OXIDATIVE STRESS AND REPRODUCTIVE FUNCTION

Reactive oxygen species in the mammalian pre-implantation embryo

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Abstract

In brief: Reactive oxygen species are generated throughout the pre-implantation period and are necessary for normal embryo formation. However, at pathological levels, they result in reduced embryo viability which can be mediated through factors delivered by sperm and eggs at conception or from the external environment.

Abstract: Reactive oxygen species (ROS) occur naturally in pre-implantation embryos as a by-product of ATP generation through oxidative phosphorylation and enzymes such as NADPH oxidase and xanthine oxidase. Biological concentrations of ROS are required for crucial embryonic events such as pronuclear formation, first cleavage and cell proliferation. However, high concentrations of ROS are detrimental to embryo development, resulting in embryo arrest, increased DNA damage and modification of gene expression leading to aberrant fetal growth and health. In vivo embryos are protected against oxidative stress by oxygen scavengers present in follicular and oviductal fluids, while in vitro, embryos rely on their own antioxidant defence mechanisms to protect against oxidative damage, including superoxide dismutase, catalase, glutathione and glutamylcysteine synthetase. Pre-implantation embryonic ROS originate from eggs, sperm and embryos themselves or from the external environment (i.e. in vitro culture system, obesity and ageing). This review examines the biological and pathological roles of ROS in the pre-implantation embryo, maternal and paternal origins of embryonic ROS, and from a clinical perspective, we comment on the growing interest in combating increased oxidative damage in the pre-implantation embryo through the addition of antioxidants.

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Introduction

The formation of a healthy embryo occurs through the tight regulation of cellular processes combining the genetic and cellular components of eggs and sperm at fertilisation to form a multitude layer of specialised fetal, placental and extra-embryonic cells (blastocyst) prior to implantation. Reactive oxygen species (ROS) are generated throughout the pre-implantation period as a by-product of mitochondrial metabolism. ROS are generally thought of as damaging, causing oxidation of lipids, nucleic acids and macromolecules resulting in embryonic arrest/death. However, at lower levels, ROS are essential signalling molecules capable of cellular communication through redox modifications to generate specific cellular responses critical to early embryo development. Pre-implantation embryonic ROS may originate from eggs, sperm and embryos or from the external environment (i.e. in vitro culture). In this review, we will examine both biological and pathological roles of ROS in the pre-implantation embryo, maternal and paternal origins of embryonic ROS and from a clinical perspective, we comment on the growing interest for combating increased oxidative damage in the pre-implantation embryo through the addition of antioxidants.
ROS formation in the pre-implantation embryo

ROS refer to oxygen-containing molecules that have a spare electron or an unstable bond such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻), hydroxyl (OH⁻) and peroxy radicals (Shields et al. 2021). The spare electron or unstable bond in these molecules allows them to detrimentally interact with macromolecules such as lipids, proteins, carbohydrates and nucleic acids (Shields et al. 2021). However, vital early embryonic events required for successful implantation and ongoing pregnancy are dependent on biological concentrations of ROS, including sperm-mediated oocyte activation and pronuclear formation (Lopes et al. 2010, Morado et al. 2013), cellular proliferation and first embryonic cleavage events, compaction and blastulation through regulation of key transcription factors (i.e. NF-κB) (Leite et al. 2017, Jamil et al. 2020) as well as blastocyst hatching (Nasr-Esfahani & Johnson 1991, Thomas et al. 1997).

The redox balance within the early stages of embryo development is tightly controlled for the optimal formation of a healthy conceptus (Fig. 1). Many forms of ROS are found in embryos all of which have different biological targets with their own spectrum of reactivity (Jamil et al. 2020). Approximately, 90% of ROS produced in human and mouse embryos arises as a by-product of mitochondrial metabolism through oxidative phosphorylation, although other ROS-generating pathways including NADPH oxidase (NOX) and xanthine oxidase are also present in the pre-implantation embryo (Guerin et al. 2001, Leese 2012) (Fig. 1). Oxidative phosphorylation produces O₂⁻ through the one-electron reduction of oxygen. Within this pathway, the final electron acceptor is O₂ and in perfect circumstances will be reduced to H₂O; however, as this process is not completely efficient some O₂⁻, peroxide dianions and OH⁻ radicals are created as by-products, of which, O₂⁻ is readily dismutated into H₂O₂ (Murphy 2009, Cobley 2020). Inhibition of this

![Figure 1](https://rep.bioscientifica.com)

Figure 1 ROS production in the pre-implantation embryo. Biological concentrations of ROS in the pre-implantation embryo are required for mediating important early embryo events including: (1) sperm-mediated oocyte activation, (2) cleavage and embryo proliferation, (3) regulation of transcription factors and (4) blastocyst hatching. This is tightly controlled, with majority of ROS in embryos produced through oxidative phosphorylation in the mitochondria, which produces superoxide (O₂⁻) as a by-product. This can be reduced by superoxide dismutase (SOD) into hydrogen peroxide (H₂O₂) and hydroxyl radical (OH⁻) through the Fenton reaction. Glutathione (GSH) is the main non-enzymatic defence system for ROS in embryos reducing H₂O₂ through the reduction of 2GSH to GSSG. H₂O₂ and O₂⁻ can also be produced by NADPH oxidase through catalysed by NADPH to oxygen (O₂) and O₂⁻ and as a by-product through the hydroxylation of hypoxanthine to xanthine then xanthin to uric acid by xanthine oxidase.
pathway through antimycin A and NaN3 is known to reduce ROS formation and oxygen consumption in bovine pre-implantation embryos (Thompson et al. 2000). Similarly, other enzymes in embryos produce ROS as a by-product, including xanthine oxidase and cytochrome P450 reductase (Filler & Lew 1981, Alexiou & Leese 1992). In bovine embryos, inhibitors of xanthine oxidase, an enzyme catalysing the production of O$_2^-$ through hydroxylation of hypoxanthine to xanthine then xanthine to uric acid, such as allopurinol and oxypurinol, are able to reduce the peroxide rise that occurs throughout development, without causing any adverse morphological changes to the cleaving embryo (Iwata et al. 1999). Unlike other ROS-generating pathways, NOX specifically makes ROS in the form of H$_2$O$_2$ or O$_2^-$ as the intended output rather than as an accidental by-product (Maraldi et al. 2021). NOX catalyses the production of O$_2^-$ through the transfer of electrons from NADPH to oxygen and has been described to produce O$_2^-$ and H$_2$O$_2$ on rabbit blastocyst cell surfaces (Manes & Lai 1995). Inhibition of NOX by diphenylene iodonium resulting in a concentration-dependent reduction in intracellular embryonic ROS concentrations in mice (Nasr-Estahani & Johnson 1991). Despite the importance of understanding redox balance in the pre-implantation embryo, this area of research continues to be neglected. The reasons for which are uncertain but may include the shift from discovery to translational research in recent years.

The level of ROS produced by a pre-implantation embryo changes throughout cleavage, compaction and blastocyst formation, and mirrors the changing metabolic requirements of the developing embryo (Hajian et al. 2017). Carboxylic acid-based metabolism is the main metabolic pathway used in early cleavage stage mouse and human embryos, where pyruvate uptake is high, lactate production is high and oxygen consumption and carbon dioxide production is low (Houghton et al. 1996, Gardner et al. 2000, Trimarchi et al. 2000). As such, the developing embryo prior to compaction is relatively metabolically quiet, which is reflective in its moderate to low ROS production. This is in contrast to the compacting/blastocyst embryo where glucose uptake and lactate production increase as well as a number of aerobic pathways, causing an overall net increase in oxygen consumption and oxidative phosphorylation (Houghton et al. 1996, Gardner et al. 2000, Trimarchi et al. 2000). This is thought to be due to the increasing energy demands of cells within the developing blastocyst as they rapidly proliferate and begin to differentiate into their embryonic cell lineages requiring high levels of ATP and biosynthesis. This increase in metabolic activity coincides with an increased ROS production in the blastocyst (Fig. 1).

In vivo embryos seem to be protected against oxidative stress by oxygen scavengers present in follicular and oviductal fluids, with these fluids rich in antioxidant-producing compounds such as vitamin A, C and E, pyruvate, taurine, GSH and cysteamine (Carbone et al. 2003, Oyawoye et al. 2003, Ozkaya & Naziroglu 2010). In vitro, derived embryos have to rely on their own antioxidant defence mechanisms to protect against oxidative damage outside what is provided in current culture media, including SOD, catalase (CAT), GSH and glutamylcysteine synthetase (Guérin et al. 2001), with concentrations of GSH in bovine blastocysts positively correlated to their viability (de Matos et al. 1996). Interestingly, the persistent, embryonic two-cell (mouse), four-cell (human and pig) and eight-cell (cow) block at zygotic genome activation (ZGA) in many mammalian culture systems is suggested to be due to an increase in ROS from in vitro culture conditions (Memili & First 2000, Meirelles et al. 2004, Kimura et al. 2010), as the genome is most sensitive to ROS at this time point (Mu et al. 2011). Prior to ZGA, the zygote remains transcriptionally silent while epigenetic reprogramming of the maternal and paternal genomes takes place (Li et al. 2013, Xu & Xie 2018). Following this, transcriptional control returns to the developing embryo (maternal-to-zygotic transition) which coincides with the degradation of maternal and paternal products (Li et al. 2013) and slowing down of cellular division to allow time for replication. This allows cells enough time to prepare for their next division and ultimately gastrulation and as such embryos at this stage are more sensitive to ROS (Zhang et al. 2014). The addition of antioxidant mimicking molecules to the modern culture system coupled with low oxygen tension has been shown to be successful in preventing the ZGA block (Legge & Sellens 1991, Meirelles et al. 2004, Svoboda 2018). Thus, tightly controlled redox balance is vital for normal embryo cleavage and blastocysts formation which is primarily regulated through embryonic metabolic pathways.

**ROS on embryo and fetal development**

Any deviation from redox homeostasis in the form of either excess accumulation of ROS or highly reduced conditions can result in oxidative stress and pathological development of the early embryo and fetus (Guérin et al. 2001). An increase in embryonic oxidative stress can occur from both endogenous and exogenous factors, such as inflammation, mitochondrial impairment, maternal diabetes, ageing, environmental agents and xenobiotic compounds (Guérin et al. 2001). This increase in cellular ROS ultimately leads to excess production of oxygen radicals, and oxidation and glycation of macromolecules and antioxidant enzymes, limiting the cellular capacity for ROS detoxification and impeding the antioxidant defence systems needed to maintain normal function (Matough et al. 2012).

Studies from animal models, which allow manipulation of the embryonic environment, have shown that GSH...
specifically appears to be the main non-enzymatic defence system against ROS in mammalian embryos (Yoshida et al. 1993, de Matos et al. 1996, Sutovsky & Schatten 1997, Gardiner et al. 1998, Perreaud et al. 1988, de Matos et al. 2002, Luciano et al. 2006). Gardiner and Reed (1994) found there is a 10-fold decrease in the total GSH content during mouse pre-implantation embryo development, with maximum levels in the pre-fertilisation oocyte, then decreasing to minimal levels by the blastocyst stage, while ROS levels remain relatively constant (Nasr-Eslahani et al. 1990), suggesting that this developmental phase is highly vulnerable to oxidative insults. Indeed, oxidative distress during this window reduces the blastocyst’s inner cell mass (Lane & Gardner 1997), decreases embryo viability after transfer (Lane & Gardner 1997) and alters the early establishment of the different cell lineages (Kwong et al. 2000), which are then linked to altered fetal development (Lea et al. 1996, Brown et al. 2018). Further evidence comes from a study by Bueno et al., where even a mild state of maternal diabetes caused abnormal redox levels that were linked to embryonic loss pre- and post-implantation in rats (Bueno et al. 2020). These embryos had lower GSH concentrations but high H$_2$O$_2$ levels at the morula stage and an increased frequency of abnormal morphology, namely empty zona pellucidas, and delayed and degenerating blastocysts (Bueno et al. 2020), indicating a reduction in antioxidant capacity and therefore, their ability to counteract oxidative insults.

Not surprisingly, only a few genes have influence in maintaining redox homeostasis in the early embryo (Ufer et al. 2010); however, their impairment by ROS can have dramatic consequences. For instance, mouse embryos where the small antioxidant protein Trx gene has been knocked out do not develop to the blastocyst stage (Matsui et al. 1996), while those lacking the redox-modulating enzyme Ref1 die shortly after blastocyst formation and are unable to implant (Xanthoudakis et al. 1996). Several FOXO proteins (FOXO1, 3 and 4) have recently been identified as essential in maintaining healthy embryonic ROS levels (Kuscu et al. 2019). Kuscu et al. showed that the downregulation of these transcription factors induced an almost 80% increase in cytoplasmic ROS levels in embryos, which was linked to developmental blocks at the 2-cell and blastocyst stages, and an upregulation of apoptotic genes at the 2-cell stage in mice (Kuscu et al. 2019). The influence of ROS on epigenetic mechanisms is also arising as a key factor in developmental pathologies. Abnormal methylation of Pax3, essential for neural tube closure during embryogenesis, has been linked to increased pre-implantation embryo ROS levels in mice and humans (Wei & Loeken 2014, Lin et al. 2019).

There is increasing evidence that exposure to oxidative stress during the pre-implantation period can alter embryo programming (Loeken 2004, Ornoy 2007), increasing the likelihood of disease later in life, as well as have transgenerational effects. Excess ROS exposure during pre-implantation embryonic development is linked to fetal growth restriction (Morrison 2008, Nowaczyk et al. 2022) and fetal hypoxia (Zhu et al. 2021), which then correlates with deleterious effects to fetal brain (Tachibana et al. 2019, Yawno et al. 2019), heart (Baschat et al. 2000, Morrison 2008, Nowaczyk et al. 2022), liver (Radford & Han 2020) and kidney (Kamianowska et al. 2019) in mice, sheep, humans and cows.

A major consequence of increased ROS in embryos is DNA damage. ROS can interact with both the pyrimidine and purine bases in DNA leading to radical products. One product, 8-hydroxyguanine, is especially detrimental as it is able to interact with the 2’-deoxyribose in DNA causing double- and single-strand breaks (Cooke et al. 2003). These lesions are usually repaired in the embryo through various mechanisms including base excision repair (BER) (Olsen et al. 2001, Smith et al. 2013), mismatch repair (Zheng et al. 2005, Tichy et al. 2011), nucleotide excision repair (Jaroudi et al. 2009), single-strand break repair (Zheng et al. 2005), double-strand break repair (Derijck et al. 2008, Jaroudi et al. 2009) (homologous recombination, non-homologous end joining) and nucleotide excision repair dependent on the nature of the damage. Following fertilisation, the early pronuclear embryo upregulates enzymes from the BER repair pathway to allow any oxidative DNA damage delivered by the oocyte/sperm to be repaired prior to the initiation of DNA replication (S-phase) (Lord & Aitken 2015, Khokhlova et al. 2020). Unrepaired DNA damage at this stage of development has been shown to cause delays in demethylation and mosaicism and heterogeneous cleavage in bovine embryos and DNA replication in mice embryos (Gawecka et al. 2013, Wyck et al. 2018, Middelkamp et al. 2020). Following ZGA (2-cell mouse, 4-cell (human, pig) and 8-cell (cow) (Svoboda 2018), the embryo utilises robust apoptotic pathways including TRP63, TRP53, TRP21, BCL--like protein 4 and RAD18 to prevent the inheritance of DNA damage (Musson et al. 2022). In addition to DNA damage repair and apoptotic pathways, the embryo can also utilise autophagy (Tsukamoto et al. 2008), with autophagy closely linked with DNA repair, and used frequently to combat mitochondrial DNA damage (Rambold and Lippincott-Schwartz 2011). There are three main outcomes of DNA damage in embryos: (i) it is either repaired, (ii) transferred to offspring or (iii) is so severe that it caused embryonic arrestment, failed implantation or miscarriage (Musson et al. 2022). While the latter is the more severe outcome, the accumulation of de novo mutations in offspring has been attributed to a number of developmental disorders and congenital malformations in humans (Deciphering Developmental Disorders 2017), making the formation of DNA damage in the pre-implantation embryo sub-optimal.
Taken together, the evidence indicates that perturbations to the redox balance during early development can severely impact embryo growth, implantation, pregnancy and offspring health.

**Maternal origins of increased ROS in embryos**

Factors delivered by sperm and oocytes at fertilisation are also able to modify both ROS production and susceptibility in the pre-implantation embryo. Oocyte developmental competence (ODC) and ability to achieve pregnancy are highly responsive to changes in maternal physiology. In particular, both maternal obesity and advanced maternal age are associated with increased time to pregnancy, lower rates of assisted reproduction technology (ART) success and poorer ODC as a result of oxidative stress.

Mouse models of obesity have shown that increased embryo ROS levels originate in the oocyte and persist throughout embryo development even when the oocyte is taken out of the obesogenic environment and cultured in vitro. Oocytes from obese females have altered mitochondrial activity (indicated by mitochondrial membrane potential) (Igosheva et al. 2010, Junghem et al. 2010) and increased ROS concentrations causing the amplified incidence of spindle and/or chromosomal abnormalities (Wu et al. 2015, Zhang et al. 2015, Han et al. 2017, Yang et al. 2021), irregular oocyte mitochondrial morphology (abnormal clustering/localisation, greater observations of broken membranes, decreased cristae, increased swelling, greater number of vacuoles) (Junghem et al. 2010, Luzzo et al. 2012, Hou et al. 2016) and decreased mitochondrial mass (Andreas et al. 2019), leading to reduced oocyte viability (Wu et al. 2015). These deficits in mitochondrial function can also be transferred to offspring causing lasting effects over generations (Saben et al. 2016). Further, oocytes derived from obese females also display redox states depictive of oxidative stress, including decreased GSH levels (Yang et al. 2021), lower NADPH and higher FAD2+ levels (Igosheva et al. 2010), increased SOD, glutathione peroxidase and CAT gene expression (Hou et al. 2016). This results in delayed on-time two-cell, four-cell, and blastocyst development rates, decreased blastocyst cell number, decreased mitochondrial mass, and increased mitochondrial membrane potential (Igosheva et al. 2010, Wu et al. 2015, Zhang et al. 2015, Han et al. 2017, Andreas et al. 2019, Yang et al. 2021).

Further, evidence implicating ROS as one of the main drivers in obesity-related poorer ODC and reduced pre-implantation embryonic quality comes from studies supplementing in vivo culture media or administering in vivo treatment (while maintaining obese state) with antioxidants. Oocytes analysed after in vivo antioxidant treatment (melatonin or cerium oxide nanoparticles) have decreased oocyte ROS levels, reduced incidence of spindle/chromosome abnormalities and improved cellular redox status (increased GSH level) (Han et al. 2017, Yang et al. 2021). Importantly, whether treatment was given in vivo or in vitro (mito-esculein), antioxidant supplementation improved embryo development rates, as well as normalising mitochondrial mass and membrane potential in blastocyst, while increasing blastocyst cell number (Andreas et al. 2019). Thus, strengthening the argument for increased ROS and its associated damage to oocytes from maternal obesity as one of the primary sources of oxidative stress in embryos, with either in vivo or in vitro antioxidant use able to ameliorate many of the negative effects of obesity on pre-implantation embryo health.

Similar to obesity, advanced maternal age is also associated with changes to ODC that results in increased ROS production throughout pre-implantation embryo development. Increased maternal age has been widely associated with alterations to oocyte mitochondria, including swelling, vacuolisation and cristae abnormalities, which are understood to impair function and contribute to high levels of ROS in pre-implantation embryos (Muller-Hocker et al. 1996). Studies using mouse models of advanced maternal age found that oocytes derived from older females have higher levels of cytosolic ROS and lipid peroxidation resulting in embryos with lower mitochondrial membrane potential and higher ROS concentrations at the two-cell and four-cell stage, and higher ROS concentrations at the morula, and blastocyst stage embryo (Mihalas et al. 2017). Aged mice show a decline in nicotinamide adenine dinucleotide (NAD+), an important factor in maintaining redox homeostasis; this decline leads to lower oocyte numbers and fertilisation rates which can be rectified with the addition of NAD+ precursors at low concentrations (Bertoldo et al. 2020, Guo et al. 2022). Similar to that for maternal obesity, antioxidant supplementation in vitro to both eggs and embryos generated from women of advanced maternal age is able to decrease both oocyte and embryonic ROS levels (Mihalas et al. 2017). The addition of in vitro maturation media with melatonin was able to decrease oocyte ROS concentrations compared with controls. Studies adding antioxidants (including α-lipoic acid, α-tocopherol, hypotaurine, N-acetylcysteine and sirtuin, or resveratrol) to in vitro embryo culture media were able to improve two-cell and four-cell mitochondrial membrane potential, rates of blastocyst development, as well as pregnancy and implantation rates upon transfer to surrogates from embryos derived from female mice of advanced age (Silva et al. 2015, Yoon et al. 2020). Further, antioxidant treatment normalised ROS concentrations in four-cell, morula and blastocyst embryos to that of young controls, with decreased blastocyst expression of genes activated in response to oxidative stress (Foxo3a, Glrx2 and P38k) (Silva et al. 2015). Thus, advanced maternal age negative effects to both ODC and pre-implantation...
embryonic health is in part related to an unbalanced redox state.

Therefore, the oocyte is a major source of embryonic ROS and oxidative stress, with maternal states (such as obesity or advancing age) being a major influence. Importantly, supplementation of antioxidants, whether in vivo or added to culture media, can negate many of these adverse effects further implicating the role of ROS in many maternal reproductive pathologies.

**Paternal origins of increased ROS in embryos**

As the main source of ROS in the pre-implantation embryo occurs as a by-product of mitochondrial metabolism, it is widely believed that there would be little effect of fathers/sperm in the generation of embryonic ROS, as mitochondria are inherited from mothers. However, there is emerging evidence in animal models that suggests paternal pathologies can alter non-genetic components delivered by sperm at fertilisation which can modify early pre-implantation metabolism and induce oxidative stress phenotypes in embryos (Chen et al. 2016, Champroux et al. 2018).

One such example is paternal obesity. Male obesity at conception is not only associated with perturbed embryo development (Mitchell et al. 2011, Binder et al. 2012a, McPherson et al. 2013) but also associated with disrupting cell segregation and proliferation (McPherson et al. 2013), embryo metabolism (Binder et al. 2012a, b), placental gene expression (Binder et al. 2015, McPherson et al. 2015, Mitchell et al. 2017) and fetal growth in mice (Mitchell et al. 2011, Binder et al. 2012a, McPherson et al. 2013). While concentrations of ROS themselves in embryos from paternal obesity have not been measured directly, other cellular indicators suggest that ROS concentrations would be increased. For example, Binder et al. (2012b) found that four-cell embryos produced from paternal obesity have reduced mitochondrial membrane potential (MMP), with MMP driving embryonic energy production through oxidative phosphorylation. This coincided with an increase in pyruvate uptake, which could be a compensatory mechanism for reduced mitochondrial function, or alternatively sequestered by the embryo due to its antioxidant properties to combat increased oxidative stress from low MMP and electron leakage (Binder et al. 2012a, b). Further, we have evidence from our paternal obesity model that changes to embryo metabolism and associated oxidative stress in blastocysts arise following in vitro culture (Fig. 2). Using a radioactive glucose isotope coupled with a hanging drop and TUNEL assay paternal obesity-derived blastocysts showed increased glucose metabolism even after controlling for their reduced cell number (Fig. 2) and this coincided with an increased apoptotic cell number (an indicator of oxidative stress; Fig. 2). High rates of glucose metabolism in blastocysts are seen in response to oxidative stress in embryos and indicate reduced embryo viability (Lane & Gardner 1996). In another study, paternal obesity at conception was associated with upregulation of Samd4 in the blastocyst which is a known transcription factor and a tumor suppressor, with its transcription previously shown to be strongly associated with oxidative stress and ROS concentrations (Bernhardt et al. 2021). This provides evidence that paternal lifestyle/environmental insults such as obesity are able to modify pre-implantation metabolism, oxidative stress and subsequent embryo

**Figure 2** Paternal obesity increases blastocyst glucose metabolism (A) which coincides with an increase in blastocyst apoptotic cells (B). Male C57BL6 mice (5–6 weeks) were fed either a high-fat diet (HFD, 21% saturated fat, n = 4) or a control diet (CD, 6% monosaturated fat, n = 4) for 10 weeks. Males were then mated with superovulated C57BL6 female mice (3–4 weeks) and zygotes were collected 22–24 h post superovulation. Embryos were cultured for 92 h in Vitrolife G-Series at 37°C at 5% CO2 and 6% CO2. Glucose metabolism (activity of the glycolysis pathway) was assessed by blastocyst incubated in a G1 medium lacking glucose but with the addition of 3.15 mM (0.25 μCi/μL) [U-53H]-glucose. Glucose metabolism was calculated based on the recovery efficiencies of the radiolabelled substrate and expressed as picomoles per embryo per cell over 3 h. Blastocyst cellular apoptosis was measured by the TUNEL assay. Individual dots represented a different blastocyst.
quality in vitro which, must be controlled through components delivered by sperm at fertilisation.

How components in sperm are able to modify early embryonic events and increase oxidative damage to the pre-implantation embryo has yet to be elucidated. Although, it is known that sperm deliver a suite of non-genetic components including a long list of RNAs, proteins and histone and DNA modifications at conception which are necessary for early embryonic events such as, first cleavage and embryonic genome activation (Chen et al. 2016, Champroux et al. 2018). As many of the enzymes that control early epigenetic remodelling of the male and female pronucleus are dependent on mitochondrial-derived metabolic co-factors (i.e. Tets, KDMs, DNMTs; (Santos 2021), any modifications to the metabolic control of the early embryo could have profound effects to its epigenetic signature. For instance, in our mouse model of paternal obesity, the ratio of active (H3K4me3) to repressive histone (H3K27me3) marks was increased in two-cell embryos generated from males fed an HFD diet, and this coincided with reduced mitochondrial function, perturbed embryo development, aberrant expression of key metabolic genes in fetal placentas and reduced fetal weights at embryonic day 18 (McPherson et al. 2015). Further evidence for the role of sperm in the generation of ROS in the pre-implantation embryo comes following sperm-oocyte binding. Oocyte activation following fertilisation is ROS-dependent and is triggered by sperm through the stimulation of mitochondrial respiration from calcium oscillations (Morado et al. 2013). The same ROS-dependent increases following fertilisation are not seen in parthenogenetically activated oocytes (Morado et al. 2013). Thus, the health and quality of the sperm delivered at fertilisation plays an important role in the early activation of embryo metabolism and the control and regulation of epigenetic signatures. Perturbations to these sperm cellular components likely contributes to increased susceptibility of the embryo to oxidative stress. Although, further research is warranted in this area.

ROS in the IVF laboratory

An optimal culture environment for gametes and embryos is essential for a successful ART laboratory. As mentioned, ROS production occurs mainly as a by-product of embryo metabolism; however, embryo culture conditions are also known to modify ROS production and cause oxidative stress. This in turn can result in reduced fertilisation rates, poor embryo development and ultimately reduced pregnancy rates following uterine transfer (Guerin et al. 2001, Bedaiwy et al. 2004). Culture conditions such as oxygen tension, light, pH, temperature and cell media content can lead to increased ROS production (Fig. 3).

Interestingly, the level of oxygen used to culture embryos can vary between clinics and continents (7–60%) culturing embryos at atmospheric levels (20%) instead of the recommended hypoxic conditions (5%); (Christianson et al. 2014). At standard incubator conditions of 37°C, media equilibrated to atmospheric conditions contain significantly higher concentrations of O2 compared with physiological levels (du Plessis et al. 2008). Embryos cultured at high oxygen levels have shown to cause mass gene deregulation in mice, including genes required for cell growth and gastrulation (Belli et al. 2020) as well as modifications to methylation and lower blastocyst rates in bovine embryos (Li et al. 2016, Belli et al. 2020). Culturing embryos at atmospheric oxygen concentrations also enhances the activity of oxygen-dependent oxidase enzymes, generating ROS at a faster rate inducing oxidative stress and reducing embryo on-time development (Guerin et al. 2001, Kovacic & Vlassavljevic 2008).

Another cause of ROS in the ART lab is the exposure of embryos to light. Throughout the in vitro culture process, exposure of the embryos to light is unavoidable as it is required for the handling process. Animal studies have demonstrated that light at wavelengths of 400–500 nm can induce embryo photodynamic stress and increase ROS production, creating negative impacts on embryo development resulting in fewer blastocysts being formed, while light at longer wavelength appears to have less of an effect on human embryos (Goto et al. 1993, Oh et al. 2007). A recent study in mice found that embryos exposed to yellow light (590 nm) had reduced blastocyst rates, while those exposed to green (520 nm), blue (470 nm) or red (620 nm) light had an increase in γH2AX-positive cells (an indicator of DNA integrity), which was associated with reduced pregnancy rates and fetal weights following embryo transfer (Campugan et al. 2022). Therefore, the use of filters on microscopes and minimising handling of embryos and gametes are important strategies to minimise the effect of light on embryo development (Guerin et al. 2001).

The type of embryo culture media used by an IVF clinic can assist in decreasing the amounts of ROS produced by the embryo. Metallic ions present in embryo culture media enhance ROS production through increased NOX activity (Shahid et al. 2014), whereas proteins such as serum albumin, chelate these heavy metals and act as antioxidants, reducing oxidative stress (Iwata et al. 1998). The pH and temperature of media are also important, as pH affects how enzymes, such as those required for oxidant control, function; this is especially important in the low CO2 environment and as such, a buffer is required (Guerin et al. 2001). Currently, embryo culture media is based on a balanced salt solution, a sodium bicarbonate buffering system, serum albumin, amino acids and vitamins (Morbeck et al. 2014). However, these differ with some being more effective than others in minimising the amount of ROS produced in the media (Shih et al. 2014).
Oxygen concentration, minimising light exposure and media selection are all important components in an ART lab to consider in order to minimise ROS and optimise embryo development (Fig. 3). Another important component of an IVF lab is embryo selection for transfer which is currently determined on morphology assessment. The limitations with this are that there is inter-observer variability; thus, non-invasive tools for embryo selection that can be utilised prior to embryo transfer, in conjunction with morphologic assessment could be of benefit. The development of a thermochemiluminescence assay which measures oxidative stress in biological fluids has been used in multiple studies to determine if there is an association between embryo quality and ROS levels in spent culture media (Wiener-Megnazi et al. 2011, Lee et al. 2012). Most of the studies concluded that ROS concentrations may be an effective tool to assist in embryo selection. However, a more recent study published by Lan et al. (2019) suggested a limited relationship between ROS concentrations as measured by chemiluminescence with embryo quality and blastocyst formation. As such, larger prospective randomised studies are warranted to determine the clinical utility of embryo ROS concentrations as a marker of embryo viability.

**The role of in vivo and in vitro antioxidants in pre-implantation embryo development**

Antioxidants are natural (such as those present in fruits and vegetables) or synthesised molecules that inhibit or delay the oxidation of molecules either through scavenging or by chelation of redox radicals (Neha et al. 2019). Their use orally to improve gamete health and pre-implantation embryo development is growing in popularity, especially in those couples undergoing ART (Agarwal et al. 2014). They are usually given as a single or combined daily supplement as a first-round treatment for couples experiencing infertility as they are low cost and readily available (Agarwal et al. 2014, 2021). Common antioxidants consumed by couples experiencing sub/infertility include melatonin, Coenzyme Q10, vitamins E, B and C, zinc, folate, resveratrol, carotenoids, selenium and cysteine (Ruder et al. 2014, Majzoub &
Agarwal 2018). Despite their routine use, the evidence supporting their benefit is still controversial. While positive effects for improving sperm ROS concentrations in sub-fertile men are well documented (Agarwal et al. 2021), the recent Cochrane review shows no effect on embryo development or pregnancy outcomes (OR 1.38, 95% CI 0.89–2.16) after removal of studies with high bias (Smits et al. 2019). Similar outcomes have also been reported in women, where findings were unsure whether oral antioxidant supplementation would be of any benefit to fertility in sub-fertile women, although clinical pregnancy rates may be increased (OR 1.65, 95% CI 1.43–1.89) (Showell et al. 2020). This was partially due to the poor-quality studies, with most studies reporting a serious risk of bias due to poor reporting of methods (imprecisions and inconsistencies) (Showell et al. 2020). Therefore, well-designed randomised placebo-controlled trials are needed in both men and women to determine the utility of oral antioxidants for improving embryo and pregnancy outcomes in couples undergoing ART. Further, we still need a better understanding of the types, combinations and doses of oral antioxidants and their interactions prior to prescription of their clinical ART use.

The use of in vitro antioxidants in embryo culture has gained much more attention in recent years, due to the simple composition of embryo culture media that lack a number of antioxidant-containing molecules normally present throughout the female reproductive tract. The inclusion of compounds, including resveratrol, melatonin, Coenzyme Q10, B-Vitamins, Vitamin C and Vitamin E, ascorbic acid, alpha-lipoic acid, baicalin, apigenin, β-cryptoxanthin, C-phycocyanin, quercetin, retinol, Royal Jelly and l-carnitine, to embryo culture media has been shown to be beneficial for embryo on-time development, blastocyst survival post cryopreservation and blastocyst cell numbers in a wide variety of mammals (i.e. mouse, sheep, cows and cats), via their ability to reduce embryo intracellular ROS concentrations (Budani & Tiboni 2020, Zarbakhsh 2021). For instance, the antioxidant combination of acetyl-l-carnitine, N-acetyl-l-cysteine and α-lipoic acid, when present in embryo culture media, was able to increase mouse pronuclear, cleavage and blastocyst on-time development and increased blastocyst trophoderm cell numbers when embryos were exposed to 20% oxygen tensions (a known modulator of oxidative stress) (Truong & Gardner 2017). This antioxidant combination when added to commercially available oocyte and embryo culture media (Vitrolife Gx-Series) also increased ongoing pregnancy rates (50% vs 25.8%, P < 0.05) in women aged 35–40 undergoing ART. This was thought to occur through the increased generation of good-quality blastocysts (Gardner et al. 2020). Further, the addition of melatonin in sheep embryo culture media resulted in an increase in blastocyst formation and cell numbers mediated through stimulation of the MT2 receptor (Lee et al. 2018). Yet, despite evidence for their benefits in vitro, the standard inclusion of antioxidants in embryo culture media is not widely used in clinical practice, although, the authors do note a number of current clinical trials been performed worldwide. Excessive amounts of antioxidants can be pro-oxidative and interfere with physiological ROS concentrations, leading to enhanced ROS generation in the mitochondria and further oxidative injury to the embryo (Rahal et al. 2014, Sotler et al. 2019). Therefore, the concentration of antioxidants added to embryo culture media needs to be carefully and thoroughly researched, as a single concentration may not be suitable for all couples especially if their gamete quality and the culture conditions and the laboratory environment are optimal. For instance, high concentrations of coenzyme C10 (100 µM) in oocyte maturation media significantly reduced blastocyst development rates in sheep (Maside et al. 2019). Further, many of the studies assessing the benefits of in vitro antioxidants in the pre-implantation tend to use suboptimal culture conditions (i.e. 20% oxygen concentrations) or addition of chemicals (i.e. H2O2) to create an environment in which the embryo displays oxidative stress (Budani & Tiboni 2020, Zarbakhsh 2021). As such the concentrations of antioxidants needed to supplement culture media of someone with normal embryo redox status would likely be much lower or not even required than that of someone who has an increased risk of oxidative stress to their embryos due to factors such as obesity or advanced age, or embryos grown in a clinical embryology laboratory who are unable to maintain ideal culture conditions. So, while the addition of antioxidants to in vitro embryo culture media seems promising for reducing ROS concentrations in the pre-implantation embryo, we must first thoroughly understand each antioxidant mechanism of action and optimal doses, before their widespread inclusion to embryo culture media (Castleton et al. 2022).

Conclusion

The redox state of embryos resides in a carefully orchestrated balance, heavily influenced by the production of ATP through oxidative phosphorylation and enzymes such as NOX and xanthine oxidase. They are counterbalanced by various compounds both intracellularly (i.e. SOD, CAT, GSH) and those present throughout the female reproductive tract (i.e. vitamins A, C and E). Biological levels of ROS are necessary for normal embryo formation including embryonic cleavage events and cellular proliferation with high concentrations of ROS throughout pre-implantation embryo development causing arrestment of development, aberrant fetal growth and transgenerational problems. This can be influenced by sperm and eggs that are delivered at fertilisation and through maternal and paternal biological and lifestyle factors (i.e. age and obesity). Although, a less clearer
mechanism for how sperm contribute to altered redox balance in embryos is noted indicated further research is warranted in this area. Redox balance is especially important in the *in vitro* scenarios as embryos are exposed to different conditions (i.e. light, culture media, pH, temperature) not found *in vivo* that can modify ROS production or increase their exposure. Research into oral antioxidants and their addition to *in vitro* culture to gain increased understanding of how effective they can be, not only in high ROS situation but also in healthy individuals, as to not upset embryo homeostasis.

**Declaration of interest**

L P and N O M are paid employees of Repromed. The other authors declare no conflict of interest.

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**References**


Cobleyn JN 2020 Mechanisms of mitochondrial ROS production in assisted reproduction: the known, the unknown, and the intriguing. Antioxidants 9 933. (https://doi.org/10.3390/antiox9090933)


de Matos DG, Furnus CC, Moses DF, Martinez AG & Malkovic M 1996 Stimulation of glutathione synthesis in vitro matured bovine oocytes

Reprom (2022) 164 F95–F108


https://rep.bioscientifica.com


Ornsto A 2007 Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy; Reproductive Toxicology 24 31–41. (https://doi.org/10.1016/j.reprotox.2007.04.004)


Ozkaýa MO & Naziroğlu M 2010 Multivitamin and mineral supplementation modulates oxidative stress and antioxidant vitamin levels in serum and follicular fluid of women undergoing in vitro fertilization. Fertility and Sterility 94 2463–2466. (https://doi.org/10.1016/j.fertnstert.2010.01.066)


Radford BN & Han VKM 2020 Evidence of increased hypoxia signaling in fetal liver from maternal nutrient restriction in mice. Pediatric Research 87 450–455. (https://doi.org/10.1038/s41390-019-0447-z)


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