OXIDATIVE STRESS AND REPRODUCTIVE FUNCTION

Oxidative stress and in vitro ageing of the post-ovulatory oocyte: an update on recent advances in the field

Jacinta H Martin1,2, Brett Nixon1,2, Shenae L Cafe1,2, R John Aitken1,2, Elizabeth G Bromfield1,2 and Tessa Lord1,2

1Priority Research Centre for Reproductive Science, School of Environmental and Life Sciences, College of Engineering, Science and Environment, The University of Newcastle, Callaghan, New South Wales, Australia and 2Hunter Medical Research Institute Program in Infertility and Reproduction, New Lambton Heights, New South Wales, Australia

Correspondence should be addressed to J H Martin; Email: Jacinta.martin@newcastle.edu.au

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Abstract

In brief: Post-ovulatory ageing of oocytes leads to poor oocyte and embryo quality as well as abnormalities in offspring. This review provides an update on the contributions of oxidative stress to this process and discusses the current literature surrounding the use of antioxidant media to delay post-ovulatory oocyte ageing.

Abstract: Following ovulation, the metaphase II stage oocyte has a limited functional lifespan before succumbing to a process known as post-ovulatory oocyte ageing. This progressive demise occurs both in vivo and in vitro and is accompanied by a deterioration in oocyte quality, leading to a well-defined sequelae of reduced fertilisation rates, poor embryo quality, post-implantation errors, and abnormalities in the offspring. Although the physiological consequences of post-ovulatory oocyte ageing have largely been characterised, less is known regarding the molecular mechanisms that drive this process. This review presents an update on the established relationships between the biochemical changes exhibited by the ageing oocyte and the myriad of symptoms associated with the ageing phenotype. In doing so, we consider the molecular events that are potentially involved in orchestrating post-ovulatory ageing with a particular focus on the role of oxidative stress. We highlight the mounting evidence that oxidative stress acts as an initiator for a cascade of events that create the aged oocyte phenotype. Specifically, oxidative stress has the capacity to disrupt mitochondrial function and directly damage multiple intracellular components of the oocyte such as lipids, proteins, and DNA. Finally, this review addresses emerging strategies for delaying post-ovulatory oocyte ageing with emphasis placed on the promise afforded by the use of selected antioxidants to guide the development of media tailored for the preservation of oocyte integrity during in vitro fertilisation procedures.

Introduction

Following ovulation, the mammalian oocyte has a finite window of vitality, beyond which it will succumb to a degenerative process known as ‘post-ovulatory oocyte ageing’. In mice, this diminishment of oocyte quality is thought to occur from approximately 15 h post-ovulation (Marston & Chang 1964, Tarin et al. 1999, Lord et al. 2013), resulting in decreased receptivity to fertilisation as well as an increased propensity to give rise to poor quality embryos and offspring with compromised health (Lord & Aitken 2013). The cumulative impact of post-ovulatory ageing on oocyte quality documented in this review compounds the natural deterioration of oocyte quality arising from maternal ageing. In this context, it is well recognised that the oocyte, as with all long-lived cells, faces the accumulation of a legacy of exposure from both exogenous and endogenous insults even prior to those accrued during post-ovulatory ageing. Studies conducted on maternal ageing (see recent reviews by Mihalas et al. 2017, Secomandi et al. 2022) also suggest that oocytes may have an impaired capacity to repair the damage they incur (Mihalas et al. 2018, Peters et al. 2020). This heightened susceptibility and inability to correct damage reflects the fact that transcription ceases when oocytes complete their growth phase and remains dormant until such time as the embryonic genome is activated (Gosden & Lee 2010). Such a situation means
that the fidelity of endogenous stores of oocyte mRNAs, proteins, and lipids is placed at heightened vulnerability during stressful events such as post-ovulatory ageing (Cafe et al. 2021).

Post-ovulatory oocyte ageing can occur both in vivo and in vitro; however, the consequences of this phenomenon are arguably most problematic in an assisted reproduction setting. The reproductive strategy of a number of species is such that ovulation and intercourse are synchronised events, significantly diminishing the likelihood that fertilisation will be delayed. For instance, species such as cattle experience behavioural oestrus, while others such as cats and camels are induced to ovulate by the act of intercourse itself (Dixson 2021). In our own species, ovulation and intercourse are not synchronised; however, biochemical alterations to the oolemma and zona pellucida (ZP) likely reduce the probability that fertilisation of aged oocytes will occur within the female reproductive tract. Contrastingly, in an in vitro setting, assisted reproductive technologies (ARTs) such as intra-cytoplasmic sperm injection (ICSI) override these biochemical barriers, thus elevating the likelihood that fertilisation of a degenerating oocyte will occur.

The impetus to fertilise oocytes within a short window of time of post-ovulation creates considerable challenges in the context of a busy in vitro fertilisation (IVF) clinic. Thus, it is of little surprise that strategies to attenuate in vitro ageing of oocytes have been the basis of numerous investigations, which will be reviewed throughout this manuscript. Additionally, the ability to delay in vitro ageing would enhance the feasibility of techniques aimed at re-inseminating oocytes that have failed to fertilise via IVF, using a technique referred to as ‘rescue ICSI’ (Beck-Fruchter et al. 2014). Given that <20% of initiated ART cycles result in a live birth (Newman et al. 2021), rescue ICSI has the potential to maximise the likelihood of success within a single cycle, thus minimising the need for future invasive ART procedures.

Beyond the benefits to our own species, the ability to attenuate in vitro ageing of oocytes would also be a valuable asset in the context of animals of ecological and agricultural significance. This is particularly the case for livestock species such as pigs and cattle, where oocytes require long periods of in vitro maturation (IVM) before IVF can be performed. For instance, bovine oocytes require >20 h of IVM before the metaphase II (MI) stage is reached (Xu et al. 1986, Koyama et al. 2014). Additionally, it has been shown to take several hours for bovine spermatozoa to penetrate the oocyte following the initiation of IVF (Takahashi & First 1993). Thus, this prolonged period of in vitro incubation initiates a delicate balance between sufficient oocyte maturation and the onset of degeneration (Koyama et al. 2014).

In this manuscript, we endeavour to provide a literature update that follows our 2013 Reproduction review on post-ovulatory oocyte ageing (Lord & Aitken 2013), with the aim of highlighting advances in our understanding of the mechanisms underpinning post-ovulatory ageing and the functional consequences of this process. Developments over the past decade include improved understanding of the epigenetic effects associated with post-ovulatory ageing, the establishment of a key role for sirtuins in the maintenance of oocyte quality, and characterisation of the demise of key proteins involved in sperm binding (sperm-egg fusion protein Juno; astacin-like metalloendopeptidase (ASTL; also known as ovastacin)). Importantly, this updated review of the literature continues to demonstrate the intricate association between oxidative stress, mitochondrial dysfunction, and pathologies of the post-ovulatory aged oocyte, again supporting the notion that reactive oxygen species (ROS) are key instigators of this process. In accompaniment to this, we review several exciting strategies to attenuate the degeneration of oocytes in vitro, with a subset of antioxidant compounds showing particular promise for future therapeutic use.

Implications of post-ovulatory oocyte ageing

At a molecular level, oocytes exhibit numerous symptoms of ageing with extended periods of time following ovulation (Fig. 1). These include reductions in cell cycle factors required to maintain meiotic arrest and a concomitant susceptibility to spontaneous activation, partial or premature cortical granule exocytosis, ZP hardening, loss of chromosomal and spindle integrity (reviewed below), increased oolemma rigidity, decreased inositol 1,4,5-trisphosphate (IP₃) receptor sensitivity, and a related decrease in calcium (Ca²⁺) homeostasis (Szollosi 1971, Longo 1981, Dodson et al. 1989, Ducibella et al. 1990, Xu et al. 1997, Takahashi et al. 2000, Kikuchi et al. 2002, Zhang et al. 2011, Liu et al. 2019). It is not difficult to infer how such widespread biochemical and structural changes, especially to those molecules with intimate roles in the fertilisation and oocyte activation cascades, affect the oocyte’s ability to support normal fertilisation and subsequent embryo development. Importantly, the underlying basis for a multitude of these biochemical aberrations is intertwined with the accumulation of ROS and subsequent oxidative stress.

Decreased fertilisation rate

A progressive loss of fertilisation capacity and disturbed embryonic development are among the major phenotypical and functional consequences of post-ovulatory oocyte ageing (Fig. 1). Since the proposition by Lord and Aitken in 2013 that oxidative stress is one of the key initiators of post-ovulatory ageing in mammalian oocytes, significant advances have been made in linking excessive ROS production, post-ovulatory oocyte ageing, and an attendant decline in oocyte fertilisation competence (Dai et al. 2017). Dai et al. reported that post-ovulatory ageing compromises the fertilisation ability
of mouse oocytes by inducing precocious exocytosis of ovastacin (Dai et al. 2017), a component of cortical granules and an oocyte-specific metalloendoprotease that cleaves ZP sperm-binding protein 2 (ZP2) after fertilisation (Quesada et al. 2004, Burkart et al. 2012). Premature exocytosis of ovastacin, in turn, causes premature cleavage of the sperm binding domain of ZP2 and consequently reduces sperm-binding capacity to the ZP (i.e. 58.3 ± 9.0 sperm bound/control ZP vs 11.5 ± 0.7 in those with a cleaved ZP2 N-terminus). Notably, immunoblotting experiments using an antibody capable of detecting ZP2 cleavage have indicated that a substantial portion of the ZP2 protein is cleaved prior to fertilisation in post-ovulatory aged oocytes, suggesting that these oocytes lose ZP2 sperm-binding sites even prior to fertilisation taking place (Dai et al. 2017). In linking these phenomena to the detrimental impact of excessive ROS, it has been shown that the incubation of post-ovulatory aged oocytes with the antioxidant, melatonin, is capable of decreasing the ratio of cleaved:uncleaved ZP2, permitting partial restoration of their capacity to support sperm binding (Dai et al. 2017).

In examining the expression and localisation of ovastacin (ASTL) in a cohort of post-ovulatory aged oocytes, Dai et al. (2017) detected a significant decrease in ovastacin expression accompanied by striking spatial abnormalities in the distribution of the protein among aged oocytes. Together, these data suggest that at least a
portion of the ovastacin protein had been prematurely exocytosed, likely accounting for the partial cleavage of ZP2 witnessed in the extracellular ZP matrix of the aged cells. It is known that post-fertilisation proteolytic cleavage of ZP2 is mediated by ovastacin, a key component of cortical granules (Burkart et al. 2012). Thus, declining ovastacin may account for the numerous observations of partial or premature cortical granule exocytosis, ZP hardening, and increased oolemma rigidity occurring during post-ovulatory oocyte ageing (Fig. 1) (Szollosi 1971, Dodson et al. 1989, Ducibella et al. 1990, Xu et al. 1997). It is also notable that the frequency of abnormally located ovastacin was reduced in response to melatonin treatment, thus further strengthening the putative role of ROS as a driver of these events (Dai et al. 2017).

In extending these observations, it was revealed that post-ovulatory ageing also disrupts the abundance of JUNO (Dai et al. 2017), a GPI-anchored protein expressed in the oolemma and implicated in gamete recognition and membrane adhesion (Bianchi et al. 2014). This post-ovulatory ageing defect was similarly ameliorated by the incubation of ovulated oocytes in media supplemented with melatonin (Dai et al. 2017). These data raise the prospect that the reduced abundance of JUNO within the membrane of the post-ovulatory aged oocyte is a likely contributor to the decreased fertilisation potential of these cells, but promisingly, such defects may be circumvented via antioxidant supplementation to mitigate the impact of ROS generation.

**Reduced embryo quality and developmental potential**

Despite the significant challenges faced by post-ovulatory aged oocytes in undergoing fertilisation, there are circumstances when this process can occur unimpeded, such as during the use of rescue ICSI. Rescue ICSI refers to the practice of performing ICSI on oocytes that did not show signs of fertilisation 18–24 h after conventional insemination (Paffoni et al. 2021). Rescue ICSI has evolved as a plausible method to reduce the occurrence of total fertilisation failure, a devastatingly frustrating condition that affects 5–20% of couples undergoing ART (Combelles et al. 2010). Despite its increasing utility, rescue ICSI continues to generate unsatisfactory clinical pregnancy (10%) and implantation rates (5%) (Paffoni et al. 2021). These data reflect the findings of a recent meta-analysis of 22 original studies encompassing 1686 rescue–ICSI cycles with 12,945 inseminated oocytes (Paffoni et al. 2021) and correlate well with observations that embryos originating from post-ovulatory aged oocytes are generally of poorer quality and exhibit delayed or impaired development during embryogenesis (Lord et al. 2013) (Fig. 1).

It has also been shown that this cohort of embryos are plagued by a significantly diminished ability to undergo uterine implantation and support a pregnancy to term (Fig. 1). Further supporting this premise, clinical studies assessing the effects of in vivo post-ovulatory oocyte ageing have reported significant correlations with delayed fertilisation and early pregnancy loss (Wilcox et al. 1998). The mechanisms underlying these low implantation and ensuing pregnancy rates have been attributed to time- and ROS-dependent deterioration in oocyte quality but may also be influenced by the loss of synchronisation between endometrial growth and embryo development (Paffoni et al. 2021). The latter of these may allude to the evolutionary advantage of post-ovulatory ageing, whereby limiting the fertilisation of low-quality oocytes arising from desynchronisation of ovulation and insemination assists in avoiding complicated or ill-fated pregnancies and a possible wasted investment of resources.

In recognising the cumulative impacts of maternal and post-ovulatory ageing, the mammalian oocyte is highly susceptible to exogenous oxidative stress and has little capacity to repair this damage (Fig. 1). One example of this susceptibility lies in the damage of the mitochondrial protein, succinate dehydrogenase (SDHA), which has been identified as a target for precocious 4HNE adduction during post-ovulatory ageing (Lord et al. 2015). This form of covalent modification irrevocably dysregulates the structure and function of SDHA, leading, in turn, to elevated levels of mitochondrial ROS generation and enhancing lipid peroxidation, effectively setting in train a cycle of declension that ultimately culminates in the demise of the oocyte (Fig. 2) (Lord et al. 2015). Indeed, such changes in oocyte biochemistry result in a loss of mitochondrial membrane potential and a corresponding increase in DNA damage and apoptosis, likely as a consequence of collapsing the mitochondrial respiratory chain (Lord et al. 2015). Notably, oocytes do not appear to possess an innate mechanism to correct such damage beyond that of their endogenous antioxidants, which are also prone to depletion following protracted post-ovulatory periods (Boerjan & de Boer 1990, Yoshida et al. 1993). Certainly, it is well established that oocytes have only a restricted capacity for DNA repair (Lord & Aitken 2015, Martin et al. 2016, 2018, 2019). Taken together, the ensuing mitochondrial dysfunction and concomitant reduction in ATP levels, likely hinder the capacity of the oocyte to support the high energy demands of embryogenesis and orchestrate a normal pattern embryonic development (Babayev & Seli 2015).

In mice, natural fertilisation of in vivo post-ovulatory aged oocytes has been correlated with longer between-labour intervals, decreased litter frequency, and lower total number of litters and offspring born (Tar et al. 1999). Moreover, first-generation offspring experience delayed development of the righting reflex, higher spontaneous motor activity, and decreased life expectancy (Tarin et al. 1999). Importantly, second-generation pups often displayed teratogenic defects, higher pre-weaning mortality, and decreased body
weight at weaning; however, they did not display developmental delays equivalent to the F1 generation (Tarin et al. 1999, Tarín et al. 2002). Regardless, these findings support the premise that oocyte ageing has long-lasting repercussions on progeny, leading to premature mortality and decreased reproductive fitness in the offspring (Tarín et al. 2002). There is also evidence that such ageing may rouse a heightened susceptibility to certain disorders later in life (Tarin et al. 1999, Tarín et al. 2002) (Fig. 1).

**Aneuploidy, spindle defects, and the spindle assembly checkpoint**

In addition to not being able to meet the metabolic demands of embryogenesis, another contributing factor instigated by post-ovulatory ageing that may lead to the progressive loss of fertilisation capacity and disturbed embryonic development is chromosomal instability and aneuploidy (Miao et al. 2005, Lacham-Kaplan & Trounson 2008, Shimoi et al. 2019), both of which now appear to be among the major phenotypical and functional consequences of post-ovulatory oocyte ageing (Fig. 1). In fact, chromosomal instability is a relatively common consequence of oocyte ageing both in vitro and in vivo (i.e. maternal ageing) (Miao et al. 2005, Selesniemi et al. 2011, Camlin et al. 2017, Mihalas et al. 2018).

Chromosomal instability leads to chromosomal segregation errors, including early segregation and nondisjunction, which can coalesce to instigate aneuploidy. Indeed, evidence now suggests that post-ovulatory ageing might also be a risk factor for aneuploidy, irrespective of maternal age (Shimoi et al. 2019). In this context, Shimoi et al. discovered that after 6 h of in vitro culture, 26.2% of mouse oocytes displayed abnormal chromosome number (either aneuploidy or polyploidy), while by 12 h this number had almost doubled, such that 40% of oocytes presented with abnormal chromosome numbers (compared with 17.2% in fresh oocytes) (Shimoi et al. 2019). Such abnormalities largely fell into

![Figure 2 Mechanisms of post-ovulatory oocyte ageing. With increasing periods of time following ovulation, the metaphase II-stage oocyte experiences an overproduction of reactive oxygen species (ROS) and elevated levels of lipid peroxidation. These ROS, together with the by-products of lipid peroxidation, promiscuously adduct to proteins, lipids, and DNA, which feedback into the cascade causing additional damage, cytoplasmic fragmentation, and the eventual apoptosis of the oocyte.](image-url)
the category of aneuploidy (6 h = 23.8%; 12 h = 40.0% vs fresh = 10.3%), with only 2.4% displaying polyplody (more than two complete sets of chromosomes). Of concern, these aneuploidies persisted through embryogenesis and further increased in incidence. To this end, chromosome spreads from eight-cell embryos derived from the aged oocytes revealed 33.7% (6 h) and 44.1% (12 h) of embryos carrying aneuploidy vs 28.2% in control oocytes (Shimoi et al. 2019). While the authors did not extend their analysis beyond fertilisation and pre-implantation development, both of which were significantly reduced, it is well borne out that embryos carrying aneuploidy are at an elevated risk of implantation errors, spontaneous abortion, and congenital abnormalities (Faralli et al. 2007). Indeed, in fish, ploidy anomalies in progeny have been commonly reported among the detrimental outcomes of oocyte ageing (Nomura et al. 2013, Samarin et al. 2016, do Nascimento et al. 2018, Waghmare et al. 2021) and have been shown to significantly affect fertilisation, embryo survival, and hatching rates. Thus, in contrast to fresh oocytes, which routinely achieve rates of ≥80% for each of these developmental stages, after 6 h of in vitro ageing, there was an almost complete loss of oocyte viability and the corresponding death of more than 90% of the embryos derived from these cells prior to hatching. Among the limited number of larvae that originated from oocytes aged between 4 and 6 h, each displayed clear evidence of corporal malformation and skeletal abnormalities (vs 5% in the control group) (Waghmare et al. 2021).

Appropriate spindle formation, chromosome segregation, and distribution fall under the jurisdiction of the spindle assembly checkpoint (SAC), which itself has recently been revealed to be vulnerable to oxidative stress (D’Angiolella et al. 2007). In oocytes, the SAC is responsible for monitoring microtubule/kinetochore attachment and dictates the progression of anaphase. In the event that a kinetochore is not fully connected to a spindle fibre, mitotic arrest deficient 2 (MAD2 or MAD2L1) will bind to MAD1 on the kinetochore and signal inhibition of anaphase progression until each chromosome is appropriately attached to microtubules and aligned on the equatorial plane (Vogt et al. 2008, Sun & Kim 2012, Jones & Lane 2013). In situations such as those described in the article by Shimoi et al. (2019), the SAC should act to arrest development until proper kinetochore/chromosome/spindle attachment has been formed. However, it appears that despite the significant abnormalities present in the post-ovulatory aged oocyte, the SAC fails to activate appropriately, thereby increasing the frequency of aneuploidy in the embryos generated from these oocytes (i.e. 17.2% aneuploidy in eight-cell embryos derived from freshly isolated oocytes vs 40.0% aneuploidy in their counterparts derived from oocytes aged for 12 h prior to fertilisation) (Shimoi et al. 2019).

By examining the key components of the SAC, Shimoi et al. (2019) have shown that the MAD2-dependent system, which monitors the connection between microtubules and kinetochores, might be disturbed by the process of post-ovulatory ageing. Support for this conclusion rests with the observation that MAD2 localisation was retained on kinetochores that were appropriately connected to spindles fibres and, in some instances, was absent on others despite failed spindle fibre–kinetochore connections and distinct misalignment (Shimoi et al. 2019). Such findings raise the prospect that, even if several chromosomes lacked a stable kinetochore–microtubule connection, these might not be enough to prevent the advance to anaphase and thus may underlie the resulting chromosome distribution errors (Nagaoka et al. 2011) seen in embryos derived from post-ovulatory aged oocytes.

In addition to MAD2, abnormalities have also been reported in cohesin (a ring-shaped multi-subunit complex that mediates sister chromatid cohesion), REC8 (a meiosis-specific subunit of the cohesion complex that is specifically cleaved by separase), and securin (a protein involved in the control of the metaphase–anaphase transition and anaphase onset) (Shimoi et al. 2019). The cohesin complex mediates cohesion between replicated sister chromatids during chromosome segregation and is itself regulated by the SAC. Numerous reports have identified a distinct relationship between the deterioration of sister chromatid cohesion, increasing maternal age, and age-related aneuploidy (Chiang et al. 2010, Revenkova et al. 2010, Jessberger 2012, Yun et al. 2014). However, the same relationship has not yet been fully borne out in the context of post-ovulatory ageing. Shimoi et al., demonstrated a time-dependent deterioration of REC8 in in vitro aged MII oocytes (Shimoi et al. 2019). The overexpression of securin, which normally acts as a suppressor of separase, may be one of the reasons for REC8 disruption. Thus, it is possible that post-ovulatory oocyte ageing, like maternal ageing, leads to a weakening of sister chromatid cohesion and might be a causative factor in the aneuploidy commonly seen in these cells. This hypothesis needs to be comprehensively explored in future studies of post-ovulatory oocyte ageing.

Epigenetic modifications during post-ovulatory oocyte ageing

In line with the unmistakable importance of nuclear integrity, we have begun to understand that epigenetic modification of chromatin also has a role of considerable importance in developmental potential (reviewed in Beaupin 2014). It is therefore concerning that animal models have shown that the establishment of epigenetic modifications in oocytes can be affected by insults as diverse as maternal diets (Steegers-Theunissen et al. 2019). 

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Oxidative stress and post-ovulatory oocyte ageing

Since the proposition by Lord and Aitken in 2013 that oxidative stress is a key initiator of post-ovulatory ageing in mammalian oocytes (Lord & Aitken 2013), the field has expanded rapidly, with novel studies evaluating the sources and consequences of ROS on protein (Mihalas et al. 2017, 2018) and DNA quality (Horta et al. 2020) in oocytes, with considerable attention given to the application of antioxidants as a means to expand the window of fertilisation (Wang et al. 2019, Zhang et al. 2019, Zhou et al. 2019). In the following sections of this review, we will discuss several aspects of ROS-mediated post-ovulatory oocyte ageing, with a focus on new studies that have driven forward our understanding of this paradigm over the past decade.

ROS are a key requirement for successful folliculogenesis, oocyte meiosis, ovulation, and embryonic development (Wang et al. 2021). However, as time passes in the post-ovulatory period, ROS, such as H$_2$O$_2$, peroxynitrite (ONOO$^-$), and superoxide anion (O$_2^-$), can accumulate within MII oocytes, leading to decreased fertilisation potential, the induction of cellular senescence pathways, and eventually cell death. While ROS are largely produced through damage or disruption to the mitochondrial electron transport chain (ETC), particularly to complexes I, III, and IV, leading to electron leakage at both sides of the inner mitochondrial membrane (Wang et al. 2021), in a clinical setting, oocytes can also be sensitised to oxidation processes due to their maturation in conditions of oxygen tension that are supraphysiological (between 2% and 8%; (Guarneri et al. 2015)), thereby enhancing the production of ROS. While standardisation of oxygen tension at 5% has been achieved for some clinics or even whole geographical regions (Herbemont et al. 2021), establishing correct oxygen tension to limit the production of ROS during oocyte maturation and storage remains an issue of contention (Guarneri et al. 2015, Morin 2017, Herbemont et al. 2021). In addition to this, the factors that lead to ROS production during clinical procedures are extensive and further complicated by an individual's lifestyle that can culminate in the accumulation of ROS within follicles, even in younger women (Mihalas et al. 2017). For comprehensive reviews focused on the sources of ROS and their consequences in mammalian oocyte function and fertilisation, see Mihalas et al. (2017) and Peters et al. (2020). Herein, we will focus on the contribution of the mitochondria and mitochondrial derived ROS to post-ovulatory oocyte ageing.

Mitochondrial dysfunction and post ovulatory ageing

It is now well regarded that preservation of mitochondrial integrity throughout oocyte maturation is vital for successful embryonic development. This is largely due
to the suppression of mitochondrial replication during oocyte development whereby mitochondrial DNA (mtDNA) count is not altered from the MII stage until the hatching blastocyst stage (St. John et al. 2010, St John 2014). Degradation of paternal structures prior to embryonic genome activation also means that sperm mtDNA is eliminated and does not contribute to the embryo’s mitochondrial content (Sutovsky et al. 2004). Taken together, this means that the mitochondria that are derived from the MII oocyte provide the majority of ATP to support the entirety of preimplantation embryonic development (Babayev & Seli 2015). Beyond the production of ATP, oocyte mitochondria also regulate the calcium oscillations that accompany fertilisation (Wakai et al. 2013). For this reason, mitochondrial function is a key determinant of fertilisation outcomes in mammals (Santos et al. 2006), and unfertilised or degenerated oocytes are known to present with mitochondrial dysfunction and/or a lower mtDNA copy number (Babayev & Seli 2015). In further support of this notion, the overexpression of mitochondrial fusion proteins can lead to the generation of mitochondrial aggregates in the cytoplasm of oocytes and interfere with spindle organisation and endoplasmic reticulum distribution during development (Wakai et al. 2014). Conversely, removal of factors involved in mitochondrial fission can impair calcium signalling and lead to abnormal follicle development and dysregulated ovulation (Udagawa et al. 2014).

Despite their essential roles, the mitochondria, and their DNA, are exceptionally susceptible to oxidative damage. Notably, this susceptibility is greatly exacerbated during both in vitro and in vivo oocyte ageing where mitochondrial structural abnormalities have been observed, accompanying reduced mtDNA copy number and decreased ATP production in aged oocytes. This susceptibility is due to its housing of the ETC, a key site for electron leakage and the generation of ROS. These elevated levels of mitochondrial ROS cause a loss of mitochondrial membrane potential and a corresponding collapse of the mitochondrial respiratory chain (Lord et al. 2015), which, in turn, leads to the generation of more ROS and lipid peroxidation products (reviewed below). This ultimately instigates significant damage to proteins, lipids, and DNA (mitochondrial and nuclear), culminating in the demise of the oocyte (Fig. 2) (Lord et al. 2015). As a result, the mtDNA, in particular, is prone to the accumulation of mutations and deletions which, over time, may reduce fitness (Bentov & Casper 2013). This situation is then worsened since mtDNA lacks protective histones further enhancing its vulnerability (Babayev & Seli 2015) and the oocytes are unable to replace these damaged organelles.

Beyond their profound effects on metabolism within the cell, mitochondrial mutations/disruptions are also known to result in pronounced spindle abnormalities, aneuploidy, and decreased developmental competence (Schon et al. 2000, Zhang et al. 2006), all hallmark features of post-ovulatory oocyte ageing. This is likely due to the dysregulation of ATP-dependent biological processes either by disabling the inherent surveillance and repair systems that would otherwise detect and correct such errors (i.e. the SAC) or through direct adduction and damage of cytoskeletal proteins. In support of this theory, it has been demonstrated that aneuploidy in post-ovulatory aged oocytes is accompanied by significant abnormalities in spindle shape (Shimoi et al. 2019) (Fig. 1). Thus, in contrast to fresh oocytes that contained slightly pointed barrel-shaped spindles, their post-ovulatory aged counterparts possessed elongated spindles characterised by a wider ‘pole-pole’ length. Such defects in the aged oocytes were accompanied by examples of collapsed or ‘obscure’ spindles, and in some instances distinct chromosome misalignment was detected wherein spindle fibres did not appear to be connected to their kinetochores (Shimoi et al. 2019). It therefore seems that post-ovulatory aged oocytes, like their maternally aged counterparts, experience difficulty in maintaining spindle morphology and appropriate connections between spindles and chromosomes (Fig. 1), likely caused by defective microtubule stability and tubulin polymerisation (Mihalas et al. 2017).

**Lipid peroxidation, oxidative stress, and post-ovulatory ageing**

In contrast to the long-held paradigm that mitochondria are the leading cause of ROS production contributing to oocyte degeneration (Zhao et al. 2011, Lord et al. 2013, Bradley & Swann 2019), the mechanisms by which the generation of ROS then culminates in widespread cellular (and tissue) damage remain in its relatively infancy. While this topic is undeniably complex, discovery of direct modification and damage to proteins by lipid-derived reactive mediators, the lipid aldehydes, has formed an important foundation for our understanding of ROS propagation and cellular degeneration in aged mouse oocytes (Lord et al. 2015, Mihalas et al. 2017, 2018). Lipid aldehydes (otherwise known as reactive carbonyl species) are reactive products generated through both enzymatic and non-enzymatic lipid peroxidation (Walters et al. 2020). Their production is cyclical in nature such that they can be generated via initial oxidation of polyunsaturated fatty acids (PUFA) by ROS produced via electron leakage from the ETC (and other sources), but once produced they can then amplify ROS production and contribute to the propagation of further lipid oxidation events (Fig. 2) (Lord et al. 2015, Walters et al. 2020). Furthermore, the production of lipid aldehydes from PUFA substrates is particularly deleterious due to their ability to covalently modify (adduct) macromolecules including proteins and DNA at sites that are remote from their site of origin (Ayala et al. 2014). This can result in a disruption to
cellular homeostasis and the onset of cell death and degeneration (Ayala et al. 2014).

While large-scale studies assessing the protein targets of lipid aldehydes within the oocyte are yet to be performed, in the context of post-ovulatory ageing, it is known that 4-hydroxynonenal (4HNE), malondialdehyde, and acrolein all increase within the MII oocyte as a function of time in culture (Lord et al. 2015). Moreover, in vitro exposure of mouse MII oocytes to increased aldehyde concentrations resulted in mitochondrial ROS production, a loss of mitochondrial membrane potential, lipid peroxidation, and eventually apoptosis (Lord et al. 2015). One of the key theories behind the propagation of mitochondrial ROS by aldehydes involves the modification of the ETC protein constituent SDHA, evidence for which was provided through direct evaluation of the ability of 4HNE to adduct SDHA within MII oocytes (Lord et al. 2015). This is reminiscent of the deleterious interaction of 4HNE with proteins in somatic cells where cytochrome c oxidase and mitochondrial ATPase have also been revealed as critical targets of 4HNE-induced damage (Chen et al. 2001, Bunik & Sievers 2002). Importantly, interference in the function of the ETC is known to result in an escalation of lipid peroxidation and radical generation itself, leading to oxidative stress and cell senescence (Lord et al. 2013, 2015). Notably, however, these deleterious cellular responses to 4HNE within the ageing post-ovulatory oocyte could be ameliorated by supplementing culture media with the electrophile scavenging antioxidant penicillamine (Lord et al. 2015). Penicillamine supplementation resulted in an increase in fertilisation rates and improved embryo development (Lord et al. 2015), indicating an inextricable link between the production of lipid aldehydes and the onset of cellular decline and fertility loss in the context of post-ovulatory oocyte ageing.

Therapeutic interventions for preventing/delaying oocyte ageing

The ability to attenuate post-ovulatory oocyte ageing, thus prolonging the window for optimal fertilisation and improving feasibility of re-insemination techniques, holds enormous potential for human medicine and agriculture alike. As such, it is unsurprising that considerable effort has been attributed to identifying techniques that can prevent oocyte demise, particularly in an in vitro setting (Table 1). The striking ability to retain developmental competence of oocytes when post-ovulatory ageing is abrogated has been eloquently demonstrated in a recent study that utilised genome transfer techniques (Yamada & Egli 2017). Specifically, genome transfer from a 20 h in vitro aged oocyte into a ‘fresh’ oocyte cytoplasm restored embryo development rates and embryo quality to levels equivalent to non-aged, non-genome transferred controls. Contrastingly, nuclear transfer from fresh oocytes into an ‘aged’ cytoplasm did not support developmental competency, suggesting that post-ovulatory ageing is largely attributed to the dysfunction of cytoplasmic factors rather than inherent issues with the genome itself. Although such genome transfer techniques are able to produce remarkable outcomes to restore ageing oocyte function, they are limited by their highly invasive and technically challenging nature. Thus, the ability to achieve similar outcomes via modulation of oocyte culture conditions has been widely explored.

Historically, studies aimed at preventing post-ovulatory ageing have largely been focused on modulating culture temperature, altering the concentration of metabolites in culture media, and supplementing compounds such as caffeine that can prevent the decline in M-phase promoting factor (MPF) activity that is linked to parthenogenesis (MPF is essential for maintaining MII arrest post-ovulation) (Fig. 1). Such approaches have been explored in our previous review article (Lord & Aitken 2013) and will not be revisited here, except to mention that more recent publications using novel compounds such as RO-3306 (Prasad et al. 2016) have reinforced that maintenance of MPF activity prevents spontaneous activation of aged oocytes, while further evidence has been produced to suggest that reducing temperature may lessen the sensitivity of porcine oocytes to activating stimuli (Li & Cui 2016) (Table 1).

Antioxidant supplementation

Since the publication of our review in 2013 (Lord & Aitken 2013), significant interest has accumulated surrounding the capacity for antioxidants to mitigate post-ovulatory oocyte degeneration by targeting multiple facets of cytoplasmic ageing, beyond a sole focus on regulating MPF activity. Certainly, numerous potential benefits of these compounds to the oocyte have been recently uncovered, including the stabilisation of oocyte membrane and ZP proteins important for sperm binding, the maintenance of sirtuin expression, and sustaining a normal epigenetic profile with increasing post-ovulatory age (Table 1). Moreover, an extensive suite of antioxidants has now been assessed across a number of species. Below we will focus primarily on antioxidant agents that have been repeatedly and independently reported to delay the onset of post-ovulatory ageing and, as such, have predominated in the literature, including resveratrol, melatonin, coenzyme Q10, and quercetin. However, a summary of all literature surrounding ‘post-ovulatory ageing’ and ‘antioxidants’ retrieved from PubMed for years 2013–present is provided in Table 1.

Following the publication of our own manuscript demonstrating the capacity of melatonin to delay the onset of post-ovulatory ageing in mouse oocytes (Lord et al. 2013), there has been considerable additional investigation into the activity of this potent antioxidant.
Table 1  Interventions used to delay the onset of post ovulatory oocyte ageing from literature published between 2013 and 2022.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Action/mechanism</th>
<th>Effect on quality of post-ovulatory aged oocytes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resveratrol</td>
<td>Antioxidant – ROS scavenging; activation of mitophagy; prevents decrease in SIRT expression</td>
<td>Reduction in spindle abnormalities; Normal cortical granule distribution maintained; Improved maintenance of mitochondrial quality and function; Delayed apoptosis; Normal localisation of sperm binding proteins maintained (JUNO); Improved fertilisation rate and blastocyst formation rate; Attenuated epigenetic changes</td>
<td>Zhou et al. (2019), Sun et al. (2019), Ma et al. (2015)</td>
</tr>
<tr>
<td>Melatonin</td>
<td>Antioxidant – ROS scavenging; prevents decrease in SIRT expression</td>
<td>Attenuated cytoplasmic fragmentation and apoptosis; Improved mitochondrial function; reduction in spindle abnormalities and meiotic errors; normal localisation of sperm-binding proteins maintained (JUNO and Ovastacin); Improved fertilisation rate and blastocyst formation rate</td>
<td>Lord et al. (2013); Chen et al. (2017); Yang et al. (2018); Dai et al. (2017); Nie et al. (2018)</td>
</tr>
<tr>
<td>Coenzyme Q10 (and ubiquinol-10)</td>
<td>Antioxidant – ROS scavenging; prevents decrease in SIRT expression; activation of mitophagy</td>
<td>Attenuated cytoplasmic fragmentation; reduction in spindle abnormalities; improved mitochondrial function; normal localisation of sperm-binding proteins maintained (JUNO and Ovastacin); increased blastocyst formation rate</td>
<td>Zhang et al. (2019), Niu et al. (2020)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Antioxidant – ROS scavenging; sustains MPF activity in ageing oocytes; prevents decrease in SIRT expression</td>
<td>Attenuated cytoplasmic fragmentation and apoptosis; reduction in spindle abnormalities; attenuated epigenetic changes; increased blastocyst formation rate</td>
<td>Wang et al. (2017a)</td>
</tr>
<tr>
<td>Allicin</td>
<td>Antioxidant – ROS scavenging; activation of autophagy</td>
<td>Reduction in spindle abnormalities; increased blastocyst formation rate</td>
<td>Park et al. (2019)</td>
</tr>
<tr>
<td>Artemisia asiatica</td>
<td>Antioxidant – ROS scavenging</td>
<td>Attenuated cytoplasmic fragmentation; normalisation of sperm-binding proteins maintained (JUNO and Ovastacin); Increased mitochondrial function</td>
<td>Jeon et al. (2017)</td>
</tr>
<tr>
<td>N-acetyl-cysteine</td>
<td>Antioxidant – ROS scavenging</td>
<td>Reduction in spindle abnormalities; improved mitochondrial function</td>
<td>Wang et al. (2019); Li &amp; Cui (2016)</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>Antioxidant – ROS scavenging</td>
<td>Prevented 4HNE-induced decline in fertilisation- and blastocyst-formation rate</td>
<td>Lord et al. (2015)</td>
</tr>
<tr>
<td>4,4’-dimethoxychalcone</td>
<td>Antioxidant – ROS scavenging; activation of autophagy</td>
<td>Delayed apoptosis; improved mitochondrial function; improved fertilisation rate, and blastocyst formation rate</td>
<td>Liu et al. (2022)</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>PPARA agonist, PPARGC1A co-activator; ROS scavenging; activation of mitophagy</td>
<td>Delayed apoptosis; improved blastocyst quality; improved mitochondrial function</td>
<td>Kim et al. (2021)</td>
</tr>
<tr>
<td>Reduced temperature</td>
<td>Dampened metabolism/redox homeostasis</td>
<td>Reduced sensitivity to activating stimuli</td>
<td>Li &amp; Cui (2016)</td>
</tr>
<tr>
<td>Injection of Sirt1 RNA</td>
<td>Prevents decline in SIRT expression</td>
<td>Attenuated cytoplasmic fragmentation; reduction in spindle abnormalities; Improved mitochondrial function; attenuated epigenetic changes</td>
<td>Xing et al. (2021)</td>
</tr>
<tr>
<td>Genome transfer to fresh oocytes</td>
<td>Degraded/dysfunctional cytoplasmic factors are replaced</td>
<td>Reduction in spindle abnormalities and abnormal chromosome segregation; increased blastocyst formation rate and improved blastocyst quality</td>
<td>Yamada &amp; Egli (2017)</td>
</tr>
<tr>
<td>RO-3306</td>
<td>Inhibition of Cdk1/prevention of MPF destabilisation</td>
<td>Reduction in spontaneous activation</td>
<td>Rat: Prasad et al. (2016)</td>
</tr>
</tbody>
</table>
Specifically, data have been produced that consolidate our findings that melatonin supplementation in vitro reduces ROS accumulation in ageing mouse oocytes to attenuate cytoplasmic fragmentation and mitochondrial dysfunction (Lord et al. 2013, Yang et al. 2018), delay the onset of apoptosis (Lord et al. 2013, Dai et al. 2017, Yang et al. 2018), and consequently improve fertilisation rate (Lord et al. 2013, Dai et al. 2017) and the capacity to support embryo development to the blastocyst stage (Lord et al. 2013, Yang et al. 2018). Importantly, equivalent effects have also now also been demonstrated in porcine oocytes (Chen et al. 2017, Wang et al. 2017b, Nie et al. 2018), and preliminary investigations suggest that melatonin supplementation may improve mitochondrial membrane potential during IVM of human oocytes (Liu et al. 2019). Excitingly, recent investigations suggest that the benefits of melatonin span beyond our original observations, with demonstration that spindle abnormalities are also reduced in aged mouse oocytes following melatonin treatment (Yang et al. 2018), while proteins that are essential to regulate sperm binding (JUNO and Ovastacin) are rescued from mis-localised expression (Dai et al. 2017). Finally, it has been suggested that the beneficial activity of melatonin may extend beyond its capacity to scavenge ROS, with Yang et al. (2018) postulating that melatonin may also exert benefits by driving sustained sirtuin expression in ageing oocytes where it would otherwise experience decline. Certainly, maintenance of sirtuin expression throughout post-ovulatory ageing via the microinjection of Sirt1 mRNA has been shown to attenuate ROS accumulation and cytoplasmic fragmentation, reduce the occurrence of spindle abnormalities, improve mitochondrial function, and delay the increase in H3K9ac and H3K4me2/3 levels that accompany post-ovulatory ageing (Xing et al. 2021). Thus, given the similarity in phenotype between this paradigm and that of melatonin supplementation, it seems apparent that sirtuin modulation is a key component of melatonin’s mechanism of action to prevent in vitro oocyte ageing.

In recent years, considerable interest has also been generated surrounding the capacity of the polyphenol resveratrol to attenuate post-ovulatory ageing. A number of studies have demonstrated that resveratrol can significantly reduce ROS levels in mouse and porcine oocytes, following either supplementation of in vitro culture media (Ma et al. 2015, Sun et al. 2019, Zhou et al. 2019, Abbasi et al. 2021) or i.p. injection in vivo (Liang et al. 2018). As with melatonin, in vitro supplementation of resveratrol has been demonstrated to improve fertilisation rates (Sun et al. 2019) and blastocyst formation rates from aged oocytes (Sun et al. 2019, Zhou et al. 2019, Abbasi et al. 2021). The biochemical mechanism behind this increase in oocyte functionality is thought to include a reduction in spindle abnormalities and aberrant cortical granule distribution, as well as prolonged maintenance of mitochondrial membrane potential, and delayed onset of apoptosis (Ma et al. 2015, Zhou et al. 2019, Abbasi et al. 2021).

Interestingly, evidence has been produced to suggest that resveratrol treatment may promote mitophagy in ageing oocytes to maintain normal mitochondrial quantity and quality (Ma et al. 2020, Peters et al. 2020), with an increase in autophagosome formation being observed in resveratrol treated vs untreated aged oocytes (Zhou et al. 2019). Specifically, the success of this compound in improving the developmental competence of oocytes may be regulated, in part, by forkhead box O3 (FOXO3) (Zhou et al. 2019). FOXO3 regulates mitophagy by targeting several autophagy-related genes such as microtubule-associated proteins 1A/1B light chain 3A (MAP1LC3A; previously LC3). Resveratrol treatment of mouse oocytes was found to increase the expression of FOXO3 and promote autophagy signalling, whereas the inhibition of mitophagy resulted in a subsequent decrease in FOXO3 expression in oocytes (Zhou et al. 2019). Finally, it has also been suggested that the increase in fertilisation rate in resveratrol-treated aged oocytes may be attributed to the stabilisation of JUNO on the oocyte membrane (Sun et al. 2019) and that resveratrol treatment may also prevent a decline in Sirt1 expression (Ma et al. 2015), as was previously reported for melatonin (Dai et al. 2017, Yang et al. 2018); however additional investigation is required to consolidate these findings.

Coenzyme Q10 and its reduced form, ubiquinol-10, have also featured as antioxidants of interest for delaying post-ovulatory oocyte ageing across species. Coenzyme Q10 has been shown to be present in human follicular fluid (Akarsu et al. 2017), suggesting it may play a role in the endogenous promotion of oocyte quality. Indeed, coenzyme Q10/ubiquinol-10 have been shown to reduce ROS levels in mouse oocytes (Zhang et al. 2019) and porcine oocytes (Niu et al. 2020). In both species, this resulted in a reduction in the occurrence of cytoplasmic fragmentation, cytoskeletal/spindle abnormalities, and mitochondrial dysfunction (Zhou et al. 2019, Niu et al. 2020). In mouse oocytes, a connection was again established between the alleviation of oxidative stress and sustained localisation of the sperm-binding proteins JUNO and ovastacin (Zhang et al. 2019). Experimentation with this antioxidant has also added credence to the role of mitophagy in the maintenance of oocyte function (Niu et al. 2020). Work conducted in porcine oocytes demonstrated that ubiquinol-10 can also promote mitochondrial ‘renewal’ via mitophagy as well as decrease oxidative stress (Niu et al. 2020). Consistent with this hypothesis, post-ovulatory ageing induced a significant decrease in mitophagy-related proteins Parkin RBR E3 ubiquitin protein ligase (PRKN) and PTEN-induced putative kinase 1 (PINK1) as well as proteins involved in mitochondrial biogenesis such as sirtuin 1 (SIRT1) (Niu et al. 2020). Cumulatively, the benefits of coenzyme Q10/ubiquinol-10 supplementation resulted in improved

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bластоцистные образования от стареющих яйцеклеток (Zhang et al. 2019, Niu et al. 2020).

Although melatonin, resveratrol, and coenzyme Q10 have been the most widely investigated antioxidants for the amelioration of post-ovulatory oocyte ageing, a number of alternative compounds have also been trialled for this purpose with varying degrees of success. Since 2013, investigators have examined the merits of the garlic extract Allicin (Park et al. 2019), the traditional East Asian medicine Artemisia asiatica (Jeon et al. 2017), the l-cysteine derivative, N-acetyl-cysteine (Li & Cui 2016, Wang et al. 2019), the reactive thiol penicillamine (Lord et al. 2015), and the plant-derived flavonoids 4,4'–dimethoxychalcone (Liu et al. 2022) and quercetin (Wang et al. 2017a) (Table 1). Particularly impressive results have been produced using quercetin, with blastocyst formation rates of treated oocytes aged for 12 h in vitro closely resembling that of ‘fresh’ oocytes (Wang et al. 2017a). Common to the effects of other antioxidants discussed in detail above, quercetin supplementation quenched ROS accumulation and thus the onset of morphological abnormalities and apoptosis. Quercetin also sustained MPF activity in ageing oocytes, decreased the incidence of spindle abnormalities and abnormal mitochondria, and appeared to attenuate epigenetic changes in the oocyte, preventing a decline in H3K9me3 levels with post-ovulatory ageing (Wang et al. 2017a).

Finally, a recent study has heralded the benefits of bezafibrate to prevent in vitro ageing in porcine oocytes (Kim et al. 2021). Although not an antioxidant itself, bezafibrate acts as an agonist for the transcription factor peroxisome proliferator activated receptor alpha (PPARA), or as a coactivator of peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (PPARGC1A), which are known to influence biological pathways involved in lipid oxidation (Nikolić et al. 2012), mitochondrial biogenesis (Nikolić et al. 2012), and ROS scavenging (Baldelli et al. 2014). As such, bezafibrate treatment was shown to reduce ROS accumulation in in vitro aged porcine oocytes, leading to an attenuation of the onset of oocyte apoptosis and improved blastocyst quality following delayed fertilisation (Kim et al. 2021).

It is strikingly apparent that antioxidant supplementation holds exciting potential to delay the onset of post-ovulatory oocyte ageing in vitro and that these benefits translate across a multitude of species. Further, recent findings have demonstrated that the utility of antioxidants such as melatonin and resveratrol transcend their ability to directly scavenge ROS, with the modulation of sirtuin expression and mitophagy/autophagy pathways also appearing to play key roles. With further development of such antioxidant supplementation strategies, the feasibility of re-insemination techniques in the clinic (i.e. rescue ICSI) may be greatly improved, holding potential to increase the live-birth rate per initiated IVF cycle. Further, the ability to prevent oocyte degeneration during the long periods in vitro culture required for maturation and fertilisation of bovine and porcine oocytes would hold significant benefit in the agricultural sector, again maximising the live-birth rate per oocyte collection.

**Conclusion**

In contrast to their inherent longevity within the ovarian microenvironment, oocytes display a limited post-ovulation functional lifespan before succumbing to a well-defined degenerative process that culminates in reduced fertilisation rates, poor embryo quality, post-implantation errors, and abnormalities in the offspring. This progressive demise in oocyte quality, which occurs in the contexts of both maternal and in vitro post-ovulatory ageing, has increasingly been linked with oxidative stress, an insult that initiates and propagates damage to the integrity of the oocyte's cargo of organelles, lipids, proteins, and DNA. As a susceptible link between the environment and genes, the oocyte's epigenome has also recently been recognised as being vulnerable to the post-ovulatory ageing phenotype, with alterations to DNA methylation and histone modification profiles having now been implicated in the control of nuclear architecture, regulation of transcription, and even chromosomal alignment in the oocytes of a variety of species. Importantly, such molecular insights into the aetiology of post-ovulatory ageing are helping to inform the development of effective therapeutic interventions centred on the application of reagents that diminish the burden of oxidative stress and thus prolong the functional lifespan of the post-ovulatory oocyte. Ultimately, the continued refinement of such strategies holds considerable promise in terms of improving oocyte integrity during ART procedures.

**Declaration of interest**

R J A is scientific director of the biotechnology company, Memphasys Ltd. R J A is an associate editor of *Reproduction*. R J A was not involved in the review or editorial process for this paper, on which he is listed as an author. The other authors have nothing to disclose.

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