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Review

Oxidative stress and antioxidant use during *in vitro* mammal embryo production. Review

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Abstract:

Of the many animal reproduction biotechnologies, *in vitro* embryo production has developed most over the past twenty years. Procedure success depends on many factors, including the presence of reactive oxygen species in adequate proportions. Both *in vitro* fertilization and gamete and embryo manipulation exposes cells to endogenous and/or exogenous factors that can affect antioxidant defense mechanisms and quality. This review discusses some sources of reactive oxygen species, the use of enzymatic, non-enzymatic and polyphenolic antioxidants to reduce oxidative stress in *in vitro* embryo production processes, and their effects on oocyte and embryo quality, gene expression and embryo developmental competence.

Key words: Antioxidants, Reactive oxygen species, Oxidative stress, *In vitro* culture, Embryo development.

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Introduction

In vitro embryo production (IVEP) involves three steps: 1) *in vitro* maturation (IVM) of oocytes obtained from antral follicles; 2) coincubation of male and female gametes, or *in vitro* fertilization (IVF); and 3) *in vitro* culture (IVC) of the presumed zygotes to blastocyst stages. Under normal physiological conditions mammal oocytes grow and are fertilized in the ideal protective environment, the ovary and the female reproductive tract. Under *in vitro* conditions, however, the gametes and embryos must be manipulated during maturation, fertilization and embryo development in environments that generate oxidative stress. The conditions causing this stress include high oxygen concentration (20 %) compared to the *in vivo* environment (3 to 5%)[(1)]; exposure to light[(2)]; culture medium composition[(3)]; changes in pH[(4)]; centrifugation processes[(5)]; and many others[(6)]. These can negatively affect both gametes and embryos, altering the functionality of biomolecules such as lipids, nucleic acids and proteins, and thus influencing embryo development[(7)].

Cells have an enzymatic and non-enzymatic antioxidant defense system, but antioxidant molecules have been used to supplement culture media and thus decrease reactive oxygen species (ROS) production in gametes and embryos. This improves their quality and reproductive potential by reducing intracellular ROS[(8)], and protects against damage to DNA and other biomolecules, raising embryo developmental competence[(9-11)]. The present review is aimed at analyzing the effect of oxidative stress and antioxidant use in *in vitro* production of embryos on gamete and embryo quality at the metabolic level, as well as gene expression and epigenetic marks.

Reactive oxygen species and oxidative stress

Reactive oxygen species (ROS) constitute a group of molecules generated through partial reduction of molecular oxygen. Most of these species (except hydrogen peroxide) have one or more unpaired electrons, a configuration called a free radical. Under basal conditions, aerobic metabolism is linked to production of ROS such as hydrogen peroxide (H_2O_2), the superoxide anion (O_2-) and the hydroxyl radical (OH-), while reactive nitrogen species (RNS) such as nitric oxide (NO•) form through conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS). Oxidative stress occurs when ROS production exceeds cellular defenses[12], generating oxidative damage to biomolecules such as lipids, proteins, carbohydrates and nucleic acids, and consequently inducing structural and functional changes such as lipid hydroperoxides[13], carbonylated proteins[14] and DNA with oxidized bases (7, 8 dihydro-8-oxoguanine)[15].

The mitochondrial respiratory chain is susceptible to oxidative damage (mainly to complexes I and II), by production of superoxide and nitrile radicals. These can affect mitochondrial proteins and alter the function of many metabolic enzymes in the mitochondrial electron transporter chain[16]. Mitochondrial DNA (mtDNA) is also known to be more sensitive to oxidative stress than nuclear DNA[17]. Possible reasons are that it lacks histones, which protect against damage from free radicals, does not have a suitable repair system, and is located near the internal mitochondrial membrane, the largest ROS production site[18,19]. Oxidative damage to the mtDNA can induce mutations and alter mitochondrial function and integrity[20]. In humans this can manifest in degenerative mitochondrial diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis[21,22].

ROS production during *in vitro* embryo production

Some cellular processes in the reproductive tract are regulated by ROS, which act as second messengers generating a specific cellular response. Macromolecules sensitive to redox modifications (phosphatases, kinases, transcription factors) are important during cell development stages such as proliferation, differentiation and cell death. In the latter, different ROS levels generate different types of cell death; for example, low concentrations promote apoptosis, intermediate concentrations generate autophagocytosis and high concentrations promote cell necrosis[12,23]. During IVM,

physiological levels of ROS are needed to reinitiate meiosis of the oocytes arrested in diplotene, and to stimulate release of intracellular CA^{2+} in the oocyte and the protein kinase activated by mitogen (MAPK)[24,25].

Physiological levels of ROS are also required for the training, hyperactivation and acrosomal reaction of mammalian sperm[26]. During sperm training, ROS such as the anion peroxynitrate, H_2O_2 and NO• have a dose-dependent effect on sperm function. Low ROS concentrations are required to promote cholesterol flow, AMPc production, hyperactivation and oocyte-sperm fusion[27,28]. In contrast, excess ROS may affect sperm functionality because the spermatozoa cell membrane is rich in polyunsaturated fatty acids, making it susceptible to lipid peroxidation. It can also negatively affect mitochondrial function in the sperm-zona pellucida interaction by reducing sperm motility, and compromising sperm DNA and therefore male fertility[29-31]. Use of antioxidant molecules is thus vital to protecting cells from high ROS levels and their negative effects.

Simulation of *in vivo* conditions in assisted reproduction techniques has improved immensely although two main factors continue to contribute to *in vitro* ROS generation and accumulation: absence of endogenous defense mechanisms and gamete and embryo exposure to environments which generate ROS. There are two main ROS sources (Figure 1). Endogenous ROS are accumulated by oocytes, sperm and embryos via various metabolic pathways and enzymes, mainly oxidative phosphorylation, NADPH oxidase and xanthine oxidase[32]. Exogenous ROS sources include environmental factors such as cryopreservation, oxygen concentration, energy source, culture medium, and light[6,33].

Oxygen is a vital component of oviduct and uterine environments and is involved in embryo development regulation, specifically through metabolism regulation. Oxygen tension found in the oviduct and uterus ranges from 5 to 8.7% in several species[34]. Levels used in oocyte maturation and cultivation with good results span from 5 to 20 %[35,36]. However, the trend is to use 20% O_2 during oocyte maturation because the energy production route at low O_2 concentrations (5 %) reduces the proportion of oocytes in IVM, which affects oocyte developmental competence[1]. In contrast, 5 % O_2 during cultivation favors embryonic developmental competence, cellularity and gene expression related to oxidative stress[37]. However, exposure to high O_2 concentrations (20 % in air) has been reported to intensify ROS level increases and thus reduce embryonic development percentages in rodents[38], swine[39,40], goats[41], bovines[42] and humans[43]. This in turn can cause arrested development, DNA damage, apoptosis and lipid peroxidation, which undermine embryo competence[44]. Studies evaluating the relative abundance of mRNA in oocytes have found a pattern of better quality when oocytes are matured at low O_2 concentrations (5 %)[45,46]. Incorrect atmospheric oxygen concentrations can clearly have detrimental effects in mammalian embryo cultivation.

Depending on composition and supplements, culture media can contribute to ROS production in IVEP systems[3,47]. Culture media contain metal ions, such as Fe^{2+} and Cu^{2+}, which are inducers of ROS formation through Fenton and Haber-Weiss reactions; iron

can also act on lipids by generating lipid peroxidation initiated by free hydroxyl radicals[48]. Supplementation with biological fluids such as fetal bovine serum (FBS) may increase ROS levels more than other supplements such as bovine serum albumin (BSA). Presence of the enzyme amino oxidase in serum[49], which participates in oxidation of primary amines, generates hydrogen peroxide as a secondary product[50], which could explain the effect of serum amino oxidase concentration on apoptosis percentage[51], cryotolerance and the gene expression pattern in bovine embryos produced *in vitro*[52]. However, this protein supplement improves bovine embryo production rate and quality[53]. Maintaining developmental competence in bovine oocytes during *in vitro* maturation requires control of medium glucose content. High glucose concentrations in the maturation medium raise ROS levels and lower intracellular glutathione (GSH) content in bovine oocytes. This inhibits the enzymes responsible for GSH synthesis, negatively affecting oocyte capacity to reduce ROS[54,55], which, in early embryonic development, can lead to lipid peroxidation of the cell membrane, DNA fragmentation and improper protein synthesis[32,56].

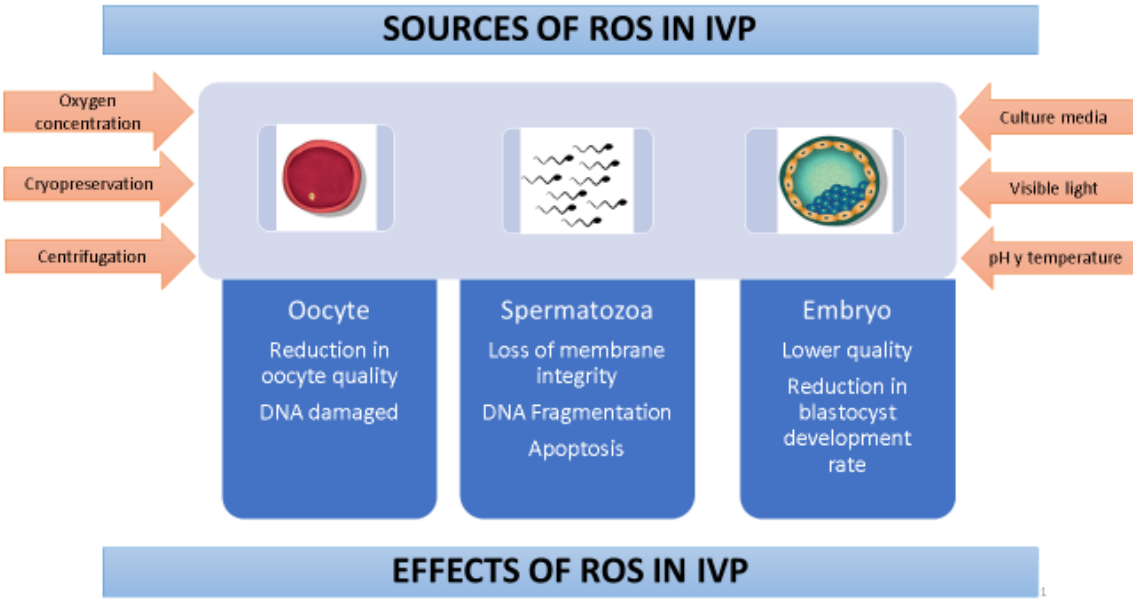
Visible light also induces ROS production by generating base oxidation, breaking down DNA chains, and causing oxidative damage in other biomolecules[2]. Sperm motility and hyperactivation are affected by excess ROS production caused by exposure to visible light[57]. Excess ROS production has also been reported *in vitro* in embryos transiently exposed to visible light. White fluorescent light, the most common type used in laboratories, was found to generate the most ROS in mouse and hamster zygotes, as reflected in the blastocyst apoptosis index, although use of filters helped to diminish these effects[58]. A study assessing the effects of daylight and laboratory light and different exposure times in culture media and porcine embryos found both types of light to reduce embryo quality and parthenogenetic blastocyst percentages[59]. This suggests that culture media and embryos need protection from light during *in vitro* production processes.

Centrifuging is a necessary step in semen preparation and training protocols for IVF. However, centrifuge time and force contribute to raising ROS levels, causing oxidative damage and affecting sperm function[5,60]. Centrifuging sexed and unsexed sperm for long periods (45 min) at 700 x g caused loss of plasma membrane integrity and DNA fragmentation[61,62]. This suggests that the sperm plasma membrane experiences a lipid peroxidation process in response to high ROS levels, thus reducing membrane fluidity and functionality for fertilization. For this reason different sperm preparation techniques have been developed (e.g. swim-up and density gradients) to obtain spermatozoa with higher motility and DNA integrity percentages to improve spermatozoa fertilization capacity during IVEP processes[63].

Cryopreservation significantly increases ROS production in spermatozoa, affecting motility, viability and training, and enhancing lipid peroxidation of the spermatic membrane, affecting potential fertility[64]. Low fertilization rates in cryopreserved oocytes have been related to freeze damage, including hardening of the zona pellucida due to premature release of cortical granules, spindle disorganization and microtubule

loss or agglutination[65,66]. Use of antioxidants may therefore be a vital factor in sperm survival and function before, during and after cryopreservation.

Figure 1: Effects of oxidative stress and sources of reactive oxygen species during embryo production



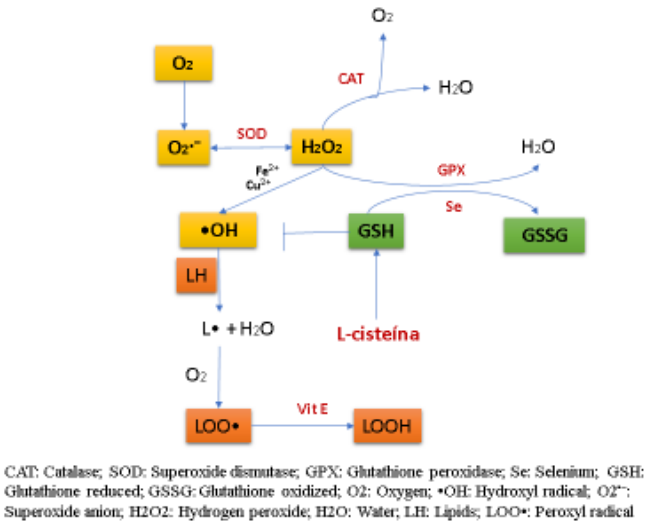
IVP = *In vitro* produced embryos

Enzymatic and non-enzymatic antioxidants

An antioxidant with biological function is defined as a substance that decreases or prevents substrate oxidation, resulting in a more potent reducing agent[67]. Reactive oxygen species (ROS) can be inactivated by a defense system consisting of antioxidant enzymes and molecules[2]. These antioxidant mechanisms can be mediated by iron- and copper-binding proteins such as transferrin, ferritin and albumin[68], and small antioxidant molecules derived mainly from fruits and vegetables. Enzymatic mechanisms can also mediate them, and include enzymes such as superoxide dismutase (SOD), which catalyzes dismutation of the superoxide anion in oxygen and hydrogen peroxide; catalase (CAT) and glutathione peroxidase (GPX), which convert hydrogen peroxide into water (and oxygen for CAT reactions); hydrophilic molecules such as ascorbate, urate and GSH; and lipophilic molecules such as tocopherols, flavonoids, carotenoids and ubiquinol

(Figure 2). Other enzymes involved in reduction of oxidized forms of small antioxidant molecules are also part of cell antioxidant mechanisms, including GSH reductase and dehydroascorbate reductase, as well as molecules responsible for maintenance of thiol groups in proteins (thioredoxin)[12].

Figure 2: Action mechanisms of enzymatic and non-enzymatic antioxidants



CAT: Catalase; SOD: Superoxide dismutase; GPX: Glutathione peroxidase; Se: Selenium; GSH: Glutathione reduced; GSSG: Glutathione oxidized; O2: Oxygen; •OH: Hydroxyl radical; $O_2^{\bullet-}$: Superoxide anion; H2O2: Hydrogen peroxide; H2O: Water; LH: Lipids; LOO•: Peroxyl radical

Reduced glutathione (GSH) is the largest non-protein sulfhydryl component in mammalian cells, and is known to protect the cell from oxidative damage and to regulate the intracellular redox balance[69]. Several studies suggest that GSH plays an important role in many biological processes, including DNA and protein synthesis and cell proliferation during embryo development[70]. In bovine oocytes, it is considered a vital biochemical marker of oocyte viability and quality[71]. Synthesis of GSH has been reported during IVM[72,73], and is associated with formation of the male pronucleus after fertilization[72,74], and early embryo development[70]. Intracellular GSH levels are therefore considered a marker of oocyte quality and embryo developmental competence after IVF.

The most evaluated antioxidants as culture media supplements are ascorbic acid (AA) and alpha tocopherol (AT). Ascorbic acid decreases ROS production in bovine oocytes, improving their potential to develop embryos[9], while AT benefits blastocyst rate and cellularity[75,76]. Alpha tocopherol (AT) can prove useful in IVEP because its hydrophobicity allows it to cross the lipid bilayer, intersperse in it and decrease ROS in the cell. In contrast, AA is hydrosoluble which allows it to act synergistically with tocopherol in some conditions, regenerating tocopherol from tocopheroxyl radicals in a redox cycle[77]. It has also been reported to reduce ROS production in the culture medium and augment bovine embryo developmental competence by lowering intracellular ROS

in oocytes matured with AA[78]. Ascorbic acid (AA) can also increase blastocyst rate and cellularity[11], raise intracellular GSH levels and lower ROS production in bovine oocytes[10]. During the IVC, AA is also reported to decrease ROS production and expression of pro-apoptotic genes in pig embryos, thus enhancing embryo development[79], and improving the survival rate and quality of vitrified embryos[80,81]. Embryo culture medium supplemented with AT or AA is reported to increase development and cellularity capacity, and reduce the proportion of apoptotic cells in porcine blastocysts derived from IVF or somatic cell nuclear transfer (SCNT); however, this effect was not observed with combined supplementation[76].

Melatonin has been reported to have beneficial effects in IVEP due to its ability to trap free radicals, reduce ROS concentration, and increase expression of antioxidant enzymes (SOD and glutathione reductase)[82,83], as well as suppress expression of pro-oxidant enzymes and improve mitochondrial function[84,85]. Like many antioxidants, melatonin can have positive or negative effects depending on the concentration at which it is administered in a culture medium. When supplemented in IVF medium at low concentrations it improves sperm quality and motility, decreases ROS levels and lipid peroxidation, and acts as an anti-apoptotic agent in bovine sperm[86] and ejaculated human sperm[87-89]. In contrast, high concentrations induce fragmentation and oxidation of sperm DNA, decrease the number of viable spermatozoa and generate a decrease in blastocyst rates, without affecting embryo quality[90,91]. In embryo culture medium, melatonin augments cleavage, blastocyst and hatching rates, increases embryo cellularity and promotes activation of antioxidant enzymes[92,93].

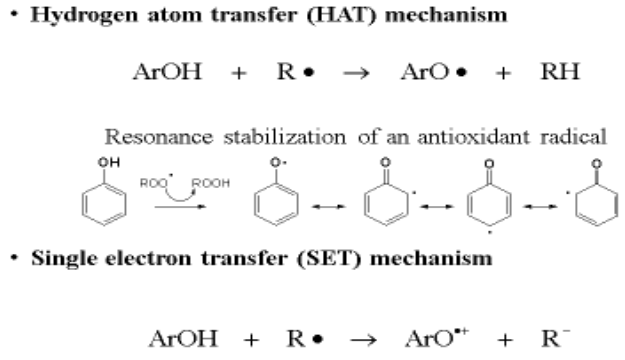
Phenolic antioxidants

Impressive progress has been made in identification, purification and evaluation of natural-origin antioxidant molecules[94], such as phenolic antioxidants. Because their structure includes aromatic rings and hydroxyl groups they are very stable and can inhibit oxidation of biologically and commercially important compounds[94,95]. As a result, they have been suggested as potentially useful for prevention and treatment of diseases caused by free radicals, such as ischemia, atherosclerosis, and neuronal and cardiovascular diseases[96,97].

Phenolic antioxidants (ArOH) have two action mechanisms: hydrogen atom transfer (HAT) or electron transfer (SET). In the first (HAT), the free radical (R•) removes a hydrogen atom from the antioxidant (ArOH), transforming it the radical ArO•. This is more stable and more efficient because its hydrogen bonds, conjugation and resonance make it a non-reactive phenoxyl radical. In the second mechanism (SET), the antioxidant

can donate an electron to the free radical, forming, among other products, a radical cation of the antioxidant ($ArO\bullet^{+}$) which is stable and does not react with substrates (Figure 3). Both mechanisms can always occur in parallel, although they have different reaction rates[98].

Figure 3: Action mechanisms of phenolic antioxidants



As part of the search for new antioxidants and evaluation of their activity in *in vitro* reproduction, green tea extract (the principal components of which are polyphenols) has been evaluated in oocytes matured *in vitro*. It favored blastocyst rate and reduced glutathione concentrations within the oocyte, but exhibited limited repeatability probably due to variability in extract composition[99]. Anthocyanins are another type of biological molecule with antioxidant capacity. These were evaluated in pig and bovine oocytes in maturation medium[100,101], and different oocyte quality parameters tested such as free radical production level, intracellular glutathione levels, relative mRNA abundance associated with embryo development, and capacity for *in vitro* embryo production. The benefits of phenolic compounds from grapes have also garnered attention. Resveratrol (3,5,4'-trans-trihydroxystilbene) and pterostilbene (natural antioxidant analog of resveratrol) are abundant in plants and fruits such as blueberries, blackberries, peanuts, grapes and red wine[102,103]. Both compounds have various *in vivo* and *in vitro* biological properties, such as antioxidant capacity, cardiac protection, anti-inflammatory, chemoprevention in some cancer models and some positive effects in metabolic diseases[102,104]. Given these properties, research has been done into their *in vitro* biological effects in other animal models and systems, such as IVEP.

When added to embryo culture medium, pterostilbene has been reported to reduce ROS levels and the percentage of lipids in embryos[105]. However, very few studies have been done on this type of antioxidant in reproductive biotechnology, highlighting the need for more studies on the molecular mechanism by which pterostilbene exerts its effect on embryo metabolism.

Various studies have been done on resveratrol in IVEP (Table 1). There are studies on the use of resveratrol in *in vitro* oocyte maturation in swine[106], bovines[10,107-109], and goats[110], which indicate that it increases GSH concentration within the oocyte, decreases ROS production and raises the blastocyst rate. Use of resveratrol during *in vitro* embryo culture also has a positive effect on embryo development[111], and increases blastocyst cryotolerance[112,113], showing that its antioxidant capacity improves oocyte quality and resistance to cryopreservation processes. High resveratrol concentrations (20 and 40 µM), however, do not provide benefits and are reported to decrease the percentage of bovine oocytes capable of completing the maturation process up to metaphase II[114].

Resveratrol's physiological effect appears to be related to its ability to enhance cellular processes dependent on Sirtuin 1 (*SIRT1*). This in turn is also associated with adenosine monophosphate-activated protein kinase (AMPK), an energy sensor which controls cell metabolism, including oxidative phosphorylation and fatty acid oxidation[115]. Activation of AMPK by resveratrol increases levels of NAD^+ (the *SIRT1* cofactor), which decreases the acetylation of *SIRT1* substrates and activates PGC-1α (coactivator 1 α of the peroxisome proliferator-activated receptor gamma)[116,117]. However, despite AMPK activation for observation of resveratrol's metabolic effects, this compound's direct target is upstream from AMPK. One proposed mechanism is that resveratrol activates AMPK through competitive inhibition of phosphodiesterases (PDEs), thus increasing AMPc levels[118]. This second messenger plays a vital role in oocyte maturation in mammals, which occurs after bonding FSH and LH to their specific receptors in the plasma membrane of granulosa cells through activation of adenylate cyclase[119]. Intracellular AMPc levels are regulated by PDEs, which hydrolyze it to 5'-AMP. Use of PDE inhibitors delays meiosis reinitiation and slows cumulus expansion kinetics, which prolongs maintenance of the gap bonds between the oocyte and cumulus cells[120]. This extension of gap bonds during *in vitro* maturation in the presence of PDE4 inhibitors could allow passage of metabolites, ions, nucleotides and amino acids, which improve oocyte cytoplasm maturation, bringing it near synchronization of nuclear and cytoplasmic maturation, which would favor blastocyst production and quality.

Table 1: Studies of resveratrol as a supplement in culture media for *in vitro* embryo production

Culture	Gametes	Species	Resveratrol Concentrations	Results	Reference
		Porcine	0.1, 0.5, 2.0 and 10.0 Mm	Improved development in *in vitro* parthenogenic and fertilized embryos; increased intracellular GSH and decreased ROS levels.	(106)
		Porcine	20 µM	Increased *SIRT1* expression; improved mitochondrial functioning and oocyte developmental capacity.	(142)
		Porcine	2 µM	Improved oocyte resistance to cryopreservation-induced damage.	(113)
		Bovine	0.1, 1 and 10 µM	Induced progesterone secretion; increased intracellular GSH; decreased ROS levels; promoted oocyte maturation and subsequent embryo development.	(107)
		Bovine	20 µM	Increased ATP content and expression of SIRT1 protein in mature oocytes; improved fertilization by reinforcing mechanisms for blocking polyspermia.	(143)
IVM	**Oocytes**	Bovine	2 µM	Lowered ROS levels; raised embryo development rates and cellularity.	(10)
		Bovine	20 and 40 µM	Regulates expression of CYP1A1 gene involved in meiosis reinitiation.	(114)
		Bovine	1, 10, 20 and 40 µM	Increased embryo development and intracellular GSH, and decreased ROS levels	(108)
		Bovine	0.2 µM, 1 µM and 20 µM	Improved oocyte developmental competence; increased maturation and blastocyst rates.	(109)
		Bovine	2 µM	Affected expression of SIRT1 protein in oocytes and blastocysts of donors of different ages.	(144)
		Bovine	2 µM	Lowered ROS levels; increased GSH levels and cleavage and blastocyst rates; decreased expression of pro-apoptotis genes.	(145))
		Caprine	0.1, 0.25, 0.5, 2.0 and 5.0 µM	Decreased ROS levels; increased GSH levels and embryo development rates; decreased expression of pro-apoptosis genes in cumulus cells, mature oocytes and blastocysts.	(110)
IVF	**Spermatozoids**	Mouse	15 µg/ml	Increased oocyte fertilization, decreased ROS generation, glutathione peroxidase activity and lipid peroxidation concentration.	(146)
		Human	0.1, 1.0 and 10.0 µM	Prevented damage to DNA caused by cryopreservation in sperm from fertile males.	(147)
IVC	**Embryos**	Porcine	0.05, 0.1, 0.5, 1.0 and 25 µM	0.5 µM in culture had positive effect on embryo development.	(111)
		Bovine	0, 0.25, 0.5 and 1 µM	0.5 µM improved embryo quality and cryotolerance.	(112)

IVM= *in vitro* maturation; IVF= *in vitro* fertilization; IVC= *in vitro* culture.

Changes in gene expression and epigenetic disorders induced by ROS

Oocyte developmental competence is defined as the ability of an oocyte to reinitiate meiosis, be fertilized, divide and attain the blastocyst stage[121]. This competence or quality is acquired progressively during folliculogenesis as the oocyte grows and matures through a series of cellular (mitochondrial activity), molecular (gene expression profile) and functional (protein kinase activity) changes[55,122]. During oocyte growth and maturation mRNA and proteins are synthesized, which contribute to early development before and after activation of the embryo genome. This mRNA storage occurs during oocyte growth, and a polyadenylation event develops in each transcript, which is a key gene expression regulator and known to be an important step in mammalian embryo development[123]. However, IVM conditions can affect polyadenylation levels in maternal mRNA, with implications for embryo quality[124]. This suggests that deficiencies in developmental competence in most *in vitro* matured oocytes are reflected in the composition and abundance of specific RNA transcripts in the oocyte.

For this reason, different transcripts have been evaluated in the oocyte in search of associations between them and oocyte quality or embryo developmental competence. Among the most studied are *NLRP5* (NLR Family Pyrin Domain containing 5, known as *MATER*), a maternal effect gene specific to the oocyte which is required for early embryo development in bovines, mice and humans[125-127]. Another is *POU5F1* (POU domain Class 5, transcription factor 1, also known as *OCT-4*), which has been validated as a marker for epigenetic and pluripotency reprogramming, and is crucial for normal embryo development[128]; increased *POU5F1* expression has been reported in pig embryos derived from SCNT treated with vitamin C[129]. In an effort to predict fertilization success and maintain oocyte viability, expression of genes such as hyaluronic acid synthetase 2 (*HAS2*), cyclooxygenase 2 (*COX2*; *PTGS2*) and gremlin (*GREM1*) have been studied in cumulus cells and correlated with oocyte competence and subsequent embryo development[111,130].

In the development of bovine embryos, cell viability is determined by alterations in the expression of metabolism-related genes such as *GLUT-1* (glucose transporter-1 transcripts)[131], growth factors such as IGF-2 (insulin-like growth factor 2) and *IGF-2R* (insulin-like growth factor 2 receptor), early differentiation and trophoblastic functions such as *IF* (interferon tau) and *Mash2* (mammalian achaete-scute homologue)[132,133]. Changes occur in gene expression during IVEP processes, and epigenetic disorders can arise that alter DNA methylation patterns in some genes (DNA methyltransferase, *DNMT1a*, *DNMT3a* and *DNMT3b*), affecting the gene expression profiles that encode for a specific tissue[134].

Evaluations have been done on the effect of antioxidant supplements in culture media on gene expression regulation. Supplementation with resveratrol during IVM of oocytes from pigs[106,111,135] and goats[110] decreased the transcription levels of genes related to apoptosis (e.g. *BAX*, *BAK* and caspase-3) but caused no changes in expression of the *BCL-2* gene. This suggests that resveratrol suppresses expression of pro-apoptotic genes in matured oocytes, and exerts a protective effect on embryos produced *in vitro*. Medium supplementation with AA has been reported to positively regulate pluripotent gene expression in porcine parthenogenetic blastocysts, and decrease expression of the pro-apoptotic gene *Bax*[79]. Ascorbic acid (AA) supplementation in culture medium and vitrification-thawing media increases expression of the *GPX1* and *SOD1* genes, both associated with oxidative stress, thus improving survival rates and decreasing peroxide levels 24 h post-thaw[136]. A study in bovines found that the relative abundance of *GPX1* is higher in excellent quality blastocysts (Grade 1) than in good blastocysts (Grade 2), suggesting that less expression of *GPX1* is associated with lower embryo quality[137].

Reactive oxygen species (ROS) produced either endogenously or exogenously during IVEP may also induce epigenetic changes. Culture conditions that include changes in pH, osmolarity, temperature, visible light exposure, oxygen concentration and cell centrifuging can influence the epigenetic pattern during the *in vitro* process thus affecting gamete and embryo quality[134]. Oxidative stress may produce alterations in DNA methylation patterns and modification of histone proteins in the gametes; these are transmissible from gametes to embryos, and generate variations in the epigenome that could alter subsequent embryo development[138-140]. The mechanisms for transmission of these alterations to the embryo during fertilization and cleavage have not yet been elucidated. It has been suggested that oxidative stress damage in the gamete epigenome -which increases the adducts in DNA and alterations in the methylation profiles– is transferred to the embryo, manifesting in phenotypic alterations that can be observed in newborns[141]. For example, induction of oxidative stress in spermatozoa using H_2O_2 causes oxidative damage in the spermatozoa epigenome, which subsequently reduces embryo development rates and alters cell differentiation in blastocysts. This causes reductions in implantation rates, reduced fetal growth, increased adipose tissue, decreased lean mass and lower glucose tolerance. These findings implicate ROS as one of the mechanisms responsible for transmitting health signals from parents to children[141].

Conclusions

In vitro embryo production techniques are used commercially in animal production, but myriad factors can generate oxidative stress and potentially affect the quality of matured oocytes and consequently embryo development rates. The environment and the procedures to which embryos and gametes are subjected generate increases in ROS levels that surpass the physiological levels required to regulate various cellular functions, thus affecting cell morphology and functionality. These factors can affect different biomolecules, causing damage to DNA, lipid peroxidation, changes in gene expression levels and epigenetic disorders. Use of molecules with antioxidant activity can ameliorate *in vitro* maturation conditions, but novel substances of natural origin are still needed to reduce oxidative stress during *in vitro* embryo production processes and improve oocyte quality and embryo developmental competence.

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