphorylation of
Fertilization is associated with a shift in the post-translational modification profile of the oocyte. (A) Immunocytochemistry using a phospho-serine antibody revealed a change in expression from the MII stage to the zygote. MII stage oocytes exhibited phospho-serine fluorescence throughout the ooplasm and localized to the plasma membrane, while fluorescence within the zygote was prominently associated with the pronuclei. (B) Similarly, fluorescence associated with the phospho-threonine antibody showed distinct localization to the pronuclei following fertilization, whilst fluorescence was uniform throughout the cytoplasm in unfertilized MII stage oocytes. (C) Pixel intensity analysis revealed a significant increase in levels of global phospho-threonine expression in zygotes, however no significant difference between levels of phospho-serine expression was detected. Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 60 oocytes per replicate. Scale bar = 50 µm. ***P < 0.001.

pronuclei-associated XRCC1 was accompanied by a significant reduction in levels 8OHdG excision by the zygote (P < 0.05) (Fig. 7E). These results suggested that the elevated levels of 8OHdG excision detected post-fertilization are primarily a result of post-translational modifications to the BER enzymes associated with the nucleus. However, these results do not account for the significantly lower levels of mitochondrial 8OHdG observed in the zygote (when compared to the MII stage oocyte) following H₂O₂ treatment (Fig. 2B, C, E). Thus, we investigated the possibility that an increase in antioxidant protection may accompany fertilization in addition to the pronuclear localization of enzymes involved in the BER pathway.
Figure 7: Post-translational modification of the BER enzymes may co-ordinate increased 8OHdG excision from the nucleus post-fertilization. (A) A proximity ligation assay (PLA) inferred that phosphorylation of OGG1 at serine residues occurs within the genetic material following fertilization, as demonstrated by co-localization between the red punctate fluorescent signal and the DAPI-stained pronuclei of the zygote. Contrastingly, no distinct association could be detected between phosphorylated OGG1 and the metaphase plate in unfertilized oocytes. (B) Similarly, fertilization stimulated phosphorylation of OGG1 at threonine residues within the pronuclei, while no association between phosphorylated OGG1 and the genetic material was detected in MII stage oocytes. Control images depict a lack of red punctate fluorescence emanated by the Duolink assay when one antibody was omitted, or when an unrelated antibody was utilized (α-tubulin) in conjunction with our antibody of interest (OGG1). (C) Phosphorylated XRCC1 (S518, T519 and T523) also exhibited cytoplasmic distribution within MII oocytes, while fertilized oocytes showed prominent localization of P-XRCC1 to the pronuclei. (D) In order to establish the effects of impaired phosphorylation and nuclear localization of XRCC1, a CK2 inhibitor (TBB) was utilized. A dose-dependent study revealed that concentrations of TBB below 200
µM were non-lethal to oocytes/zygotes over a 5 h incubation, and at a concentration of 100 µM did not impair fertilization rate. (E) Exposure to 100 µM TBB throughout fertilization did however result in reduced nuclear localisation of P-XRCC1 in zygotes (C), in conjunction with a significant decline in levels of 8OHdG excision. Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 60 oocytes per replicate. Scale bar = 50 µm. *P < 0.05.

Post-fertilization changes improve antioxidant capacity to protect the zygote from oxidative damage

In order to assess cellular antioxidant capacity, MII stage and fertilized oocytes were exposed to 1 mM H₂O₂ for 1 h before a further 30 min incubation with DFF DA; a live-cell assay that omits fluorescence upon interaction with intracellular oxidants. Interestingly, fertilized oocytes exhibited significantly lower levels of DFF DA fluorescence than MII stage oocytes (P < 0.01), suggesting a more rapid breakdown of H₂O₂ (Fig. 8A). An increased capacity for neutralization of harmful oxidants post-fertilization would provide explanation as to the previously described decrease in susceptibility of zygotes to H₂O₂-induced mitochondrial oxidative DNA damage (Fig. 2B, C, E) as well as the reduced likelihood for vitality loss after a 3 h recovery period following H₂O₂ treatment (P < 0.001) (Fig. 8B). An analysis of peroxisome abundance using an antibody against peroxisomal membrane protein (PMP70) did not reveal any significant changes within the oocyte following fertilization, suggesting that these catalase-rich organelles were not responsible for the increased H₂O₂ scavenging potential of the fertilized oocyte (Fig 8C). Contrastingly, antioxidant activity of the enzyme glutathione peroxidase (GPx) was found to be significantly upregulated post-fertilization, with populations of zygotes exhibiting an average rate of activity of 14.7 (± 3.9) nmol/min/ml compared to only 7.4 (± 2.6) nmol/min/ml in equivalent populations of MII oocytes (P < 0.01) (Fig. 8D).

The increase in GPx activity associated with fertilization also prevented the accumulation of cytoplasmic ROS that normally occurs in vitro with increasing periods of time from ovulation (8 h) (P < 0.01) (Fig. 8E) and acts as a trigger for oocyte degeneration and apoptosis (Lord et al., 2013; Lord et al., 2015).

DISCUSSION

Repair of oxidative DNA lesions such as the highly mutagenic 8OHdG adduct prior to initiating S-phase of the first mitotic division in the zygote is crucial for the preservation of embryonic integrity and the production of healthy offspring.
Figure 8: Fertilization stimulates GPx activity within the oocyte. (A) Populations of MII stage oocytes and zygotes were subjected to a 1 h H₂O₂ challenge, followed by a 30 min recovery in the presence of the fluorescent probe DFF DA. Fertilized oocytes exhibited significantly lower levels of fluorescence when compared to MII stage oocytes, suggesting a more rapid breakdown of H₂O₂. (B) Zygotes were also found to be less susceptible to oxidative stress-induced vitality loss; exhibiting significantly elevated levels of viability above populations of MII stage oocytes following 1 mM H₂O₂ treatment and 3 h recovery time. (C) The improved capacity for H₂O₂ breakdown in zygotes was not associated with increased abundance of catalase-rich peroxisomes, as no change in peroxisome levels were detected following fertilization using an anti-PMP70 antibody. (D) Contrastingly, activity of the antioxidant enzyme GPx was doubled in populations of zygotes when compared to equivalent populations of MII stage oocytes, as determined by a GPx ELISA assay. (E) The characterised up-regulation of GPx activity was also found to prevent the accumulation of ROS within the ooplasm known to be associated with extended periods of in vitro culture (8 h). Mean ±SEM values are plotted in histograms. Independent replicates were conducted with a minimum of 50 oocytes per replicate. **P < 0.01, ***P < 0.001. Scale bar = 50 µm.
Unfortunately, 8OHdG can be abundant within spermatozoa, particularly within sub-fertile populations, elevating the likelihood that damaged DNA will be contributed to the zygote post-fertilization (Aitken et al., 2010). Further to this, the zygote has been demonstrated to possess a restricted capacity for 8OHdG repair, allowing progression through S-phase in the presence of unrepaired oxidative DNA damage (Ronen and Glickman, 2001; Vinson and Hales, 2002; Aitken et al., 2009; Chabory et al., 2009; Takahashi, 2012; Lane et al., 2014). The current manuscript has characterized BER in the mouse pre- S-phase zygote, revealing a low abundance of the first enzyme in the BER pathway, OGG1, thereby explaining the limited capacity for 8OHdG repair at this early developmental stage. Despite the under-representation of OGG1, we have demonstrated BER activity within the oocyte/zygote, the efficiency of which has been found to increase in response to fertilization. Specifically, post-translational modifications of the BER enzymes post-fertilization appear to increase association between these enzymes and the male and female pronuclei, promoting 8OHdG excision from oxidized nuclear DNA. This elevation in 8OHdG repair, although limited, along with a previously undescribed increase in oxidant scavenging activity in fertilized oocytes, are likely to be important not only for minimising levels of mutagenesis in the resulting embryo, but also for redirecting female germ cells away from inevitable senescence and apoptotic death to a developmental pathway associated with cell survival and a commitment to embryogenesis.

The observation that OGG1 is poorly represented at both the transcript and protein level in C57/BL6 x CBA oocytes and zygotes was surprising, as the BER pathway is known to be the primary orchestrator of 8OHdG repair in eukaryotic cells [reviewed by David et al. (2007)]. Additionally, these DNA repair proteins are required within the oocyte throughout oogenesis to control levels of DNA damage, as embryonic gene expression is not initiated until the 2-4 cell stage of embryo development (Flach et al., 1982). Repair of oxidative lesions such as 8OHdG is particularly important at the early stages of zygote development prior to the initiation of mitosis in order to prevent irreversible changes in genetic integrity including transversion mutations (Wood et al., 1992; Bruner et al., 2000; Ohno et al., 2014). As such, the limited availability of this crucial enzyme, OGG1, is likely a contributor to the vulnerability of early mammalian embryos to oxidative DNA damage (Meseguer et al., 2007). Indeed, recent studies have suggested that oxidative DNA damage contributed to the zygote by the fertilizing spermatozoon can directly affect embryo development and quality (Chabory et al.,
2009), as well as increase the vulnerability of offspring to pathologies such as metabolic syndrome and obesity (Meseguer et al., 2007; Lane et al., 2014); presumably a consequence of these paternal 8OHdG lesions evading repair by the zygote. It is important to note that although enzymes within alternative DNA repair pathways, such as those of the global genomic NER pathway, are known to be present within the mammalian oocyte (Zheng et al., 2005; Menezo et al., 2007) and are also capable of 8OHdG excision, these enzymes perform long patch repair [up to 13 nucleotides (Melis et al., 2013)] that is normally reserved for 8OHdG lesions that are causative of structural distortions [reviewed by Brierley and Martin (2013)]; thus, these mechanisms are unlikely to significantly contribute to minimising oxidative DNA damage in the zygote. Furthermore, no changes in levels of 8OHdG excision could be identified in this study within the zygote upon inhibition of global genomic and transcription coupled NER using the xeroderma pigmentosum group A (XPA) inhibitor X80 (Neher et al., 2010).

Although there do appear to be limitations to the capacity of the BER enzymes to excise 8OHdG in the oocyte, the current study has characterised a series of mechanisms that are likely to be important for minimising oxidative DNA damage following the union of gametes, thus decreasing the potential for mutagenesis in the embryo. Significantly, within a short window following fertilization (< 5 h), the rate of 8OHdG excision by the BER enzymes was found to be dramatically accelerated. This elevation in BER activity was not related to changes in expression of any of these enzymes, in keeping with the fact that embryonic gene expression is not initiated until the 2-4 cell stage (Flach et al., 1982). Furthermore, although OGG1 was identified within mouse spermatozoa in this study, as has previously been described in human spermatozoa (Smith et al., 2013b), use of the OGG1-deficient SAMP8 mouse (Smith et al., 2013a) suggested that the sperm-derived enzyme was not significantly contributing to the elevated rate of 8OHdG excision following fertilization. Despite this, the high level of OGG1 expression within spermatozoa is clearly important for maintaining low levels of 8OHdG within the paternal chromatin prior to fertilization. As demonstrated by our expression studies, the oocyte/zygote has an abundance of APE1 and XRCC1 that would – following the union of gametes - allow for swift hydrolysis of abasic sites created by OGG1 in the sperm chromatin, followed by insertion of a new base by DNA polymerase [reviewed by David et al. (2007)].
The mechanism we propose for increasing BER activity following fertilization is via post-translational modification of the BER enzymes. Certainly, the post-translational modification of proteins has been previously shown to be stimulated or accelerated by post-fertilization changes within the murine oocyte allowing for the ‘reprogramming’ of this cell away from senescence and apoptosis towards embryogenesis in the absence of active gene expression and de novo protein translation (Howlett and Bolton, 1985). An abundance of post-translational modifications to the BER enzymes have been characterized previously; some of which increase BER activity, while others exert an inhibitory effect [reviewed by (Almeida and Sobol, 2007)]. For the purpose of this study, we have focused on phosphorylation of OGG1 and XRCC1. Phosphorylation of OGG1 by cyclin-dependent kinase 4 (Cdk4) at serine/threonine residues is known to accelerate 8OHdG excision twofold (Hu et al., 2005), whilst phosphorylation of XRCC1 at Ser518, Thre519, Thre523 by CK2 is known to instigate nuclear localisation (Parsons et al., 2010), as well as accelerated 8OHdG excision by way of increased interaction with OGG1 and APE1 (Vidal et al., 2001; Marsin et al., 2003; Parsons et al., 2010; Ström et al., 2011). Although no global increases in levels of OGG1 or XRCC1 phosphorylation were identified between the MII and zygotic stages, a distinct nuclear localisation of the signal could be detected following fertilization. This suggests that while BER within the chromatin of the MII stage oocyte is limited, fertilization-associated factors orchestrate phosphorylation of the BER enzymes associated with the nuclei, allowing for accelerated 8OHdG excision and repair prior to the initiation of S-phase and subsequent embryogenesis. By impairing phosphorylation and thus nuclear localisation of XRCC1 using the CK2 inhibitor TBB (Kubota et al., 2009), a significant decline in levels of 8OHdG excision in the zygote was observed (Fig. 7).

In addition to the fertilization-associated elevation in BER activity within the chromatin, this study has also identified an associated increase in antioxidant protection within the zygote. In contrast to unfertilized MII stage oocytes, zygotes demonstrated a superior capacity for H$_2$O$_2$ breakdown; suggesting an increase in catalase or GPx bioavailability (Pigeolet et al., 1990). Although no change in abundance of catalase-rich peroxisomes could be detected, an elevation in GPx activity was apparent following fertilization. This elevation in GPx activity was particularly beneficial for protecting the mitochondria from H$_2$O$_2$–induced oxidative DNA damage; a biological trait that is acutely important when considering that these mitochondria are to be distributed to every cell within the embryo, and consequently the offspring, and will be primarily
responsible for energy production via oxidative phosphorylation (Dumollard et al., 2007), at least until mitochondrial replication following the blastocyst stage of development (St. John et al., 2010). Further to this, the post-fertilization increase in GPx activity would provide protection to the nuclear DNA to prevent the acquisition of any further oxidative lesions prior to the initiation of embryogenesis.

The fertilization-associated mechanisms described in this manuscript are also likely to be a component of the ‘molecular switch’ that rescues the MII stage oocyte from otherwise inevitable entry into a post-ovulatory ageing/apoptotic cascade, redirecting this cell into a developmental pathway. In the absence of fertilization, the oocyte experiences an accumulation of ROS with increasing amounts of time post-ovulation (Lord et al., 2013; Lord et al., 2015). This oxidative stress and associated damage is known to be a direct trigger for degeneration and the initiation of age-associated apoptosis in the mammalian oocyte (Kujoth et al., 2006; Lord et al., 2013; Lord et al., 2015). Importantly, the current study has demonstrated that fertilized oocytes are resistant to this time-dependent accumulation of ROS, presumably as a consequence of upregulated GPx activity. By preventing the accumulation of ROS and associated oxidative damage, these fertilization-associated events effectively redirect the oocyte away from the default apoptotic pathway that inevitably occurs in the absence of fertilization, and allow for unimpeded embryo development.

In conclusion, the results depicted in this study suggest that a series of strategies are in place to minimise levels oxidative DNA damage in the mammalian zygote and thus the mutational load ultimately carried by the embryo. Firstly, expression of OGG1 in the spermatozoon will serve to minimise levels of 8OHdG contributed to the OGG1-deficient oocyte, instead presenting abasic sites that can readily be rectified by the abundance of APE1 and XRCC1 in the ooplasm. Additionally, in response to fertilization, the maternal BER machinery accelerates 8OHdG excision in the pronuclei as a consequence of post-translational modifications, while an increase in antioxidant activity protects the entire embryo from the acquisition of any further oxidative damage. Despite this, we propose that when OGG1 within the sperm cell is overwhelmed, for example, in the spermatozoa of sub-fertile men exhibiting high levels of 8OHdG (De Iuliis et al., 2009), then the oocyte has a limited capacity to excise these potentially mutagenic adducts following fertilization. When the burden of oxidative damage is too high in the OGG1-deficient oocyte, consequences can include arrest or disruption of the normal developmental process (Ronen and Glickman, 2001; Vinson and Hales, 2002;
Aitken et al., 2009; Chabory et al., 2009; Takahashi, 2012; Lane et al., 2014). In a clinical context, this study further highlights the concerns associated with using ART techniques such as ICSI on unscreened sub-fertile patients that potentially harbour high levels of oxidative DNA damage within their spermatozoa.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


Chapter 4: Supplementary Figures

Supplementary Table 1: Primer sequences used for qPCR.

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Supplementary Figure 1: A comparison of OGG1 levels between different tissue types. (A) Western blotting analysis detected a prominent band associated with OGG1 in protein lysates from spleen and ovary tissue, whilst expression within oocyte lysates was low. (B) Densitometry analysis (normalized to tubulin) confirmed this trend, with levels of OGG1 expression in oocyte lysates nearing 50% of that of spleen and ovary lysates. Mean ±SEM values are plotted in histograms.
CHAPTER 5

Final Discussion
Chapter 5: Overview

The following chapter provides an overview of the findings presented in this thesis in the context of previously published literature. This review recaps the importance of the development of techniques aimed to maximise oocyte quality in an assisted reproduction setting, particularly when considering the high cost, invasive nature, and low success rate of these increasingly popular procedures. As such, we discuss the now established role for reactive oxygen species and electrophilic aldehydes in the degeneration of oocyte quality that occurs within a matter of hours of in vitro culture, as well as the previously unbeknownst change in redox capacity that occurs following timely fertilization of the oocyte by the spermatozoon. In light of these findings, we propose that the adoption of antioxidant supplementation techniques - such as those proven to be effective throughout this thesis - in an assisted reproduction setting may improve the success rate of in vitro fertilization procedures when immediate utilization of oocytes is not possible, and may also increase the viability of re-insemination techniques such as rescue-ICSI.
Final discussion

5.1 Introduction

The use of assisted reproductive technologies (ART) has increased exponentially since the birth of the first ‘IVF baby’ in 1978, such that 1 in 25 Australian children are now a product of IVF or ICSI (Macaldowie et al., 2013). Although the utilization of these technologies by couples experiencing fertility problems is now widely accepted socially, the costs associated with treatment [approximately $4000 per initiated cycle, with an average of 1.9 cycles initiated per patient (Macaldowie et al., 2013)] result in a lack of accessibility for a significant portion of the population. In addition to the financial burden of ART treatments, ovarian stimulation and subsequent egg collection procedures can be unpleasant and invasive for patients. For these reasons, it is obviously preferential to minimise the number of initiated cycles to which patients are subjected. As less than 25% of initiated IVF cycles result in a clinical pregnancy (Macaldowie et al., 2013), research into optimising gamete and embryo quality in in vitro culture, as well methodologies associated with micromanipulation, are clearly paramount to improving IVF success rates.

Oocyte quality is a particularly defining factor for ART success, known to significantly affect outcome in terms of embryo development, pregnancy and production of healthy offspring. Following the union of the spermatozoon and oocyte at the time of fertilization, maternally derived factors play an indispensable role in orchestrating early embryo development [reviewed by Schultz and Heyner (1992)]. Prior to the initiation of embryonic gene expression – which does not occur until the 2-cell stage within the mouse (Flach et al., 1982), and the 4-cell stage in humans (Braude et al., 1988) – the zygote relies entirely on molecules accumulated throughout oogenesis
to conduct key developmental events such as demethylation (Hatanaka et al., 2013) and remodelling (Torres-Padilla et al., 2006) of both maternal and paternal genomes.

Unfortunately, oocyte quality experiences a relatively rapid decline following transition from the germinal vesicle stage to metaphase II arrest at the time of ovulation. As discussed throughout this thesis, increasing periods of time following ovulation are associated with degeneration of the MII oocyte as a result of post-ovulatory oocyte ageing [reviewed in Chapter 1, (Lord and Aitken, 2013)]. As a consequence of this degeneration, the oocyte becomes less amenable to fertilization (Marston and Chang, 1964; Ben-Rafael et al., 1986; Badenas et al., 1989; Chian et al., 1992; Kikuchi et al., 2000; Liu et al., 2009), produces poor quality embryos that are prone to erroneous development and implantation (Wilcox et al., 1998; Yanagida et al., 1998; Lord et al., 2013) and have an increased potential for producing offspring with abnormalities (Tarin et al., 1999).

In a clinical setting, it is standard procedure for oocytes to remain in culture media for upwards of 2 hours following retrieval, awaiting utilization for IVF or ICSI. However, in circumstances of increased work load; i.e. when the number of ART procedures booked in for a particular day are high; fertilization of these oocytes can be significantly delayed (Yanagida et al., 1998). Clearly, such situations exacerbate the likelihood that degeneration may occur at the hands of post-ovulatory ageing. Similarly, in livestock industry where oocytes require extended periods of *in vitro* maturation prior to IVF (upwards of 22 h for bovine oocytes), a delicate balance exists between ensuring sufficient nuclear maturation has occurred, and the instigation of *in vitro* ageing within the oocyte (Koyama et al., 2014).

As such, the development of reagents that could be added as supplements to oocyte culture/maturation medium in order to prevent degeneration would be highly beneficial. The adoption of such methodologies could also improve the chance of XXVI
success with re-insemination techniques such as next day ‘rescue ICSI’ on oocytes which have failed-to-fertilize by conventional IVF (Kuczyński et al., 2002), thereby minimising the likelihood that further ovulation cycles would need to be stimulated. In order to develop such techniques, in depth analyses of the molecular mechanisms controlling oocyte degeneration are required, as well as characterisation of the optimal window of time for fertilization of oocytes post-retrieval in an *in vitro* setting. Additionally, investigation into the molecular events that occur at the time of fertilization to redirect the oocyte away from impending post-ovulatory ageing and into a developmental pathway may also allow for the formulation of methodologies that adopt similar strategies to delay oocyte degeneration.

5.2 Molecular mechanisms – a key role for oxidative stress and electrophilic aldehydes in post-ovulatory ageing

At a basal level, ROS are important for cellular signalling. However, the post-ovulatory oocyte creates a ‘perfect storm’ in terms of vulnerability to oxidative imbalance. Firstly, *in vitro* culture conditions stimulate ROS production by way of increased oxygen tension (Mastroianni and Jones, 1965; Mass et al., 1976) and exposure to light (Goto et al., 1993). Furthermore, the abundance of mitochondria within the oocyte, its reliance on oxidative phosphorylation (Dumollard et al., 2007; Dumollard et al., 2008), as well as the decline in antioxidant protection by glutathione with increasing periods of time post-ovulation (Boerjan and de Boer, 1990; Yoshida et al., 1993) mean that superoxide anion, and, in particular, the product of its dismutation – hydrogen peroxide, accumulate within the oocyte with increasing amounts of time post-ovulation [Chapter 2, 3; (Takahashi et al., 2003; Tatone et al., 2011; Lord et al., 2013; Lord et al., 2014a)].
Although increases in levels of ROS with post-ovulatory age have been reported previously, a direct link has now between established between oxidative stress, lipid peroxidation and dysfunction of the oocyte, as well as entry into apoptosis [Chapter 2, 3 (Lord et al., 2013; Lord et al., 2014a)]. Research within this thesis has found that the accumulation of ROS post-ovulation initiates a self-perpetuating cycle of lipid peroxidation, electrophilic aldehyde production, and mitochondrial damage [Chapter 3, (Lord et al., 2014a)]. Specifically, the electrophilic aldehydes produced as a by-product of lipid peroxidation (particularly 4HNE) throughout \textit{in vitro} ageing adduct to a protein component of complex II of the ETC: succinate dehydrogenase. Covalent modification of proteins by electrophiles such as 4HNE can severely impair enzymatic function (Monroy et al., 2013). Certainly, this appears to be the case with succinate dehydrogenase, as exposure of healthy oocytes to elevated, but physiological levels, of 4HNE (as observed in \textit{in vitro} aged oocytes) stimulated electron leakage from the mitochondria (detected as superoxide anion production) and consequently, encouraged further lipid peroxidation and electrophilic aldehyde generation. Importantly, initiation of this damaging cycle with either 4HNE or hydrogen peroxide treatment culminated in a collapse of mitochondrial membrane potential and subsequent apoptosis characterised by phosphatidylserine externalisation, caspase activation and fragmentation [Chapter 2, 3 (Lord et al., 2013; Lord et al., 2014a)].

Further to identifying the mechanism by which the post-ovulatory oocyte enters into an intrinsic apoptotic cascade, work presented in this thesis has established direct links between the time-dependent accumulation of ROS and 4HNE within the oocyte and the functional deficiencies acquired during \textit{in vitro} ageing [Chapter 2 and 3, (Lord et al., 2013; Lord et al., 2014a)]. After extending \textit{in vitro} culture time of the oocyte by only 8 h, embryos produced by IVF were significantly less likely to reach the 2-cell, 4-cell and blastocyst stages, and exhibited elevated levels of apoptotic blastomeres when...
compared to embryos derived from ‘fresh’ oocytes (Lord et al., 2013). The decline in oocyte quality after 8 h culture was directly accompanied by a significant increase in levels of ROS generation. However, if such enhanced ROS generation was prevented via supplementation of the medium with an antioxidant agent, then the functionality of the oocyte was effectively preserved – allowing for the production of embryos that did not significantly differ in quality from those derived from fresh-oocyte controls [Chapter 2, (Lord et al., 2013)]. The link between ROS and 4HNE accumulation and the functional deficiencies observed within the aged oocyte - particularly the decreased capacity of the oocyte to support embryo development, may be attributed to a myriad of factors that have been discussed in detail in Chapter 1, 2 and 3 (Lord and Aitken, 2013; Lord et al., 2013; Lord et al., 2014a). Briefly, the elevation in ROS levels may impair the functionality of calcium channels (Rohn et al., 1993; Doan et al., 1994; Wesson and Elliot, 1995) and calcium signalling molecules (Gao et al., 2001) to induce the abnormal calcium oscillations observed following fertilization of aged oocytes that are known to impair progression to the 2-cell stage (Gordo et al., 2000; Gordo et al., 2002). Furthermore, oxidative damage to the mitochondria as well as alkylation of mitochondrial proteins by 4HNE may result in impaired metabolism within blastomeres. Additionally, accumulation of oxidative DNA damage (specifically 8OHdG) within the oocyte as a consequence of minimal DNA repair (Lord et al., 2014b) may result in alteration of embryonic gene expression (discussed in Chapter 4)

5.3 Molecular mechanisms – fertilization directs the oocyte away from ageing and apoptosis by upregulating antioxidant protection and DNA repair

As described previously, degeneration and apoptosis of the MII stage oocyte is inevitable in the absence of fertilization. However, in the event of timely fertilization, the spermatozoon effectively ‘rescues’ the oocyte from certain death, and redirects this
cell down a developmental pathway leading to embryogenesis. Until recently, the mechanisms controlling this post-fertilization ‘molecular switch’ that determines the developmental fate of the oocyte had remained unresolved. Certainly, a number of post-fertilization events have received attention including; the initiation of calcium oscillations by sperm-derived phospholipase C zeta (Yoon et al., 2008; Kuroda, 2010) and the subsequent reinitiation of meiosis (Steinhardt et al., 1974; Kline and Kline, 1992), cortical granule exocytosis and the prevention of polyspermy (Kline and Kline, 1992), and initiation of embryogenesis [reviewed by Runft et al., (2002)]. Despite this, limited insights have been generated to explain how the downstream events of fertilization prevent cytoplasmic ageing in the oocyte.

In establishing that the onset of oxidative stress within the oocyte with post-ovulatory age was directly responsible for the initiation of an intrinsic apoptotic cascade; as well as being associated with an elevation in levels of oxidative DNA damage (8OHdG) [Chapter 4 (Lord et al., 2014b)]; we hypothesised that fertilization may be associated with biochemical changes that either prevent the accumulation of ROS and/or actively repair oxidative damage (such as 8OHdG); thereby preventing cytoplasmic degeneration and apoptosis. Indeed, in monitoring levels of H$_2$O$_2$ within MII stage oocytes and oocytes that had been fertilized in vitro immediately following retrieval, a distinct trend could be identified. Fertilization essentially prevented the accumulation of ROS associated with extended periods of in vitro culture time (8 h) [Chapter 4 (Lord et al., 2014b)], thus circumventing oxidative stress driven apoptosis. The ability to minimise ROS accumulation in the fertilized oocyte was found to be associated with a superior capacity for H$_2$O$_2$ catabolism by way of upregulated glutathione peroxidase activity; as such, the zygote was more resistant to H$_2$O$_2$ induced 8OHdG formation and vitality loss.

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In addition to these findings, experiments within this thesis have depicted a previously uncharacterised elevation in levels of 8OHdG excision post-fertilization [Chapter 4 (Lord et al., 2014b)]. Following fertilization, an increased phosphorylation of the BER enzymes associated with the nucleus was detected; with phosphorylation of both OGG1 (Hu et al., 2005) and XRCC1 (Vidal et al., 2001; Marsin et al., 2003; Parsons et al., 2010; Ström et al., 2011) known to be associated with accelerated 8OHdG excision. This post-fertilization alteration to oocyte biochemistry is likely to be important for circumventing apoptosis, however, more likely is the imperative to produce healthy embryos by minimising the potential for mutagenesis in the zygote as a consequence of persisting 8OHdG lesions (Bruner et al., 2000; Wu et al., 2013; Ohno et al., 2014).

Cumulatively, these data suggest that at the time of fertilization, an upregulation of antioxidant activity and repair of oxidative damage aid in circumventing the initiation of oxidative stress-driven apoptosis that would otherwise be inevitable in the MII stage oocyte. These findings further highlight the integrative role for ROS signalling in the oocyte and provide additional insight to allow for the development of methodologies to prevent or delay post-ovulatory ageing of the MII stage oocyte in vitro.

5.4 Novel strategies for delaying post-ovulatory ageing in vitro

Thus far, there has been limited success in the literature in identifying compounds that can be safely utilized to supplement oocyte culture media in order to prevent or delay post-ovulatory ageing prior to ART. For instance, although caffeine supplementation is known to significantly extend the window in which murine oocytes can be fertilized (up to 34 h post-hCG injection) (Ono et al., 2011), the direct association between caffeine and elevated levels of DNA damage (Ishida et al., 1985)
means that this compound is not likely to be adopted for use in an ART setting [reviewed in Chapter 1 and 2 (Lord and Aitken, 2013; Lord et al., 2013)].

In identifying key molecules that orchestrate in vitro ageing and apoptosis of the MII stage oocyte within this thesis; namely ROS such as H$_2$O$_2$ and O$_2^-$, and electrophilic aldehydes such as 4HNE and acrolein; we hypothesised that compounds which are known to react directly with these molecules; i.e. antioxidants and reactive thiols respectively, may effectively slow or prevent the onset of oocyte degeneration and death in in vitro culture. Although a series of experiments have been conducted previously which have depicted limited success in attenuating post-ovulatory ageing using antioxidant agents such as ascorbic acid (vitamin C) and trolox (vitamin E) (Tarin et al., 1998), we hypothesised that the precise antioxidant selected for oocyte preservation will be influenced by its capacity to remain stable in culture medium in vitro, and its ability to access key intracellular compartments within the oocyte.

Thus, the potent antioxidant melatonin was selected on the basis of several important characteristics. Firstly, melatonin not only directly scavenges O$_2^-$ (Poeggeler et al., 1994) and H$_2$O$_2$ (Tan et al., 2002) but also blocks lipid peroxidation and electrophilic aldehyde production (Zhang et al., 2006). Additionally, melatonin is stable in aqueous solution (Cavallo and Hassan, 1995) and is amphiphilic; meaning it can penetrate both lipophilic and hydrophilic regions of the cell (Reiter et al., 2000a; Reiter et al., 2000b). Finally; all by-products resulting from the interaction between melatonin and ROS exhibit antioxidant activity themselves (Tan et al., 2002); rather than the pro-oxidant activity that can be possessed by by-products developed following the interaction between ROS and vitamin C for example (Riabchenko et al., 2010). As such, melatonin was found to successfully prevent the accumulation of ROS within the oocyte, delaying the onset of apoptosis, extending the optimal window for fertilization,
and improving the quality of embryos produced from oocytes that had been aged for 8 and 16 h in vitro [Chapter 2 (Lord et al., 2013)].

Experiments within this thesis have also described a capacity for penicillamine to attenuate electrophilic aldehyde-induced pathologies associated with post-ovulatory ageing. Penicillamine is a nucleophilic thiol that directly reacts with electrophilic aldehydes, ‘mopping up’ these cytotoxic compounds within the cell and thereby diminishing their capacity for intracellular damage. Additionally, penicillamine can chelate transition metals such as Cu$^{2+}$ (Flora and Pachauri, 2010) that contribute to the production of highly damaging ROS such as hydroxyl radical via the Fenton reaction (Friedrich et al., 2012). As such, penicillamine supplementation to oocyte culture media prevented the decline in fertilization rate and embryo quality associated with exposure of the oocyte to elevated levels of 4HNE [Chapter 3 (Lord et al., 2014a)].

The potential for the utilization of these antioxidants - particularly melatonin- as an oocyte culture media supplement in a clinical situation is high; primarily because of its very low toxicity (Reiter et al., 1997), and consequently high threshold for safe use in an ART setting. Despite this, there are limitations to using antioxidants for this purpose. As we have demonstrated, although antioxidant supplementation can attenuate post-ovulatory ageing – delaying the onset of damage to certain cellular components such as the plasma membrane – it cannot prevent in vitro ageing altogether. Future experiments that utilize a combination of molecules proven to safely extend the window for fertilization may attenuate in vitro ageing and apoptosis of the oocyte more definitively. For example, the inclusion of an antioxidant such as melatonin to prevent excessive ROS formation, in addition to a reactive thiol such as penicillamine to ‘mop up’ electrophilic aldehydes, added to a culture medium high in pyruvate to support ATP production (Downs et al., 2002; Liu et al., 2009; Li et al., 2011) may allow for even further attenuation of post-ovulatory ageing than has been described in this thesis.
Despite this, the complexities of post-ovulatory ageing – of which many further intricacies are likely yet to be discovered – mean it is unlikely that the occurrence of oocyte ageing in vitro could be completely prevented.

5.5 Concluding remarks

The collective findings within this thesis provide insights into the molecular mechanisms controlling post-ovulatory oocyte ageing and apoptosis, and suggest novel strategies for minimising the impact of this phenomenon in an assisted reproduction setting. Specifically, we have characterised a self-perpetuating cycle involving ROS production, lipid peroxidation, electrophilic aldehyde formation and mitochondrial damage that becomes apparent with increasing periods of in vitro culture time of MII stage oocytes. The elevation of ROS and electrophilic aldehydes within the ageing oocyte was found to be directly linked to the acquisition of functional deficiencies (impaired fertilization and embryo development), as well as the eventual initiation of an oxidative stress-driven intrinsic apoptotic cascade. Importantly, this thesis also uncovered a previously unbeknownst upregulation of antioxidant protection and repair of oxidative DNA damage occurring at the time of fertilization that is likely to be imperative for circumventing the onset of oxidative stress-driven oocyte apoptosis, and for allowing unimpeded development of the embryo. Once the key role of ROS and electrophilic aldehydes in post-ovulatory ageing had been determined, it was possible to develop techniques for extending the optimal window for fertilization of MII stage oocytes in vitro; namely, supplementation with the antioxidant melatonin and the aldehyde-reactive compound penicillamine. Utilization of such techniques in an ART setting could alleviate the concern of quality deterioration when immediate insemination following oocyte retrieval is not possible, and may also increase the viability of re-insemination techniques such as rescue-ICSI to minimise the necessity...
for initiation of additional ovarian stimulation cycles. The successful utilization of melatonin and penicillamine to prevent deterioration of oocyte quality in this study could also be translated to other facets of assisted reproduction such as cryopreservation; a process known to be hindered by oxidative stress (Tatone et al., 2010). In its entirety, this study highlights the importance of timely fertilization of oocytes following retrieval in an ART setting, and suggests that the adoption of antioxidant supplementation techniques may be a safe and effective means by which to delay deterioration of oocyte quality as a result of post-ovulatory ageing.

References


Lord, T., Smith, T.B., Aitken, R.J., 2014b. Fertilization initiates an acceleration of 8-hydroxyguanosine repair and an increase in antioxidant protection to prevent propagation of oxidative DNA damage in the embryo.

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Rohn, T.T., Hinds, T.R., Vincenzi, F.F., 1993. Ion transport ATPases as targets for free radical damage. Protection by an aminosteroid of the Ca\textsuperscript{2+} pump ATPase and Na\textsuperscript{+}/K\textsuperscript{+} pump ATPase of human red blood cell membranes. Biochem Pharmacol 46, 525-534.


