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Effect of antioxidants on preimplantation embryo development *in vitro*: a review

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Review

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Summary

In vitro culture of the embryo is a useful method to treat infertility that shows embryo potential for selecting the best one to transfer and successfully implantation. However, embryo development *in vitro* is affected by oxidative stresses such as reactive oxygen species that may damage embryo development. Antioxidants are molecules found in fruits, vegetables, and fish that play an important role in reducing oxidative processes. In the natural environment, there is a physiological antioxidant system that protects embryos against oxidative damage. This antioxidant system does not exist *in vitro*. Antioxidants act as free radical scavengers and protect cells or repair damage done by free radicals. Various studies have shown that adding antioxidants into embryo culture medium improves embryo development *in vitro*. This review article emphasizes different aspects of various antioxidants, including types, functions and mechanisms, on the growth improvement of different species of embryos *in vitro*.

Introduction

Infertility is a prevalent problem in today's society that has different causes and therapies. One of the common ways to treat infertility is assisted reproductive technology (ART) (de Waal *et al.*, 2014). ART was launched in the late 1970s and, at this time, about 4% of all births are the result of this technique. One of the most common ART methods is embryo culture that shows embryo potential for selection as the best to transfer and successfully implant (Schwarzer *et al.*, 2012; Kirby, 2018). The birth success rate with ART is approximately 30% (Sunderam *et al.*, 2019; De Geyter *et al.*, 2018).

Antioxidants are natural or synthesized molecules. Natural antioxidants are present in fruits, vegetables and fish and play an important role in reducing oxidative processes in the body (Bazinet and Doyen, 2017). Synthetic antioxidants are made based on natural compounds or are fully synthetic compounds (Augustyniak *et al.*, 2010). Antioxidants are chemical compounds or substances that inhibit or retard the oxidation of other molecules (Rozoy *et al.*, 2012). Antioxidants act as free radical scavengers and protect cells or repair the damage done by free radicals (Tebboub and Kechrid, 2019). Free radicals are defined as molecules containing one unpaired electron within an outer orbit that can be produced from two oxidant sources: endogenous oxidants and exogenous oxidants. Endogenous oxidant production occurs when cells use oxygen and naturally generate free radicals that could damage cells. Exogenous oxidants are commonly known as free radicals that are produced from environmental factors such as sunlight and pollution (Kumar *et al.*, 2017; Haida and Hakiman, 2019). Antioxidants decrease the effect of oxidants by binding together with these harmful molecules. However, antioxidants are effective at low concentrations and may act as oxidants and become adverse by increasing concentration (Iwayama *et al.*, 2017).

Embryo development *in vitro* is affected by some factors. One of the most important factors is reactive oxygen species (ROS) (Li *et al.*, 2014). It seems the mechanism that causes increasing oxygen levels and damage to development of the embryo is ROS, which are produced *in vitro* and lead to DNA damage, delay in embryo development, and ultimately embryo death (Bontekoe *et al.*, 2012). This event occurs especially during the collection, manipulation and culture of embryos (Truong and Gardner, 2017). However, low ROS levels produced by embryos are necessary for regulation of development (Sunderam *et al.*, 2014). In the natural environment of the uterus, embryo development occurs at low oxygen concentrations of about 2–8% (Truong *et al.*, 2016). For this reason, embryo culture is often carried out at 5% oxygen, a concentration that more resembles the natural environment of various mammalian species (Wale and Gardner, 2010, 2016). Ma *et al.* (2017) showed that low oxygen tension improved embryo viability by increasing the expression of antioxidant enzymes and glucose transporter activities. In the natural environment, there is a physiological antioxidant system that protects embryos from oxidative damage (Agarwal *et al.*, 2012). This endogenous antioxidant system is not available or is insufficient *in vitro*. Therefore, to obtain blastocysts with high potential for implantation, optimization of the embryo culture medium is perhaps necessary by adding exogenous

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antioxidants (Abdelrazik *et al*., 2009). Although it has been reported that adding the correct dose of antioxidants to the culture medium can protect embryos from oxidative stress (Truong and Gardner, 2017; Truong *et al*., 2016; Yu *et al*., 2014), not all reports have confirmed this finding. Maside *et al.* (2019) reported that adding coenzyme Q10 (a potent antioxidant with a critical protective role against oxidative stress) at different doses had no effect on improving porcine embryo development *in vitro*. In another study, Rincon *et al.* (2019) showed that high-density lipoprotein (HDL), which acts as an antioxidant, did not have a positive effect on bovine embryo development *in vitro*. These issues showed that the use antioxidants for human embryo development *in vitro* is promising, but more research is needed. Alternatively, human serum albumin (HSA) as a protein supplement with antioxidant effects is currently used to improve human embryo culture medium (Bungum *et al*., 2002; Labied *et al*., 2019; Lan *et al*., 2019). Several antioxidants that have been used from 2009 onwards in embryo culture medium are listed in Table 1.

Although there are many articles on the use of antioxidants in embryo culture medium, there is summary of these studies or review of articles in this field. The current review article emphasizes the different aspects of various antioxidants including type, function and mechanisms for growth improvement of different species of embryos *in vitro*.

Reactive oxygen species

All living aerobic multicellular organisms require molecular oxygen to survive. Oxygen has two unpaired electrons in separate orbits in its outer shell. This electron structure makes it susceptible to forming radicals. The sequential reduction of oxygen through the addition of electrons leads to the formation of ROS (Valko *et al*., 2007; Liu *et al*., 2018). The term 'ROS' is a phrase used to describe a number of reactive molecules and free radicals derived from molecular oxygen. ROS are formed as by-products of normal oxygen metabolism during mitochondrial electronic transport and play an important role in cellular signalling, homeostasis, physiological processes, cell proliferation, hypoxia adaptation and cell fate determination. ROS concentration is important and determines their physiological effects (Van Blerkom, 2009; Scialo *et al*., 2017; Zhao *et al*., 2019). ROS generated by mitochondria, such as superoxides, are involved in multiple cell signalling pathways that control the rates of cell proliferation and other cellular activities such as molecular responses to hypoxia (Bell *et al*., 2005; Bell and Chandel, 2007; Van Blerkom, 2008). Regarding the role of hypoxia-inducible factor-1 (HIF-1) in cell survival under hypoxic conditions, ROS can regulate HIF-1 during low oxygen conditions. ROS regulates HIF-1 directly or indirectly through the ERK and PI3K/AKT signalling pathways. ROS increase the signalling activity of ERK and PI3K/AKT, and cause HIF-1 transcription and translation. These processes lead to cell proliferation (Movafagh *et al*., 2015; Zhao *et al*., 2019). ROS produced by electron leak during electron transfer chain in the mitochondria play an important role in cellular signal transduction and the physiology of cells (Zhao *et al*., 2019). Moreover, ROS are important second messengers that mediate different intracellular pathways. ROS act through the oxidative modification of many types of proteins, receptors, phosphatases, caspases, kinases, ion channels, and transcription factors (De Giusti *et al*., 2013; Zhao *et al*., 2019), therefore small amounts of ROS are needed for the natural function of cells (Scialo *et al*., 2017). However, during times of environmental stress such as through UV radiation, heat exposure, and ionizing radiation, ROS levels can increase. At high concentrations, ROS react readily with lipids, proteins, carbohydrates and nucleic acids and may result in significant damage to cell structures (Valko *et al*., 2007; Liu *et al*., 2018). In the process of embryo development, ROS can cause lipid peroxidation, which affects cell division, metabolite transport and mitochondrial dysfunction. In addition, it causes a break in the nuclear DNA strand that is involved in inhibiting embryo development. Typically, the production of lipid peroxide formation is usually considered as an indirect indicator of free radical markers (Li *et al*., 2015).

Under normal conditions, ROS and antioxidants keep a stable ratio. Excess ROS can create a negative environment, affecting fertilization, impairing embryo development, inducing apoptosis and resulting in embryo death (Paszkowski and Clarke, 1996). Optimizing the composition of embryo culture medium is necessary for increasing embryo quality *in vitro*. There are metallic ions such as Fe^{2+} and Cu^{2+} in culture medium that have the potential to accelerate ROS production within the cell. In addition, some sera that are commonly added to culture medium, contain amine oxidase, which leads to enhanced H_2O_2 production. Moreover, ROS in the culture medium may be created from embryo metabolism, therefore it seems that ROS can play an essential role in IVF success (Agarwal *et al*., 2006). Concentrations of ROS in embryo culture medium correlate with the degree of embryo fragmentation or blastocyst formation (Lee *et al*., 2012). Embryo culture medium are often supplemented with antioxidants, therefore keeping an oxidant and antioxidant equilibrium in embryos (Agarwal *et al*., 2006). It has been demonstrated that embryos in culture medium produce ROS at various rates, depending on the compound of medium (Shih *et al*., 2014). However, Lan *et al.* (2019) have reported that the relationship between ROS levels and early human embryo development *in vitro* is limited, such that ROS levels in culture medium have no significant relationship with embryo quality and blastocyst formation. These issues have shown that the exact role of ROS in early embryo development is not yet fully distinguished.

Mitochondria are organelles for ATP production that are important for controlling cell growth, dynamic response, signalling and apoptosis in most mammalian cells. In oocytes and embryos, a high level of ATP production is necessary for maturation of oocytes, fertilization, and early embryo development *in vivo* and *in vitro*. Mitochondria in oocyte and early embryo are spherical organelles with short cristae that surround the high-electron density matrix. Despite their simple appearance, they are active in oxidative phosphorylation and are the primary source of ATP in the human oocyte and early embryo (Van Blerkom *et al*., 1995; Van Blerkom, 2011). During ATP production, various types of ROS such as superoxide, hydrogen peroxide and hydroxyl radicals are produced by oxidative phosphorylation in mitochondria. This production of ROS is related to oocyte maturation, fertilization and embryo development, so that ROS accumulation decreases embryo development and blastocyst quality. In addition, severe oxidative stress resulting from enhancing ROS causes mitochondrial fission that leads to the mitochondrion dynamic response, therefore enhancing mitochondrial fission by the accumulation of ROS decreases ATP production (Yang *et al*., 2018). During embryo culture, ROS levels enhance compared with *in vivo* embryos at similar stages. Hajian *et al.* (2017) reported that ROS production in embryos decreased from fertilization to about 8 to 16 cell stage and increased from compaction to blastocyst stage. This increase in ROS production is likely to be related to the change from anaerobic to aerobic glycolysis, because ATP production at

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Table 1. Beneficial effects of various antioxidants on the development of different embryo species *in vitro*

Antioxidant (Reference)	Embryo species	Effective dose	Results
Alpha-lipoic acid (ALA) (Truong and Gardner, 2017)	Mice	5 μM	Total cell numbers in the blastocysts increased and embryonic cell cleavage was performed faster.
Alpha-lipoic acid (ALA) (Truong and Gardner, 2020)	Mice	5 μM	ALA conveyed beneficial effects on embryo development during cryopreservation by decreasing oxidative stress and apoptosis and increasing cell numbers and acetylation levels.
Allicin (Jeong *et al*., 2017)	Porcine	0.1 μM	Rates of cleavage and blastocyst formation increased by decreasing ROS levels.
Anethole (Anjos *et al*., 2019)	Bovine	30 μg/ml	Rate of blastocyst formation and the number of embryonic cells increased by decreasing ROS levels.
Apigenin (Safari *et al*., 2018)	Mice	10 μM	Rate of blastocysts and hatched blastocysts, the number of embryonic cells, zona pellucida thickness against H_2O_2, or actinomycin D improved by decreasing apoptosis and ROS.
Ascorbic acid (Nohalez *et al*., 2018)	Porcine	50 μg/ml	Quality of blastocysts improved by decreasing ROS production.
Ascorbic acid (Torres *et al*., 2019)	Bovine	100 μM	Quality of embryos increased by downregulating Bax, GPx1, and BMP15 and upregulating Bcl-2 and CYP51A1. Moreover, reduced fat accumulation in the blastocysts by increasing lipolysis and suppressing lipogenesis.
Astragalus polysaccharide (Weng *et al*., 2018)	Boar	0.5 mg/ml	Rate of cleavage and blastocysts was increased by improving mitochondrial activity, decreasing the concentration of ROS, and improving the activity of SOD and CAT.
Astaxanthin (Li *et al*., 2015)	Bovine	0.5 mg/l	Astaxanthin improved embryo development, increased chromosomal stability, normalized the epigenetic modifications, and inhibited the overproduction of lipid peroxidation.
Baicalin (Guo *et al*., 2019)	Pig	0.1 μg ml^{-1}	Cleavage and blastocyst formation rates and the number of blastomeres increased by inhibiting the production of ROS and reducing apoptosis.
β-Cryptoxanthin (Park *et al*., 2018)	Porcine	1 μM	Blastocyst formation rate and the number of blastomeres increased by enhancing glutathione (GSH) levels and expression of the antioxidant genes superoxide dismutase 1 and peroxiredoxin 5 and by decreasing ROS levels.
C-Phycocyanin (Niu *et al*., 2017)	Porcine	5 μg/ml	Blastocyst formation and hatching rate increased by attenuating mitochondrial dysfunction, oxidative stress and apoptosis.
Canthaxanthin (Taweechaipaisankul *et al*., 2016)	Porcine	40 μM	Rate of cleavage and blastocyst formation increased by enhancing mRNA expression of Bcl2 and Oct4, and GSH levels and reducing Caspase-3 expression and ROS levels.
Carnitine (Sovernigo *et al*., 2017)	Bovine	0.5 mg/ml	Blastocyst development improved by decreasing ROS levels and increasing GSH levels.
Chlorogenic acid (CGA) (Nguyen *et al*., 2017)	Porcine	50 μM	Embryos were protected from DNA damage induced by oxidative stress.
Crocetin (Dos Santos *et al*., 2019)	Bovine	1 μM	Rate of blastocysts and the number of embryonic cells increased and intracellular levels of ROS decreased. Moreover, embryonic genes related to response to stress and lipid metabolism included *ATF4*, *BAX*, *FOXO3*, *GADD45A*, *GPx1*, *GPx4*, *HSF1*, *SOD2*, *ACACA*, *SREBF1* and *SREBF2* were upregulated.
Crocin (Chen *et al*., 2019)	Porcine	400 μg/ml	Embryos were protected against apoptosis by increasing expression levels of SOD, CAT, GPx, and Bcl-2 and level of GSH, and decreasing expression levels of Bax and levels of ROS.
Cysteamine (Sovernigo *et al*., 2017)	Bovine	100 μM	Blastocyst development improved by decreasing ROS levels or increasing GSH levels.
Ferulic acid (Tanihara *et al*., 2018)	Porcine	10 μM	Quality and development of embryos improved following *in vitro* fertilization (IVF).
Glutathione (GSH) (Sun *et al*., 2015)	Bovine	3 mM	Exogenous GSH during embryo development *in vitro* improved developmental potential and quality of embryos by the ability of GSH to maintain the redox balance.
Glutathione (GSH) (Li *et al*., 2019)	Bovine	3 mM	Exogenous GSH affected intracellular GSH levels through the γ-glutamyl cycle and improved embryo development *in vitro* by increasing the redox regulation.
Glutathione (GSH) (Ali *et al*., 2018)	Mice	1 mM	Embryo development improved by reducing endoplasmic reticulum stress.
Grape seed extract (GSE) (Karimian *et al*., 2018)	Sheep	800 μg/ml	Blastocyst formation rate increased by scavenging free radicals.

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Table 1. (*Continued*)

Antioxidant (Reference)	Embryo species	Effective dose	Results
Kaempferol (Zhao *et al.*, 2020)	Porcine	0.1 μM	Rate of blastocyst formation improved by decreasing the caspase-3 gene and ROS levels, and increasing *COX2* and *SOX2* gene expression levels (embryo development-related genes).
Kaempferol (Yao *et al.*, 2019)	Porcine	0.1 μM	Rate of formation and quality of blastocysts increased by attenuating oxidative stress and increasing mitochondrial function.
L-Carnitine (Kim *et al.*, 2018)	Human	1 mM	Number of cells in the inner cell mass and trophectoderm, embryo development, the numbers of good-quality embryos, the rate of implantation and clinical pregnancy increased.
L-Carnitine (Truong and Gardner, 2017)	Mice	10 μM	Total cell numbers of the blastocysts increased and embryonic cell cleavage was performed faster.
L-Carnitine (Abdelrazik *et al.*, 2009)	Mice	0.3 and 0.6 mg/ml	Embryos were protected against hydrogen peroxide (H_2O_2), actinomycin D and TNF-α by decreasing DNA damage, apoptosis rate and ROS levels.
L-Carnitine (Khanmohammadi *et al.*, 2016)	Mice	0.5 mg/ml	Rate of blastocysts and hatched blastocyst, zona pellucida thickness, and the number of blastocysts' inner cell mass improved.
L-Carnitine (Zare *et al.*, 2015)	Mice	0.6 mg/ml	Cleavage rate and blastocyst development rate increased.
L-Carnitine (Zare *et al.*, 2017)	Mice	0.6 mg/ml	Expression levels of *Dppa2* (an important gene for embryonic development) and *Bcl-xL* mRNA in the 2-cell stage embryos and blastocysts increased.
L-Carnitine (Shafiei *et al.*, 2020)	Mice	0.3 mg/ml	Embryo development improved by decreasing ROS levels and increasing implantation-related genes: *ErbB1* and *ErbB4* (the earliest expressed genes on preimplantation embryos).
L-Carnitine (Truong and Gardner, 2020)	Mice	10 μM	L-Carnitine conveyed beneficial effects on embryo development during cryopreservation by reducing oxidative stress and apoptosis rate and increasing the cell numbers and acetylation levels.
L-Ergothioneine (Mishra *et al.*, 2018)	Sheep	10 mM	Percentage of cleavage, morula, and blastocyst increased while there was no change in expression of the majority of apoptotic and antioxidant genes studied in the developmental stages of embryos.
Lupeol (Khan *et al.*, 2018)	Bovine	2 μM	Blastocyst quality and the number of blastomeres increased by reducing the expression of NFκB1, COX2, CASP3, and the rate of apoptosis.
Lycium barbarum polysaccharide (LBP) (Yang *et al.*, 2019)	Mice	400 μg/ml	Development of cryopreserved 2-cell embryos improved by restoring mitochondrial function and decreasing ROS levels.
Lycopene (Chowdhury *et al.*, 2017)	Bovine	0.2 μM	The quality of embryos increased by decreasing expression levels of ROS, NFκB, COX2, caspase-3, iNOS, and Bax, and increasing expression of the *Bcl-2* gene.
Melatonin (Marques *et al.*, 2018)	Bovine	10^{-9} M	Production and quality of blastocysts improved by reducing ROS levels and apoptosis and increasing GSH levels and expression of *SOD* and *HSPB1* genes.
Melatonin (Pang *et al.*, 2016)	Bovine	10^{-3} M	Rate of blastocyst development increased and the rate of apoptosis in the blastomeres decreased by reducing BAX and CASP3 and enhancing *BCL2*, *XIAP* and *CAT* genes.
Melatonin (Soto-Heras *et al.*, 2019)	Goat	10^{-7} M	The quality of embryos increased by decreasing ROS levels, increasing mitochondrial activity, and ATP concentration and regulating the expression of related genes (ACTB, SLC1A1, SOD1, GPx1, Bax, DNMT1, GCLC, and GDF9).
Melatonin (Zou *et al.*, 2020)	Human	10 μM/l	The high-quality blastocyst formation rate increased by protecting mitochondrial function and decreasing the ROS level.
Melatonin (Asgari *et al.*, 2012)	Mice	100 nM	Embryo development improved by increasing embryonic inner cell mass.
Melatonin (Tian *et al.*, 2017)	Mice	10^{-7} M	The quality of the blastocysts improved by decreasing ROS levels and cellular apoptosis.
Melatonin (Lee *et al.*, 2018)	Porcine	1 nM	The cell number of blastocysts and embryo development increased.
Melatonin (Kim *et al.*, 2019)	Porcine	10^{-7} M	Rate of blastocyst formation and the number of blastomeres increased by decreasing oxidative stress through the Nrf2/ARE signalling pathway.
N-Acetyl-L-cysteine (NAC) (Truong and Gardner, 2020)	Mice	10 μM	NAC conveyed beneficial effects on embryo development during cryopreservation by reducing oxidative stress, increasing the cell numbers, reducing apoptotic cells and increasing acetylation levels.
N-Acetyl-L-cysteine (NAC) (Truong and Gardner, 2017)	Mice	10 μM	Total cell numbers in the blastocysts increased and embryonic cell cleavage was performed faster.
Naringenin (Perez-Pasten *et al.*, 2010)	Mice	Up to 30 μM	Embryos were protected from damage caused by hydroxyurea including developmental retardation and abnormalities.

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Table 1. (*Continued*)

Antioxidant (Reference)	Embryo species	Effective dose	Results
Polydatin (Khan *et al.*, 2017)	Bovine	1 μM	Blastocyst development improved by increasing the expression of Sirt1 protein and decreasing expression of NFκB and COX2 proteins and ROS levels.
Pterostilbene (Ullah *et al.*, 2019)	Mice	0.25 μM	Embryonic cells were protected by increasing expression levels of Bcl-2, Nrf2, CAT, Heme oxygenase1 (HMOX1), GPx and SOD, and decreasing expression of Bax and caspase-3.
Quercetin (Sovernigo *et al.*, 2017)	Bovine	2 μM	Blastocyst development improved by decreasing ROS levels or increasing GSH levels.
Quercetin (Sameni *et al.*, 2018)	Mice	5 μM	Rate of blastocysts and hatched blastocysts, the number of embryonic cells, zona pellucida thickness against actinomycin D were improved by decreasing apoptosis.
Quercetin (Perez-Pasten *et al.*, 2010)	Mice	Up to 30 μM	Embryos were protected from damage caused by hydroxyurea including developmental retardation and abnormalities.
Quercetin (Fan *et al.*, 2017)	Rat	1.0 μmol/l	Embryo development was improved by decreasing ROS levels.
Quercetin (Karimian *et al.*, 2018)	Sheep	5–15 μg/ml	Blastocyst formation rate increased by scavenging free radicals.
Quercetin (Lee *et al.*, 2015)	Zebrafish	100 μM	Embryos were protected against cisplatin-induced toxicity with decreased apoptosis.
Resveratrol (Sovernigo *et al.*, 2017)	Bovine	2 μM	Blastocyst development improved by decreasing ROS levels and increasing GSH levels.
Resveratrol (Hayashi *et al.*, 2018)	Bovine	1 μM	Quality of embryos improved after cryopreservation and thawing through mitochondrial synthesis.
Resveratrol (Madrid Gaviria *et al.*, 2019)	Bovine	0.5 μM	Resveratrol helped embryos to partially restore the initial quality they had before the cryopreservation process by maintaining GSH levels.
Resveratrol (Hara *et al.*, 2018)	Bovine	0.5 μM	Development of frozen embryos after warming increased with increasing mitochondrial activity.
Resveratrol (Abe *et al.*, 2017)	Bovine	0.5 μM	Embryo development improved by decreasing ROS levels and modifying mitochondrial function.
Resveratrol (Wang *et al.*, 2018)	Mice	25 μM	Number of blastomeres and the levels of GSH increased and the levels of ROS and the abnormal mitochondrial distribution decreased.
Resveratrol (Lee *et al.*, 2018)	Porcine	2 μM	Cell number of blastocysts and embryo development increased.
Resveratrol (Zabihi *et al.*, 2019)	Sheep	0.5 μM	Rate of morula and blastocyst and the number of blastomeres increased during *in vitro* culture and *in vitro* maturation.
Retinol (vitamin A) (Elomda *et al.*, 2018)	Rabbit	1000 nM	Embryo development improved by decreasing the malondialdehyde (MDA) level, increasing SOD and GPx activities, and upregulating expression of the gap junction protein alpha 1 (GJA1).
Royal jelly (Eshtiyaghi *et al.*, 2016)	Ovine	10 mg/ml	Rate of cleavage and blastocyst formation increased by enhancing SOD and GPx activities.
Sericin (Khatun *et al.*, 2018)	Bovine	1.0% (w/v)	Rate of apoptosis and expression of *BAX* and *HSPA1A* genes decreased in the blastocysts and IFNT2 levels increased.
Syzygium aromaticum (Santos *et al.*, 2019)	Bovine	20 μg/ml	Embryo development and the number of embryonic cells were increased by reducing ROS levels and increasing mitochondrial activity.
Vitamin C (Sovernigo *et al.*, 2017)	Bovine	50 μg/ml	Blastocyst development improved by decreasing ROS levels and increasing GSH levels.
Vitamin C (Karimian *et al.*, 2018)	Sheep	25–200 μg/ml	Blastocyst formation rate increased by scavenging free radicals.

this stage depends on the Krebs' cycle, whereas prior to this stage ATP production is mainly dependent on glycolysis.

For ROS measurement, fluorescent probes are superior sensors due to high sensitivity, ease in data collection and high resolution in microscopy imaging techniques. The fluorescent probe for detecting each type of ROS is different (Gomes *et al.*, 2005). However, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) is used to detect overall ROS production and in culture medium and is used in IVF (Martin-Romero *et al.*, 2008). Although in general, detection of ROS in human early embryos using sensitive fluorescent probes, rather than exact identification of species and quantification of level, has been generally observed as a negative factor (Betts and Madan, 2008; Van Blerkom, 2009). Due to the toxic effects of these fluorescent probes on embryo development, detection of ROS cannot be carried out at the same time in cultured embryos for transfer (Kohler *et al.*, 1994; Yang *et al.*, 1998).

In the embryo culture process, ROS are generated both endogenously and exogenously. Endogenous ROS are produced by embryo metabolism, while exogenous ROS are produced spontaneously by buffers and enriched culture medium. The most important ROS produced in this manner are superoxide anions, H_2O_2, hydroxyl radicals, and alkyl hydroperoxide. Given that

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extracellular oxidation rates of ROS-sensitive dyes are higher than those measured within the intracellular environment, these data should not be interpreted as a higher rate of total ROS generation in the extracellular environment. Buffers and culture media containing cupric or ferric salts may lead to the generation of significant amounts of superoxide anions. Moreover, in the composition of serum and serum synthetic replacements (SSR), oxidase activities exist that may speed up the generation of ROS in these buffers. Also, it seems that ROS production is higher in more complex culture media compared with simple media (Martin-Romero *et al*., 2008; Menezo *et al*., 2010).

There are several types of ROS including peroxide, superoxide anions, hydrogen peroxide, hydroxyl radicals and nitric oxide.

Peroxide is a compound that possesses one or more oxygen–oxygen bonds. The most common peroxide is hydrogen peroxide. Hughes *et al.* (2010) showed that the presence of peroxide in culture medium affected mouse embryo development, such that 1-cell embryos had the highest sensitivity for peroxides in mineral oil (Hughes *et al*., 2010). The sources of peroxide in embryo culture media are embryonic mitochondria and mineral oil, which is used to cover the culture media (Burton *et al*., 2003; Otsuki *et al*., 2007).

Superoxide anions ($O_2^{\bullet-}$) are the most common type of ROS that is generated in mitochondria. In aerobic organisms, most oxygen is converted to water through the mitochondrial respiratory chain, however a small proportion of the oxygen molecules (about 1–2%) is converted to superoxide anion radicals. Increased mitochondrial activity is directly related to increased levels of superoxide anion production, which could adversely affect mitochondrial respiration. The half-life of superoxide anions is about 10^{-9} to 10^{-11} s, but in the presence of superoxide dismutase (SOD), this reduces to 10^{-15} s. (Taverne *et al*., 2013; Chen *et al*., 2018). Joo *et al.* (2001) have shown that increasing superoxide anion concentrations in embryo culture media reduces embryo development, and Nonogaki *et al.* (1992) showed that adding SOD into the culture medium has a protective effect on embryo development against oxidative stress. For detecting superoxide anions, dihydroethidium (or hydroethidine) (DHE) is used as a fluorescent probe; when DHE is oxidized by superoxide anion, it is converted to ethidium which is a fluorescent compound (Benov *et al*., 1998).

Hydrogen peroxide (H_2O_2) is a neutral molecule, which is the least reactive molecule among types of ROS and is stable under physiological pH and temperature in the absence of metal ions. It is highly diffusible and crosses the plasma membrane easily. H_2O_2 can be produced from superoxide anions by SOD. Moreover, in the presence of metal ions and superoxide anions, H_2O_2 can produce hydroxyl radicals. H_2O_2 is oxidized by catalase and peroxidase (Dickinson and Chang, 2011; Taverne *et al*., 2013). Catalase is a ROS-scavenging enzymes found in all adult organs and the embryo, although the activity of embryonic catalase is only about 5% that of adult activity, it may enhance the risk of injury during embryo development due to increased ROS. If catalase is not effective, H_2O_2 may initiate signal transduction pathways or react with iron to create highly reactive hydroxyl radicals, which can damage cells. The protective role for catalase against teratogenesis has been demonstrated in embryo culture, such that it is a specified exogenous catalase that increases embryonic antioxidant activity and protects against DNA oxidation (Abramov and Wells, 2011; Miller-Pinsler and Wells, 2015). The half-life of H_2O_2 is about 10^{-3} s in the absence of catalase and 10^{-8} s in its presence (Taverne *et al*., 2013). H_2O_2 is one of the major ROS produced in culture medium (Martin-Romero *et al*., 2008). Several studies have shown that H_2O_2 damages embryo development *in vitro* and adding antioxidants such as L-carnitine, apigenin, and quercetin into culture medium can protect the embryos against it (Abdelrazik *et al*., 2009; Yu *et al*., 2014; Safari *et al*., 2018). For detecting H_2O_2, H_2DCFDA and Amplex-red/horseradish peroxidase are used (Wang and Joseph, 1999; Martin-Romero *et al*., 2008).

The hydroxyl radical ($^{\bullet}OH$) is the neutral form of the hydroxide ion (OH^-). Hydroxyl radicals are the most reactive and dangerous radicals that can be formed from superoxide anions and H_2O_2 in the presence of metal ions. *In vivo*, the half-life of hydroxyl radicals is only about 10^{-9} s, therefore when hydroxyl radicals are produced *in vivo*, they react close to their site of formation (Dickinson and Chang, 2011). Due to their features, the presence of hydroxyl radicals is very harmful to the embryo development *in vitro* (Dumoulin *et al*., 1995). Fluorescein is used as a fluorescent compound to detect hydroxyl radicals. Fluorescein is oxidized by hydroxyl radicals to a non-fluorescent product. This reactivity is useful to assess antioxidant activity in an assay using hydroxyl radical averting capacity (Ou *et al*., 2002).

Nitric oxide (NO) is an uncharged lipophilic molecule containing a single unpaired electron, which causes it to be reactive with other molecules such as oxygen, superoxide radicals and glutathione. While NO is not a very reactive free radical, it is able to form other reactive intermediates that have an effect on protein function and on the function of the entire organism. NO is removed within seconds by diffusion from tissues and enters the red blood cells and reacts with oxyhaemoglobin. The direct toxicity of NO is modest, but is greatly increased by reacting with superoxide anion. These reactive intermediates can trigger nitrosative damage in biomolecules. Conversely, NO can act as an antioxidant. NO is a neurotransmitter and blood pressure regulator. At physiological concentrations, the half-life of NO due to its reaction with oxygen is in the range 9–900 min. In aqueous solution, the half-life of NO decrease to between 6.2 and 3.8 s (Beckman and Koppenol, 1996; Kelm, 1999). In relating the effect of NO on embryo development *in vitro*, it seems that NO is useful and acts as a regulator in preimplantation embryo development (Chen *et al*., 2001; Tranguch *et al*., 2003). NO has regulatory functions in modulating oxidative respiration by binding to the same site as oxygen in the electron transport chain. Such normal functions have been described for oocytes during their maturation and for embryos, in which for some species, endogenous NO synthase has been detected (Tranguch *et al*., 2003; Feng, 2012; Tengan and Moraes, 2017). Multi-component Hantzsch ester synthesis of 1,4-dihydropyridines (DHPs) compounds can be used via the fluorescent probes to detect NO (Wang *et al*., 2016).

Types of antioxidants

Antioxidants are divided into enzymatic and non-enzymatic antioxidants based on their catalytic activity (Haida and Hakiman, 2019). Enzymatic antioxidants are produced in cells and protect the body against free radicals via some enzymes that form a distinctive group, with detoxification. Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) are the key enzyme antioxidants of this defence system by which free radicals that are generated during metabolic reactions are removed (Jeeva *et al*., 2015). Non-enzymatic antioxidants mainly include polyphenols (flavonoids, phenolic acids, and anthocyanins), carotenoids (carotenes, xanthophylls) and vitamins (vitamins A and C) (Kumar *et al*., 2017; Xu *et al*., 2017). These types of antioxidants are found naturally in fruits, vegetables and foods such as orange,

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tomato, carrot and fish. To obtain these antioxidants, their separation and purification are needed. This means that they must be separated from feedstock to enhance their purity and antioxidant capacity (Rozoy *et al.*, 2012; Jeeva *et al.*, 2015). These types of antioxidants scavenge free radicals by donating hydrogen ions to stabilize the free radicals (Parveen *et al.*, 2016; Maarman, 2017; Xu *et al.*, 2017; Haida and Hakiman, 2019).

Genes involved with antioxidants

Antioxidants usually activate certain genes to neutralize free radicals and protect cells. Nuclear factor erythroid-derived 2-like 2 (Nrf2) is a key transcription factor that is able to activate anti-oxidative reactions; it is known as the master regulator of the antioxidant response and modulates the expression of various antioxidant genes. Nrf2 plays an important role in immune and inflammatory responses and tissue remodelling (Hybertson *et al.*, 2011). Moreover, Nrf2 signalling is a key pathway by which enzymatic antioxidants remove ROS and other harmful free radicals to protect cells from oxidative stress. Upregulation of many enzymatic antioxidants or inhibition of lipid peroxidation is mediated by Nrf2 (Chen *et al.*, 2015; Canella *et al.*, 2018).

In addition to Nrf2, Kelch-like ECH-associated protein 1 (*Keap1*) and antioxidant response element (*ARE*) or electrophile response element (*EpRE*) are also important genes involved in protecting cells, therefore the Nrf2/Keap1/ARE signalling pathway is one of the most important cellular defence mechanisms against oxidative stress. When oxidative stress injures cells, *Nrf2* expression levels are significantly increased, while Keap1, a signalling molecule that binds to motifs in the N-terminal region of Nrf2, is decreased. Subsequent stabilization and nuclear localization of ARE/EpRE binding leads to protection of cells (Kundu and Surh, 2010; Yan *et al.*, 2019).

NAD(P)H/quinone oxidoreductase 1 (*NQO1*) is an antioxidant gene that is overexpressed under certain conditions such as in cancer tumours, to suppress it. For this disease, NQO1 activates Nrf2 to initiate cellular defence mechanisms against the tumour (Pey *et al.*, 2016; Osman *et al.*, 2015).

Effect of antioxidants on apoptosis

Apoptosis or programmed cell death is essential for the normal functioning and survival of most multicellular organisms. Apoptosis is important for removing damaged or infected cells, however excess apoptosis can cause adverse biological consequences (Kannan and Jain, 2000). Apoptosis plays an important role in embryo development, If apoptosis is increased in embryonic cells, the blastocyst expands, decreasing the zona pellucida thickness, such that hatching and implantation may not occur correctly. Apoptosis could eventually lead to embryo death (Abdelrazik *et al.*, 2009; Yu *et al.*, 2014; Safari *et al.*, 2018).

Maintaining the integrity of the mitochondrial membrane is an important process to prevent apoptosis. Under oxidative stress, the permeability of mitochondria increases and leads to uncoupling of the respiratory chain, resulting in hyperproduction of ROS, cessation of ATP synthesis, and depletion of glutathione (GSH) (Kannan and Jain, 2000; Susin *et al.*, 1998; Morel and Barouki, 1999). Under these conditions, to establish osmotic balance, diffusion of H_2O leads to swelling of mitochondria. This change in mitochondrial membrane potential predisposes the cells to oxidative damage by impairing the endogenous antioxidant defence mechanisms (Marzo *et al.*, 1998; Yang and Cortopassi, 1998). This antioxidant defence system has two main ROS degrading pathways that involve GSH and thioredoxin (Trx) (Tonissen and Di Trapani, 2009; Handy and Loscalzo, 2012).

In the GSH pathway, GSH is one of the most abundant molecules among endogenous antioxidants. GSH directly reacts with ROS or indirectly scavenges ROS by revitalizing other antioxidants. Many antioxidants used to scavenge oxidative stress are converted chemically into oxidation products, such that they react with GSH to form GSH adducts during protection against free radicals such as ROS and H_2O_2 (Espinosa-Diez *et al.*, 2015; Kwon *et al.*, 2019). Mitochondria are the main intracellular sites of oxygen consumption and the chief sources of ROS production, most of which originate from the respiratory chain in mitochondria. Mitochondrial GSH (mGSH), an antioxidant enzyme existing in mitochondria, acts as the primary line of defence against oxidative modifications (Mari *et al.*, 2009). The importance of mGSH is based on its abundance and its versatility to counteract with H_2O_2, mainly as a cofactor of enzymes such as GPx (Dannenmann *et al.*, 2015). GPx is an enzyme antioxidant that is expressed in many cells and tissues during embryo formation and it protects embryos against oxidative stress. As GPx removes H_2O_2, the rate of apoptosis in embryonic cells decreases (Yu *et al.*, 2014). In embryogenesis, cytosolic GPx is highly expressed in most cells to protect embryos from oxidative stress (Baek *et al.*, 2005). It seems that some flavonoid antioxidants can affect GPx activity. Yu *et al.* (2014) showed that quercetin reduced apoptosis in mouse zygotes by maintaining the activity of GPx, preventing mitochondrial dysfunction, and decreasing intracellular ROS levels (Yu *et al.*, 2014). Other studies have shown that these antioxidants, such as apigenin and quercetin, can decrease the rate of apoptosis in mouse embryonic blastomeres by decreasing the destructive effects of H_2O_2 (Safari *et al.*, 2018; Sameni *et al.*, 2018). Lagoa *et al.* (2011) have shown that flavonoid antioxidants inhibit H_2O_2 production by increasing mitochondrial activity. Boadi *et al.* (2016) have shown that flavonoid antioxidants, such as quercetin, genistein, and kaempferol, sustained intracellular GSH levels in the cells. Therefore, it seems that flavonoid antioxidants may improve embryo development *in vitro* by affecting GSH activity and decreasing the destructive effects of H_2O_2 and ROS.

In the Trx pathway, Trx is a major antioxidant for maintaining the intracellular reduction–oxidation (redox) state. Trx acts as a redox-active protein to regulate the activity of different enzymes within the cell. Also, Trx acts as a ROS scavenger and directly inhibits pro-apoptotic proteins such as apoptosis signal-regulating kinase 1 (ASK1). Mitochondria and cytoplasm contain Trx systems and inhibition of either system can lead to activation of apoptotic pathways (Miranda-Vizuete *et al.*, 2000; Tonissen and Di Trapani, 2009). Bing *et al.* (2003) showed that Trx is involved in the improvement of the development of bovine embryos *in vitro*. Thioredoxin-2 (Trx2), is a mitochondrial protein that reduces oxidative stress, regulates apoptosis and is essential for the control of cell survival during mammalian embryonic development (Patenaude *et al.*, 2004; Hansen, 2012). Moreover, Trx2 may contribute to the development of the embryonic nervous system, as Pirson *et al.* (2015) reported that Trx2 modulated apoptosis of neurons during embryonic development of the chick spinal cord. It seems that flavonoid antioxidants can affect Trx (s) expression. Park *et al.* (2020) showed that quercetin prevents the decrease in Trx expression following neuronal cell damage. Sharma *et al.* (2007) reported that flavonoids significantly decreased the release of ROS from astrocytes stimulated with IL-1β. This decrease caused an increase in SOD and Trx1 expression levels and

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protection against oxidative stress. In this regard, Khera *et al.* (2013, 2017) showed that overexpression of endogenous antioxidants such as GPx and Trx following supplementation with selenium, an exogenous antioxidant, could protect embryo trophoblast cells from mitochondrial oxidative stress.

Action mechanisms of antioxidants on embryo development

Culture conditions during early developmental stages affect metabolic activity and the ability of embryos to adapt to the environment. In embryo development *in vitro*, oxidative stress generates excessive ROS free radicals, and leads to increased apoptosis, changes in gene expression, and reduced embryo quality (Ullah *et al.*, 2019). Oxidative stress occurs as a result of an imbalance between antioxidants and ROS production which is induced by endogenous and exogenous factors during embryo culture *in vitro*. Enhanced embryo development under lower oxygen conditions is probably due to improved embryo metabolism and reduced ROS production (Guerin *et al.*, 2001). There are some mechanisms in embryo culture that lead to a decrease in levels of ROS and protect against oxidative stress. Under oxidative stress conditions, the Nrf2 transcription factor binds to ARE to induce antioxidant and detoxification enzymes. ROS generation in the culture medium, in particular, increases in the blastocyst stage. The Nrf2-mediated oxidative stress response pathway is the main pathway in the blastocyst for which upregulation for most antioxidant-related genes is controlled by transcription factor Nrf2 (Gad *et al.*, 2012). Indeed, Nrf2 signalling has the most effect on embryo development *in vitro* (Ullah *et al.*, 2019). Expression of the *Nrf2* gene also increases during embryo cryopreservation to protect embryonic cells (Mehaisen *et al.*, 2015). Nrf2 activates enzyme antioxidants such as GPx, SOD, and CAT against excessive ROS in embryos and therefore protects embryonic cells *in vitro* (Ullah *et al.*, 2019). Moreover, antioxidants can increase the expression of the anti-apoptotic gene *Bcl-2* and reduce the expression of pro-apoptotic genes, *Bax* and *caspase-3* in blastocysts, indicating the protective effects of antioxidants on embryo development (Mishra *et al.*, 2017; Ullah *et al.*, 2019). Pterostilbene is an antioxidant that acts by the same mechanism in embryonic cells or adult cells. Sireesh *et al.* (2017) have reported that pterostilbene increases *Bcl-2* expression and reduces the expression of *Bax* and *caspase-3* in pancreatic β-cells of diabetic animals through Nrf2 activation. For embryo culture, pterostilbene protects embryonic cells by increasing expression levels of Bcl-2, Nrf2, CAT, Heme oxygenase1 (HMOX1), GPx and SOD, and by decreasing expression levels of Bax and caspase-3 (Ullah *et al.*, 2019).

The oviduct and the uterine environments contain many substances that maintain embryo development or remove toxic agents. To mimic these conditions *in vitro*, various substances that decrease ROS concentrations in embryos are added to culture media. For example, adding SOD or CAT improves embryo development by upregulating GSH synthesis as an effective antioxidant for the developmental potential of embryos (Orsi and Leese, 2001; Ali *et al.*, 2018).

Conversely, intracellular lipid contents may affect embryo quality and developmental potential. Lipid metabolism and oxidative stress response-related genes are the most affected via embryonic genes *in vitro* (Jeong *et al.*, 2009). Moreover, inhibition of fatty acid oxidation during IVM impairs embryo development and indicates the importance of lipid metabolism in embryonic development (Somfai *et al.*, 2011). During preimplantation embryo development, lipid may be sequestered within cells and used by mitochondria to increase the production of ATP required for compaction and blastocyst formation or differentiation of cell lineages (Jeong *et al.*, 2009). Excess lipids may be accumulated in the embryo by uptake from the culture environment and may impair mitochondrial activity to metabolize complex lipids (Tarazona *et al.*, 2006; Krisher and Prather, 2012; Walther and Farese, 2012). Some antioxidants such as L-carnitine, quercetin, and apigenin play a primary role in fatty acid transportation from the cytosol into mitochondria, increase mitochondrial activity, enhance lipid metabolism and improve cleavage rates in embryos, which indicates the importance of mitochondria and lipid metabolism in embryo development (Khanmohammadi *et al.*, 2016; Safari *et al.*, 2018; Sameni *et al.*, 2018; Talebi *et al.*, 2020).

Optimizing embryo culture medium

Optimizing embryo culture media is essential to obtain high-quality embryos. Various factors added to culture media can improve the oxidative state of early embryos. Weathersbee *et al.* (1995) reported that synthetic serum substitute was a suitable, standardized means of adding protein to embryo culture medium. Synthetic serum substitute is primarily a globulin-enriched protein preparation containing mostly human serum albumin (HSA). HSA scavenges ROS and protect embryos against DNA damage (Lan *et al.*, 2019). Albumin is one of the most important substances used to optimize embryo culture media. Albumin is a small protein that is present in human plasma. Its functions consist of regulation of osmotic pressure and transport of some substances (Otsuki *et al.*, 2013). Albumin acts as a surfactant that facilitates the handling of gametes and preimplantation embryos *in vitro* (Bungum *et al.*, 2002). Albumin has specific binding sites for copper ions. Copper ions can accelerate the destruction of free radical reactions. Most plasma copper is bound to the protein ceruloplasmin, which has antioxidant properties. In this regard, cytosolic SOD (SOD1) functions more than mitochondrial SOD (SOD2) because it works to scavenge ROS with the aid of metal cofactors including copper and zinc. The mechanism of scavenging ROS by SOD1 involves alternate reduction and reoxidation of the copper at the active site of the enzyme, and zinc participates in proper protein folding and stability (Fukai and Ushio-Fukai, 2011). Albumin is able to inhibit copper-stimulated peroxidation and inhibits the production of free radicals from systems containing copper ions and H_2O_2. Another antioxidant activity of albumin may be to scavenge peroxy radicals and decrease lipoxygenase activity (Halliwell, 1988). Albumin has different functions in the growth of embryos *in vitro*. It acts as a regulator of pH and osmotic pressure, a scavenger of toxins and free radicals, a stabilizer of cell membranes, a carrier of growth-promoting substances, and a nutrient (Otsuki *et al.*, 2013). Three different types of albumin are used to optimize embryo culture medium: HSA, bovine serum albumin (BSA), and recombinant HSA (rHSA):

1. HSA is known to be a multifunctional protein in the intravascular compartment (Maciazek-Jurczyk *et al.*, 2018). HSA was used in human embryo culture media for the first time by Pool and Martin (1994), who showed that albumin accelerated the growth of embryos. HSA is the primary protein supplement used in clinical embryo culture media. HSA as a macromolecule supplementation can act as a surfactant, as a nitrogen source, as a carrier molecule for other compounds, modulate the physical

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microenvironment, and stabilize membranes (Swain *et al.*, 2016).
2. BSA is the most abundant protein in bovine blood plasma. Due to its unique characteristics and known structure, is commonly used as a model protein for the culture of animal oocyte and embryo and is effective in their development (Ledesma-Osuna *et al.*, 2008; Nasrollahzadeh *et al.*, 2017).
3. rHSA has been shown to be as efficient as HSA for fertilization and embryo development. In addition, using rHSA for IVF may decrease the risk of contamination and the transmission of plasma-derived impurities. However, rHSA has not been widely applied in human embryo culture media because of the high cost of production (Bungum *et al.*, 2002; Otsuki *et al.*, 2013).

In addition to the role of albumin in optimizing embryo culture medium, studies have shown that albumin can also optimize culture media used for sperm and oocytes through its antioxidant properties. Some studies have shown that centrifugation during sperm preparation using density gradient centrifugation or swim-up without serum albumin is associated with iatrogenic damage to sperm. Free radicals produced by mitochondria during centrifugation cause membrane lipid peroxidation and DNA damage in the absence of albumin (Aitken and Clarkson, 1988; Twigg *et al.*, 1998; Aitken *et al.*, 2014; Muratori *et al.*, 2019). This damage may result from tightly packed sperm pellets through peroxide formed from superoxide radicals by MnSOD. It is released from damaged mitochondria and induces lipid peroxidation of the plasma membrane, depolarizes mitochondria, affects sperm motility and reduces ATP generation (De Iuliis *et al.*, 2006; Uribe *et al.*, 2015; Barbonetti *et al.*, 2016; Kotwicka *et al.*, 2016). Another study showed that BSA protected spermatozoa against cool storage-induced DNA damage through its antioxidant properties. (Sariozkan *et al.*, 2013). Conversely, it has been shown that increasing serum albumin improved the viability of bovine oocytes *in vitro* (Hamman *et al.*, 2019).

Despite all the benefits of using albumin in embryo culture media, Otsuki *et al.* (2009) reported that when peroxide in mineral oil (used to maintain embryos in the culture medium) is more than 0.02 mEq/kg, albumin present in culture media allows entry of free radicals into the zona pellucida, causing damage to the human embryos. In other studies, Martinez *et al.* (2017) showed that peroxidized mineral oil enhanced the oxidant status of culture media and inhibited porcine embryo development *in vitro*. Otsuki *et al.* (2007) demonstrated that peroxidation of mineral oil applied in culture was harmful to fertilization and human embryo development. In this regard, Ainsworth *et al.* (2017) reported that the standard embryo assays used by manufacturers did not detect the potential toxicity of peroxides in mineral oil. It seems that the use of mineral oil requires further studies and would depend on various factors such as brand, purity, starting material, method of production and storage. These issues indicated that optimization of human embryo culture media requires more comprehensive, complete study.

Some studies have shown that when purified serum albumin cannot be used, organic compounds such as polyvinyl alcohol (PVA) or certain dextran polymers can be adequate substitutes for IVF and preimplantation embryogenesis. PVA is a polymer and a suitable replacement for BSA in embryo culture media that supports the development of preimplantation embryos. Dextran is also a polymer chain consisting of a non-toxic branch of glucose. These polymers with their surfactant properties facilitate the handling of the embryos, probably by influencing the physico-chemical attributes of the media. Moreover, they have been shown to protect embryos against cryoinjury by avoiding the mechanical pressure that occurs during cryopreservation (Dumoulin *et al.*, 1994; Biggers *et al.*, 1997).

Comparison of the effect of different antioxidants on embryo development

Some studies have shown that different antioxidants have various differing effects on embryo development *in vitro*, therefore comparing their effects would help future studies to select the most suitable candidates. Conversely, it seems that combined and concomitant use of antioxidants can be even more effective than their separate use. In this regard, Sovernigo *et al.* compared the effects of five well known antioxidants (quercetin, cysteamine, carnitine, vitamin C and resveratrol) by the levels of ROS and GSH in bovine embryos. The results showed that quercetin, vitamin C and resveratrol significantly reduced ROS levels compared with cysteamine and carnitine. GSH levels increased in cysteamine and carnitine compared with quercetin, vitamin C and resveratrol (Sovernigo *et al.*, 2017).

Perez-Pasten *et al.* (2010) compared the effects of quercetin and naringenin on the development of mouse embryos *in vitro*. The results showed that quercetin and naringenin both reduced the abnormal development of embryos produced by hydroxyurea at doses less than 30 μM. Lee *et al.* (2018) showed that the combination of resveratrol and melatonin supported a synergistic increase in blastocyst formation rates and total cell numbers of blastocysts and improved the development of porcine embryos. Truong *et al.* (2-16) showed that the combination of different antioxidants (acetyl-L-carnitine, *N*-acetyl-L-cysteine and α-lipoic acid) in culture media had a more beneficial effect on mouse embryo development *in vitro* (Truong *et al.*, 2016).

Antioxidants in the clinic

The use of antioxidants clinically in IVF is still very limited. Currently, lipoic acid is the most common used antioxidant in human embryo culture media (Truong and Gardner, 2017), but more antioxidants are expected to be used in human embryo culture media in the future. More recently, there have been some investigations into the effects of antioxidants on human embryo development *in vitro*. Kim *et al.* (2018) reported that adding L-carnitine to culture media improved human embryo quality and pregnancy outcomes. L-Carnitine may improve embryo development *in vitro* by increasing mitochondrial activity and β-oxidation processes (Arenas *et al.*, 1998; Abdelrazik *et al.*, 2009; Kepka *et al.*, 2014; Khanmohammadi *et al.*, 2016). Truong *et al.* (2016) suggested that the combination of some specific antioxidants may improve human embryo culture media, however before the use of human embryos proper evaluation is required (Truong *et al.*, 2016; Truong and Gardner, 2017).

Prescribing the proper dose of antioxidants is one of the most important aspects of their effectiveness. Many antioxidants are dose dependent and may be harmful if consumed in too large quantities (Halliwell, 2012). Quercetin is a flavonoid antioxidant that, at appropriate doses, has favourable effects *in vitro* on the development of different species of embryos (Perez-Pasten *et al.*, 2010; Lee *et al.*, 2015; Fan *et al.*, 2017; Sameni *et al.*, 2018). Perez-Pasten *et al.* (2010) showed that quercetin at doses above 100 μM caused significant increase abnormalities such as oedema, rotation failure, neural tube defects, somite dysmorphology, and

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telencephalic hypoplasia for developing mouse embryos *in vitro*. L-Carnitine is a useful antioxidant for ovarian regeneration (Zarbakhsh *et al*., 2019) and embryo development *in vitro*, but it is toxic at high doses, (Abdelrazik *et al*., 2009; Khanmohammadi *et al*., 2016). Perez-Pasten *et al.* (2010) compared different doses of pure naringenin on developing mouse embryos *in vitro*. They reported that 30 μM pure naringenin with antioxidant and free radical scavenging activities had a protective effect against hydroxyurea-induced embryonic damage, while at doses above 100 μM it produced growth retardation, developmental defects and reduced viability in cultured mouse embryos.

As many antioxidants have beneficial effects on embryo development *in vitro*, to achieve proper supplement levels for human embryo culture media, a study on antioxidants is needed because they constitute a promising therapeutic approach.

Conclusion

Based on the reviewed literature, correct doses of most antioxidants have the potential to protect embryo development *in vitro* through mediating in signalling pathways, scavenging free radicals, increasing mitochondrial activity and decreasing apoptosis. Therefore, it seems that the use of antioxidants in human embryo culture media can be applied in the future as a non-invasive and effective method to improve human embryo development *in vitro*, although further studies including clinical trials must be conducted for confirmation. The important role of ROS in IVF and the fact that its effects have not yet been fully elucidated, more research is needed.

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