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Review Article/Artículo de revisión

THE IMPORTANCE OF ANTIOXIDANTS IN SPERM QUALITY AND *IN VITRO* EMBRYO PRODUCTION

IMPORTANCIA DE LOS ANTIOXIDANTES EN LA CALIDAD ESPERMÁTICA Y LA PRODUCCIÓN *IN VITRO* DE EMBRIONES

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ABSTRACT

Oxidative stress (OS) is the imbalance between the production of Reactive Oxygen Species (ROS) and the protective effect of the responsible antioxidant system for their neutralization and/or removal. OS has been identified as one of the main factors associated with male or female infertility. Indeed, the excessive production of ROS affects the structural and functional integrity of gametes and embryos either *in vivo* or *in vitro.* In particular, OS damages proteins, lipids, DNA and accelerates cell apoptosis. These events have been implicated with impaired sperm quality and low fertilization rates. The increased amounts of ROS have also been correlated with poor outcome in assisted reproductive techniques (ART) settings. The biological systems are equipped with antioxidant agents in order to counteract the negative effects of the ROS overproduction. Thus, ROS generation due to pathological conditions of the genital tract or the handling of gametes and embryos at high oxygen tension during ART, render the use of antioxidants essential, to protect the cells from the detrimental consequences of OS. A volume of recent published data indicates that both oral administration and *in vitro* supplementation of antioxidants are very promising strategies in order to maintain sperm quality characteristics and to ensure fertilization. Nevertheless, further studies should be addressed in order to provide answers on the safety, effectiveness, mechanism of action and combination of different antioxidants, depending on the circumstances. This review summarizes the consensus on the role of oxidative stress and antioxidants in animal and human reproduction. An emphasis is given in the critical role of plant derived antioxidants; this new knowledge may contribute in achievement of high fertilization rates.

Keywords: antioxidants, oxidative stress, spermatozoa, oocytes, embryos.
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RESUMEN

El estrés oxidativo (OS) es el desbalance entre la producción de Especies Oxigeno Reactivas (ROS) y el efecto protector del sistema antioxidante responsable de su neutralización y/o remoción. EL OS ha sido identificado como uno de los principales factores asociados con la infertilidad masculina y femenina. En realidad, la excesiva producción de ROS afecta la integridad estructural y funcional de gametos y embriones ya sea *in vivo* o *in vitro*. En particular, el OS daña proteínas, lípidos, DNA y acelera la apoptosis celular. Estos eventos han sido implicados con disminución de la calidad espermática y bajas tasas de fertilización. Altas cantidades de ROS han sido también correlacionadas con pobres resultados de técnicas de reproducción asistida (ART). Los sistemas biológicos están equipados con agentes antioxidantes con la finalidad de contrarrestar los efectos negativos de la sobreproducción de ROS. Por lo tanto, la generación de ROS debido a condiciones patológicas del tracto genital o por la manipulación de gametos y embriones bajo una alta tensión de oxígeno durante las ART, hace esencial el uso de antioxidantes, para proteger a las células de las consecuencias negativas del OS. Un volumen de información recién publicada indica que tanto la administración oral como la suplementación *in vitro* de antioxidantes son estrategias muy promisorias para mantener la calidad espermática y mejorar la fertilización. Sin embargo, estudios adicionales deber ser realizados a fin de proveer respuestas sobre la seguridad, efectividad, mecanismo de acción y la combinación de diferentes antioxidantes, según las circunstancias. Esta revisión resume el conceso sobre el rol del estrés oxidativo y los antioxidantes en la reproducción animal y humana. Se ha hecho énfasis en el crítico rol de los antioxidantes derivados de plantas; este nuevo conocimiento puede contribuir al logro de altas tasas de fertilización.

Palabras clave: antioxidantes, estrés oxidativo, espermatozoide, oocitos, embriones.
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INTRODUCTION

Aerobic processes demand oxygen for controlled oxidation of molecules with subsequent release of energy. Even under physiological conditions, aerobic metabolism entails the formation of intermediate products, called Reactive Oxygen Species (ROS), which are playing an intriguing role in cell physiology. ROS are formed during oxygen reduction and interact with all cell components (Halliwell & Gutteridge, 2007). Although most of the available studies emphasize in the consequences resulting from the overwhelming production of ROS, their involvement in important physiological cell processes, such oxidative burst, gene expression and cell proliferation, is critical (Kohen & Nyska, 2002).

Various biological functions, including reproduction are affected by ROS. Low and moderate amounts of ROS mediate physiological processes, such as spermatogenesis, sperm hyperactivation, acrosome reaction (AR), interaction and fusion of gametes, oocyte maturation and embryo development (de Lamirande & Gagnon, 1993; O' Flaherty et al., 1999; Pasqualotto et al., 2004). In bulls, superoxide anion and hydrogen peroxide are essential for *in vitro* sperm capacitation and AR, respectively (O' Flaherty et al., 1999, 2003; Rivlin et al., 2004). However, elevated levels of ROS reduced sperm motility *in vitro* directly in equine (Baumber et al., 2000) and ram spermatozoa (Bucak et al., 2007), or indirectly via the induction of lipid peroxidation (LPO) in bovine (Bansal & Bilaspuri, 2008), caprine (Bucak et al., 2009), rabbit (Alvarez & Storey, 1984) and boar (Cerolini et al., 2000) spermatozoa.

The role of OS in the control of female reproduction has not been fully elucidated in animal species, comparing to human. Nevertheless, it seems that the balance disruption between ROS production and antioxidant defense can influence reproductive processes, such as follicular atresia, selection and dominance of the graafian follicle at different levels (Pasqualotto et al., 2004; Agarwal et al., 2006). The exaggerated production of ROS is controlled by the endogenous antioxidant defense (especially superoxide dismutase-SOD), which varies according to the stage of oocyte development in bovine species (Lonergan et al., 2003). The integrity of the antioxidant defense within the different stages of oocyte development may contribute to the overall quality of oocytes (Lonergan et al., 2003). Finally, ROS overproduction is leading to embryo death, despite the fact that embryos are equipped with internal antioxidant mechanisms, while similar mechanisms are present in the oviductal fluid. ROS can lead to embryo death due to LPO, DNA fragmentation and alterations in mitochondrial structures and enzymes (Takahashi et al., 2000; Guérin et al., 2001).

TYPES AND SOURCES OF ROS

Reactive Oxygen Species are oxygen-derived, short-lived and powerful molecules, which are mainly formed as intermediate products during oxidative phosphorylation in all cell types. ROS represent a broad category of molecules, including radical (e.g. superoxide anion, hydroxyl radical) and non-radical (e.g. hydrogen peroxide) derivatives (Fuchs et al., 1997).

There is evidence that spermatozoa may generate ROS in two ways: 1) the nicotinamide adenine dinucleoatide phosphate (NADPH) oxidase system at the level of the sperm plasma membrane and 2) the NADPH-dependent oxido-reductase (diphorase) at the level of mitochondria (Gavella & Lipovac 1992; Lopes et al., 1998). However, ROS produced by mammalian spermatozoa are mainly of mitochondrial origin (Ford 2004). Moreover, the main endogenous potential sources of ROS in male reproductive system are leukocytes and immature/abnormal spermatozoa, containing cytoplasmic droplets (Garrido et al., 2004; Bansal & Bilaspuri, 2011). ROS generation in immature spermatozoa is as a consequence of a defect that takes place during spermiogenesis causing retention of cytoplasmic droplets. Interestingly, there is evidence supporting a negative effect of immature spermatozoa in boar semen used in artificial insemination programs (Larsson et al., 1984). Furthermore, in bovine semen, ROS are produced primarily by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction (Shannon & Curson, 1972).

Regarding oocytes, little is known about their contribution to OS. In human, low levels of follicular fluid ROS and LPO are potential markers for predicting pregnancy (Pasqualotto et al., 2004). In bovine, there is evidence that the production of ROS remained unaltered between immature and *in vitro* matured oocytes (Dalvit et al., 2005). Mammalian embryos produce ROS mainly via oxidative phosphorylation (Guérin et al., 2001). A gradual increase in ROS production is detected from 2-cell bovine embryo up to late morula stage (Dalvit et al., 2005). This can be attributed to the sustained consumption of oxygen, glucose and pyruvate uptake during embryo development (Thompson et al., 1996). ROS production begins to decrease in the blastocyst stage due to the contribution of glycolysis to ATP supplementation (Thompson et al., 1996) and to the induction of antioxidant defense (Harvey et al., 1995).

OXIDATIVE STRESS AND OXIDATIVE DAMAGE IN GAMETES AND EMBRYOS

Oxidative stress is the imbalance between ROS production and the protective effect of the responsible antioxidant system for their neutralization and/or removal. In general, the uncontrolled production of ROS is harmful to all cellular components of gametes and embryos including proteins, lipids, nucleic acids and affects the fertilizing capacity. There is a great deal of evidence associating an increase in OS with a decrease in antioxidant protection. However, whether such changes are a cause or a consequence of ROS generation is still under investigation. One thing is certain that in the absence of (endogenous or exogenous) antioxidant protection, gametes and embryos are vulnerable to free radical attack (Aitken & Baker, 2006; Bansal & Bilaspuri, 2011) leading to pathological processes. Some of the main pathological effects and the importance of them in the field of reproduction are discussed below.

a. DNA fragmentation

DNA damage is induced by OS (Twigg et al., 1998). The existing positive correlation between ROS production and DNA fragmentation supports the hypothesis that the ongoing impairment of DNA affects the fertilizing capacity of spermatozoa (Simões et al., 2013). Spermatozoa are mainly protected from ROS detrimental effect by the tight packaging of DNA and the presence of antioxidants in the seminal plasma (Lopes et al., 1998). However, the seminal plasma containing high concentrations of ROS scavengers is removed during *in vitro* embryo development (Agarwal et al., 2006). The oxidative damage of spermatozoa impairs not only the fertilizing capacity, but also its competence to support normal embryo development (Simões et al., 2013). Spermatozoa are unique in that they lack DNA repair mechanisms. It is important that oocytes and zygotes have been shown to repair sperm DNA damage, so the effect of sperm DNA fragmentation depends on the combined effects of sperm chromatin damage and the capacity of oocytes to repair it (Ashwood-Smith & Edwards, 1996). However, bovine spermatozoa with affected motility and extended DNA fragmentation would still be able to fertilize, but the embryo development would be arrested before reaching the blastocyst stage (Simões et al., 2013).

b. Lipid peroxidation

Spermatozoa are especially susceptible to OS due to high energy demand, abundance of polyunsaturated fatty acids (PUFAs) of their plasma membrane and low concentration of intracellular antioxidant enzymes (Kodama et al., 1996; du Plessis et al., 2008). Lipid peroxidation, a self-propagating cascade of reactions, is induced by ROS, especially by hydroxyl radicals and affects the fluidity and integrity of sperm plasma membrane and thus decreases the fusogenic ability impairing fertilization (Aitken & Baker, 2006). The main product of LPO is malondialdeyde (MDA), which is highly toxic and interacts with cell components. The model of ROS evoking loss of sperm motility and genetic integrity is further supported by evidence that LPO is a major cause of motility loss and DNA fragmentation in spermatozoa (Kodama et al., 1996; Sapanidou et al., 2016).

c. Protein oxidation

Proteins are one of the initial targets of ROS. The oxidative damage is caused directly or indirectly, via the by-products of LPO, especially MDA, which interacts with the sulphydrylic groups of proteins (Halliwell & Gutteridge, 2007). Protein oxidation affects the functionality of specific receptors and the activity of intracellular enzymes. Some of these enzymes are related to the acquisition of fertilizing capacity and ATP supplementation (Guérin et al., 2001). Another hypothesis suggests that the excessive production of ROS decreases axonemal protein phosphorylation, resulting in reduction of membrane fluidity and sperm immobilization, both of which negatively affect sperm-oocyte fusion (de Lamirande & Gagnon, 1992; Baumber et al., 2000; Bansal & Bilaspuri, 2011).

ANTIOXIDANTS

Biological systems are equipped with antioxidant defense mechanisms in order to maintain ROS levels within certain range. According to Halliwell & Gutteridge (2007) an antioxidant is any substance that when is present at low concentrations, compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate. Antioxidants are subdivided in two categories: enzymatic such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx) and non-enzymatic such as vitamins A, E, glutathione (GSH), melatonin, carotenoids, polyphenols, etc. (Al Gubory et al., 2010). The key difference between enzymatic and non-enzymatic antioxidants is that the former destroy or quench free radicals in the cellular environment, while the latter have various functions including stimulation of enzymatic antioxidants and radical scavenging (Sies 1997).

ENZYMATIC ANTIOXIDANTS

Enzymatic antioxidants are also known as natural antioxidants. They neutralize excess ROS and prevent the damage of cellular structure. These enzymes are part of the first line defense against OS. Spermatozoa possess primarily enzymatic antioxidants, with SOD being the most predominant. SOD scavenges both intracellular and extracellular superoxide radical and prevents LPO of plasma membrane. However, it should be conjugated with CAT and GPx to prevent the action of hydrogen peroxide, which promotes the formation of hydroxyl radicals (Sikka et al., 1995; Makker et al., 2009). There is a wide difference in SOD activity among spermatozoa of different mammalian species which explains the variability in sensitivity to ROS that can be encountered in mammalian spermatozoa. Donkeys, rats and stallions are those with the highest sperm enzymatic activity (Alvarez & Storey, 1984; O'Flaherty 2014). On the other hand, spermatozoa remove hydrogen peroxide with a reaction catalyzed by CAT. This enzyme has been localized in bovine sperm (Bilodeau et al., 2000). GPx is the final member of the seminal enzymatic antioxidant triad. GPx consists of a family of antioxidants (GPx1-5) that are involved in the reduction of hydroperoxides using GSH as an electron donor. The activity of GSH-Px is determined by the regeneration of reduced GSH, which is carried out by glutathione reductase (GSSG-R). The balance and interaction between GSH-Px, GSSG-R and GSH play an important role in protecting sperm from oxidative damage (Alvarez & Storey 1989).

NON-ENZYMATIC ANTIOXIDANTS

The variety of enzymatic antioxidants is enriched with other substances present in the diets that pass to the seminal plasma such as carotenoids, flavonoids, vitamin E and C (Sikka et al., 1995; Makker et al., 2009). The non-enzymatic substances, also known as synthetic antioxidants or dietary supplements, are taking part in the first line of defense (Agarwal et al., 2005). Non-enzymatic antioxidants are also participating in the second line of defense against ROS which involves molecules characterized by the ability of rapid inactivation of radicals and inhibition of LPO such as melatonin, carotenoids etc. Some of the non-enzymatic antioxidants stimulate enzymatic antioxidants indicating that both types act synergistically to maintain or reestablish redox homeostasis (Saraswat et al., 2016; Mirończuk-Chodakowska et al., 2017). Finally, non-enzymatic antioxidants are classified into two categories: hydrophilic and lipophilic (Nogushi & Nikki 1999).

ANTIOXIDANT CAPACITY OF SEMINAL PLASMA AND FOLLICULAR FLUID

Seminal plasma plays an important role in protection of spermatozoa from the oxidative attack. Spermatozoa are susceptible to ROS due to the small volume of cytoplasm, low concentration of intracellular antioxidant enzymes, as well as the inability for protein synthesis (Kodama et al., 1996; du Plessis et al., 2008). Endogenous antioxidant defense system includes three major enzymatic antioxidants (SOD, CAT and GPx), in addition to a great variety of non-enzymatic ones (ascorbate, vitamin A and E, albumin, taurin etc).

Antioxidant defense mechanisms are present in the follicular fluid, as well as in embryos and the oviductal fluid (Guérin et al. 2001). The bovine follicular fluid contains the enzymatic antioxidants, including Mn-superoxide dismutase (MnSOD), cytosolic Cu/Zn superoxide dismutase (Cu/ZnSOD), GPx (Lonergan et al., 2003), as well as lipid- and water- soluble non-enzymatic antioxidants, such as α-tocopherol, β-carotene, ascorbic acid (Dalvit et al., 1998; Hoshi 2003). Oocytes and embryos are protected by these scavengers that are present both in the follicular and oviductal fluid. Oocytes are also protected by cumulus cells, containing enzymatic antioxidants, especially SOD and GSH (Dalvit et al., 2005).

Recent data support that high levels of ROS are accompanied with suppressed seminal and follicular antioxidant capacity. The decreased scavenging capacity and the elevated ROS levels play a significant role in fertilization and embryo formation. Consequently, under *in vivo* conditions, dietary supplementation of antioxidants would be very promising to avoid the negative effects of OS (Zini & Al-Hathal 2011).

ORAL (DIETARY) SUPPLEMENTATION OF ANTIOXIDANTS

Despite the large body of literature on the effect of oral administration of antioxidants, it remains unclear which is the best antioxidant agent depending on the pathological condition. There are no studies which establish the optimal dose, the combination and the duration of treatment. In brief, a dietary antioxidant should be effectively absorbed and distributed, while it should enhance the antioxidant capacity of the genital tract. Comhaire et al. (2000) suggested that it is important to accomplish the determination of ROS levels, DNA fragmentation index and Total Antioxidant Capacity (TAC) assay before and after the administration of any antioxidant agent in order to evaluate its effect on fertilizing capacity in the seminal plasma or in the follicular fluid. Nevertheless, to date many clinical trials have demonstrated a significant improvement in semen parameters and pregnancy outcome in human. Zini & Al-Hathal (2011) discussed the rationale of oral antioxidant therapy in infertile men and suggested that antioxidant dietary supplements (such as astaxanthin, vitamin E, etc.) improved sperm quality parameters compared

to the placebo group. Similar studies have been undertaken in animals, more specifically in rabbits, where the supplementation of drinking water for 12 weeks with vitamin E (1 g/L), ascorbic acid (1.5 g/L) or a combination of them reduced lipid peroxidation levels and improved rabbit semen quality. Interestingly, the best results were obtained from vitamin E (Youssef et al., 2003).

Plant derived antioxidants have been also tested *in vivo*. Saffron (100 mg/kg) also improved sperm motility and morphology and preserved chromatin integrity after 60 days of oral administration in rats (Mardani et al., 2014), while the supplementation of diet with catechins of green tea (200 ppm/kg) alleviated the effects mediated by the enrichment of rabbit spermatozoa with PUFA by preventing LPO (Kokoli et al., 2010). Catechins also inhibited apoptosis after 40 days of oral administration in rabbits. The supplementation with 400 ppm/kg was not so effective, compared to the lower dose (Kokoli et al., 2010). Additionally, quercetin *per os* for 28 days in low doses (<20 mg/kg) augmented sperm count, motility and viability and enhanced the activity of CAT and SOD in male rats (ElMazudy et al., 2015).

The influence of antioxidants in the female reproductive tract has also been reported. For example, lack of vitamin E affected the release of gonadotrophins from adenohypophysis (Das & Chowdhury, 1999), while the oral administration of ascorbic acid stimulated their release in female rats (Lee et al., 2007). In dairy cows, the administration of vitamin E in combination with selenium has been reported to reduce the incidence of postpartum reproductive disorders such as retained fetal membranes, cystic ovaries and to improve fertility (Arechiga et al., 1994). In general, the deficiency of antioxidant minerals (e.g. Zn, Se) or vitamins (e.g. folic acid, vitamin B12) reduces the survival and growth of embryos and fetuses.

OXIDATIVE STRESS IN ASSISTED REPRODUCTIVE TECHNIQUES (ART) AND ANTIOXIDANT SUPPLEMENTATION

Despite the rapid expansion and developments achieved in *in vitro* embryo production (IVEP), the outcome remains unsatisfactory. The proportion of fertilized oocytes reaching to the blastocyst stage is still limited, ranging 30-40% in bovine (Rizos et al., 2008). The handling and culture of gametes and embryos *in vitro* renders them susceptible to high risk of OS because the oxygen tension in the oviduct is approximately one-quarter to one-third of atmospheric tension (Hoshi 2003). Hence, Catt & Henman (2000) proposed three possible ways to counteract the negative effects of OS: a) decreasing the oxygen in the gas phase used for culture, b) modification of the media with components designed to protect cells from OS and c) reducing the co-incubation period for sperm and oocytes to minimize oxidative damage due to sperm metabolism. The idea of modified media with antioxidant agents during the different stages of IVEP seems to be very promising in order to improve the blastocyst rate.

a. *In vitro* maturation (IVM)

In the majority of IVM techniques in domestic animals, cumulus cell expansion and nuclear maturation are concomitant phenomena during the culture period (Salavati et al., 2012). The role of ROS and the impact on oocyte maturation and embryonic development still remains controversial. Controlled amounts of ROS contribute to oocyte maturation in cow (Blondin et al., 1997) and swine (Taemoto et al., 2000). However, the *in vitro* conditions render the oocytes to higher oxygen concentration than *in vivo*, leading to overwhelming production of ROS, which is associated with meiotic arrest and chromosomal errors (Guérin et al., 2001). Oocytes are protected by cumulus cells, containing enzymatic antioxidants, especially SOD and GSH. However, Cetica et al. (2001) reported that under standard culture conditions, no increase was observed in ROS production during IVM of bovine oocytes and between denuded matured and immature oocytes. This implies that oocyte is capable of controlling the increase in ROS due to its own enzymatic antioxidant activity. Indeed, Lonergan et al., (2003) found that mRNA expression of enzymatic antioxidants is significantly higher in matured oocytes in cow animal model. On the contrary, cumulus cells have a critical role in protecting *in vitro* matured porcine oocytes against OS–induced apoptosis through the enhancement of glutathione content (Taemoto et al., 2000).

Despite the endogenous protection of oocytes, researchers tried to modify the medium of IVM in order to ensure a certain production of ROS during IVM which is required to increase blastocyst production (Blondin et al., 1997). There is evidence that the modification of the medium with β-mercapthoethanol (β-ME) and cysteamine enhanced GSH synthesis in bovine (De Matos & Furnus, 2000) and in buffalos (Gasparrini et al., 2000), resulting in the improvement of embryo development. Furthermore, Ali et al. (2003) have focused on the possible effect of cysteine, CAT and SOD on bovine oocytes. The addition of cysteine to the maturation medium revealed a significant improvement of the developmental competence of the oocytes. On the other hand, SOD and CAT had no effect, which can be attributed to the fact that cysteine permeates the cumulus oocyte complexes (COCs), while enzymatic antioxidants not (Ali et al., 2003).

The role of vitamins as antioxidants in the maturation medium is converging, depending on species. For example, the beneficial effect of vitamin E on oocyte maturation and embryo development was recorded in porcine (Tao et al., 2010) and ovine oocytes (Natarajan et al., 2010), but not in canine oocytes (Salavati et al., 2012).

The literature regarding the supplementation of IVM medium with melatonin is conflicting. Dimitriadis (2006) found that the presence of melatonin at the final stages of IVM improved the percentage of blastocyst production in bovine, while Tsantarliotou et al., (2007) suggested that the supplementation of maturation medium with different concentrations of melatonin did not improve cleavage and blastocyst rates, compared with the control group. Furthermore, the presence of melatonin in IVM medium of porcine oocytes resulted in lower levels of ROS and significantly higher percentage of mature oocytes, compared with the untreated group, but no increase in cleavage frequency or blastocyst cell number was observed (Kang et al., 2009).

So far, plant derived antioxidants proved to be beneficial for the oocytes during IVM. Interestingly, the addition of aqueous extract of saffron (40μg/ml) resulted in significantly higher percentage of matured oocytes *in vitro* in mice (Maleki et al., 2014), while crocin (10μg/ml), one of the main bioactive constituents of saffron, improved both nuclear maturation and subsequent developmental competence (Maleki et al., 2016). This observation has been attributed to the increase in GSH content in the matured oocytes. Interestingly, crocin had a dose-dependent effect on GSH concentration and cytoplasmic maturation (Maleki et al., 2016). Similarly, the anthocyanin treatment of porcine oocytes during IVM improved the developmental competence of embryos, most likely by increasing intracellular GSH synthesis and reducing ROS level (You et al., 2010). Furthermore, the addition of green tea polyphenols (15 μM) in IVM medium had beneficial effects on subsequent bovine embryo development, which could be attributed to the protective effect of polyphenols on oocytes against OS (Wang et al., 2007).

b. *In vitro* fertilization

i. Sperm cryopreservation, sperm preparation and antioxidant supplementation.

The use of cryopreserved sperm in farm animals IVEP is a common practice. The handling of spermatozoa in high oxygen tension compared to that of the genital tract and the removal of seminal plasma triggers the production of ROS (Bilodeau et al., 2000) and diminishes the activity of GPx and SOD (Chatterjee & Gagnon, 2001). Consequently, spermatozoa are often more susceptible to LPO and their fertilizing capacity after thawing is affected. Several studies have focused in supplementing the freezing medium with antioxidants. In bovine spermatozoa, vitamin E (1 mg/ml) supplementation in the extender medium reduced LPO, enhanced CAT and GSH activity and improved motility parameters (Hu et al., 2011). Furthermore, the addition of SOD combined with GSH in the freezing medium resulted in the improvement of post thaw quality parameters (motility, viability, hyposmotic swelling test), associated with the enhancement of SOD and GPx activity and the diminution of LPO in bovine spermatozoa (Karaji et al., 2014).

Besides, the hormone melatonin has been evaluated as antioxidant agent in the semen extenders. The supplementation of 2 mM or 3 mM melatonin in the freezing extender of bovine spermatozoa improved the quality of post thawed semen which can be attributed to the reduction of LPO and the enhancement of total antioxidant capacity and enzymatic activity (Ashrafi et al., 2013). Similarly, Succu et al. (2011) showed that 1 mM melatonin in ram freezing extender medium led to higher viability rates, higher percentages of total motile and progressive motile spermatozoa, higher intracellular ATP concentrations and significant DNA integrity, compared to the untreated group.

Likewise, the presence of plant derived antioxidants has been evaluated in semen extenders. Lycopene (1 mg/ml), a carotenoid pigment, improved post thaw motility and viability and protected DNA when added in the freezing medium of bovine spermatozoa (Bucak et al., 2015). Resveratrol has been also evaluated, but the results are conflicting among species. For example, although the addition of 50 μM resveratrol had no effect on motility and viability parameters, the supplementation of the extender reduced OS and improved membrane stability and *in vitro* fertilizing capability of buffalo spermatozoa (Longobardi et al., 2017). On the contrary, the addition of resveratrol (1 mM) in bovine sperm was accompanied with significant improvement in post thaw sperm quality parameters (motility, viability, DNA integrity) (Bucak et al., 2015). In any case, it is important to underline that the maintenance of sperm integrity and its fertilizing capacity also depends on the cryopreservation protocol.

The selection of the ideal sperm preparation technique is crucial to minimize the effects of ROS. The density gradient technique separates normal spermatozoa from the potential risk factors of OS, such as dead spermatozoa and leukocytes (Chen & Bongso, 1999). Besides, Gadea et al. (2005) proposed that the supplementation of the media with antioxidants right after thawing blocks the production of ROS or counteracts oxygen toxicity.

Furthermore, enzymatic antioxidants have been tested *in vitro* but the data are also disputable. The addition of CAT (200 U/mL) and GSH (10 mM) protected equine spermatozoa from OS-induced DNA damage and loss of motility (Baumber et al., 2005), while the addition of reduced

GSH (1 mM and 5 mM) to the thawing medium of boar spermatozoa resulted in increased penetration rate of oocytes, although not significant (Gadea et al., 2005).

The role of melatonin has also been investigated in sperm preparation medium. In bovine, the addition of melatonin (1 μM) protected spermatozoa after 1 hour incubation from the induced OS, in terms of motility (Tsantarliotou et al., 2012). Da Silva et al. (2011) reported that the incubation of equine spermatozoa with melatonin (50 pM-1 μM) for 3 h had no effect on motility parameters, assessed by Computer Assisted Sperm Analyzer (CASA), despite the fact that LPO was suppressed. Nevertheless, melatonin, probably due to the controlled amounts of lipid hydroxyperoxides, reduced changes in the spermatozoa related to apoptosis (increased sperm membrane permeability and lowered mitochondrial membrane potential-$\Delta\Psi_m$). Similar evidence exists in red deer spermatozoa, where melatonin at 0.1 mM or 1 mM did not improve sperm status after 4-h incubation (Domínguez-Rebolledo et al., 2010), and in ram spermatozoa, where melatonin failed to protect both sperm kinematic parameters and viability (Casao et al., 2010). Nevertheless, the pre-treatment of ram spermatozoa with melatonin (100 pM) for 1 h caused a significant increase in the fertilization rate following IVF (Casao et al., 2010). The ameliorative effect of melatonin has been also confirmed in porcine where the incubation of spermatozoa with melatonin (100 nM) for 6 hours protected spermatozoa from the induced OS and improved the developmental ability of IVM/IVF embryos (Jang et al., 2010).

Two main bioactive constituents of saffron, crocin and crocetin have been also evaluated *in vitro*. More specifically, the addition of crocin (1 mM) resulted in maintenance of bovine and red deer sperm quality parameters (motility, viability, DNA integrity) by regulating intracellular levels of ROS (Domínguez-Rebolledo et al., 2010; Sapanidou et al., 2015) and ongoing LPO (Sapanidou et al., 2015). The ameliorative effect of crocin on motility and viability was also observed even when exogenous OS was induced by hydrogen peroxide (Tsantarliotou et al., 2016). On the other hand, the supplementation with crocetin (2.5 μM) was accompanied with significant lower production of ROS and lipid hydroxyperoxides, resulting in maintenance of sperm fertilizing capacity (Sapanidou et al., 2016).

The addition of resveratrol (25-50 μM) in sperm preparation medium alleviated the effects of the induced OS and, consequently, prevented the decline of functional activity and antioxidant capacity of bovine spermatozoa after 6 h of incubation. The effect was attributed to the enhancement of SOD and GSH activity (Tvrdá et al., 2015). On the contrary, the treatment of ram spermatozoa with resveratrol (up to 75 μM) negatively affected plasma membrane integrity and motility parameters after 1 hour of incubation (Silva et al., 2016). Finally, the supplementation of sperm preparation medium with a polyphenol-rich grape pomace (5 μg/ml) extract suppressed MDA production and protected sperm quality parameters, motility and plasma membrane integrity of bovine spermatozoa (Sapanidou et al., 2014).

ii. Antioxidant supplementation during IVF

The antioxidant requirements of gametes during fertilization seem to be paradoxical for oocytes and spermatozoa, depending on the balance between the amounts of ROS generated and scavenged (Blondin et al., 1997). The involvement of specific ROS may depend on the incubation conditions and on the species, while the molecular mechanism of action is not well defined in all species. ROS may induce capacitation/AR either directly or indirectly, through regulation of specific enzymes, such as phospholipase A_2 (PLA_2) which is present in sperm plasma membrane (de Lamirande et al., 1997; O'Flaherty et al., 1999). Capacitation studies indicate an asynchrony in the events related to fertilization. In capacitating human spermatozoa, superoxide anion production starts at the beginning of the incubation period while sperm hyperactivation peaks 1-3 h later and capacitation progressively increases over the 6 h incubation (de Lamirande & Gagnon, 1995). One of the first changes that occur during sperm capacitation is cholesterol efflux (Davis et al., 1980), which is partially regulated by ROS (Aitken & Nixon, 2013). Consequently, the addition of any antioxidant agent should be done with respect to the fine balance between production and scavenging of ROS, as well as the adequate timing for the phenomena related to the acquisition of sperm fertilizing capacity.

Despite the fact that the data regarding antioxidant supplementation during conventional IVF are conflicting the objective of this technique is to ensure alive and highly motile spermatozoa with intact DNA and plasma membrane. The addition of enzymatic antioxidants in the IVF medium was accompanied with controversial results. More specifically, the combination of SOD and catalase in different concentrations resulted in lower morula and blastocyst production, compared to the control group in bovine species (Iwata et al., 1998; Ali et al., 2003). Similarly, the presence of β-ME and cysteine at low concentrations during IVF did not improve embryo development to the blastocyst stage (Gonçalves et al., 2010). On the other hand, the addition of taurine (50mM), the main end product of cysteine in mammals, stimulated embryonic development up to the blastocyst stage in bovine (Tsuzuki et al., 2010).

The addition of green tea polyphenols during bovine IVF at low concentration (10μM) had no effect, while higher concentrations reduced the cleavage and blastocyst rates (Wang et al., 2007). On the contrary, the addition of crocin (1 mM) or crocetin (2.5 μM) in the IVF medium resulted in significantly higher blastocyst production compared to the negative control (Sapanidou et al., 2015, 2016). Consequently, the data are

conflicting to describe whether the supplementation of IVF medium is necessary or not. However, the addition of any antioxidant should be committed with respect to all redox-regulated physiological processes.

c. *In vitro* culture (IVC)

IVC is another crucial step during IVEP. ROS production increases dramatically between 2-cell embryo and late morula in bovine species (Dalvit et al., 2005). The handling of zygotes/embryos under atmospheric oxygen tension and high glucose concentration induces in overproduction of ROS (Iwata et al., 1998). Other potential exogenous sources of ROS are the metallic cations in the media, the visible light and ROS producing spermatozoa that remain outside (Guérin et al., 2001).

In that cases where the reduction of oxygen tension from 20% to 5% is not possible, the addition of an antioxidant agent to the culture medium may offer a way of protecting the embryos. Supplementing culture media with CAT or SOD resulted in an increase in the rate of blastocyst formation in bovine (Lauria et al., 1994).

The addition of vitamins in IVC media has been also proposed and the presence of α-tocopherol (100 μM) significantly improved blastocyst production in bovine (Marques et al., 2007). This observation has been verified in porcine where α-tocopherol (100 μM) had an embryotrophic effect during IVEP (Kitagawa et al., 2004) while Hossein et al. (2007) suggested that the effect of α-tocopherol depends on the concentration and the supplementation time. The latest study also suggests that the supplementation with 100 μM ascorbic acid, 0 and 96h of culture is effective, too (Hossein et al., 2007). Furthermore, Feugang et al. (2004) showed that 400 μM Trolox (a water-soluble analogue of vitamin E) and β-ME (100 μM), added separately from the morula stage, prevented apoptosis induced by OS and improved the quality of resulting bovine blastocysts. Indeed, a previous study carried out by Takahashi and co-authors (2002) suggested that β-ME exerted the antioxidant role via the promotion of cystine uptake in embryos.

Many investigators have studied the impact of melatonin supplementation of *in vitro* culture media in porcine, bovine and ovine embryo development, overall demonstrating a beneficial effect. Papis et al., (2007) reported that the addition of melatonin (0.1 mM) had a positive or negative effect, depending on the oxygen tension during culture of bovine embryos. The ameliorative effect was observed in 20% oxygen tension. The results have been verified by Wang et al., (2014a) where the supplementation of culture medium with low concentrations of melatonin (10^{-9} M and 10^{-7} M) significantly upregulated the expression of antioxidative (GPx4, SOD1, bcl-2) and developmentally important genes, while downregulated the expression of pro-apoptotic genes. Furthermore, melatonin receptor MT1 and MT2 genes were identified in bovine embryos (Wang et al., 2014a). The most effective concentration (10^{-7} M) was also added to bovine presumptive zygotes in order to evaluate the cleavage and fertilization rates and the kinetics of embryo development. Actually, the presence of melatonin promoted blastocyst production, accelerated *in vitro* bovine embryo development and improved quality of blastocysts which was indexed by an elevated cryotolerance (Wang et al., 2014b). Moreover, melatonin (10^{-9} M) had a positive effect on porcine embryo cleavage rates and blastocyst total cell numbers (Rodriguez-Osorio et al., 2007) while it improved *in vitro* embryonic quality and survival in sheep (Abecia et al., 2002).

Resveratrol (0.5 μM) improved the cryotolerance of *in vitro* produced bovine embryos and hatching rates (Zullo et al., 2016). A positive effect of resveratrol on *in vitro* embryonic development was also demonstrated in porcine, as indicated by enhanced blastocyst formation and improved embryo development (Lee et al., 2010). The catechins of green tea have been also evaluated during IVC in bovine embryos. The supplementation with 15 μM catechins was accompanied with significant increase in blastocyst production (Wang et al., 2007). Following trials concluded that the antioxidant effect of green tea polyphenols is associated with an increase in the relative transcription of many antioxidant enzymes genes (SOD1, CAT, GPx) and decrease in the apoptotic index of bovine embryos (Wang et al., 2014b).

Ultimately, carotenoids have been also tested during IVC. Zullo et al. (2016) investigated the effect of crocetin during IVC. The authors concluded that crocetin (1μM) increased blastocyst production, improved embryo cryotolerance and reduced the incidence of apoptotic cells in the blastocysts.

CONCLUSIONS

ROS have a significant impact on gametes' and embryos' homeostasis. These metabolites of oxygen are continuously controlled by endogenous antioxidant systems creating a redox balance in all cell types. The impairment of antioxidant status disrupts this balance and favors ultrastructural and functional damages of gametes and embryos leading to subfertility. In the last decades the concern regarding subfertility, poor fertilization outcome of IVEP and inherent relationship with OS is a subject of broad scientific research in both human and animal species. The results of *in vitro* and *in vivo* studies suggest that enzymatic and non-enzymatic antioxidants can protect cells from oxidative stress. However, ROS are critical for successful fertilization and embryo development and therefore antioxidants and ROS scavengers should be used

very judiciously with respect to the physiological processes, where ROS are playing a signalling role. This review summarizes the consensus on the role of oxidative stress and antioxidants in human and animal reproduction. An emphasis is given in the critical role of plant derived antioxidants. Nevertheless, further studies should be addressed in order to elucidate the ameliorative action, the recommended dosage or concentration of antioxidants and the possible synergistic role between different antioxidants, depending on the cause of subfertility or the stage of IVEP.

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