J Anim Reprod Biotechnol 2023;38:47-53
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.38.2.47

JARB
Journal of Animal Reproduction and Biotechnology
Check for updates

Review Article

Antioxidants as alleviating agents of *in-vitro* embryo production oxidative stress

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Received March 24, 2023
Revised May 4, 2023
Accepted May 11, 2023

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ABSTRACT Despite numerous advances in *in-vitro* embryo production (IVP), many documented factors have been shown to influence the development of mammalian preimplantation embryos and the success of IVP. In this sense, elevated levels of reactive oxygen species (ROS) correlate with poor outcomes in assisted reproductive technologies (ART) due to oxidative stress (OS), which results from an imbalance between ROS production and neutralization. Indeed, excessive production of ROS compromises the structural and functional integrity of gametes and embryos both *in vivo* and *in vitro*. In particular, OS damages proteins, lipids, and DNA and accelerates cell apoptosis. Several *in-vivo* and *in-vitro* studies report an improvement in quality-relevant parameters after the use of various antioxidants. In this review, we focus on OS and the source of free radicals and their effects on oocytes, sperm, and the embryo during IVP. In addition, antioxidants and their important role in IVP, supplementation during oocyte *in vitro* maturation (IVM), *in vitro* culture (IVC), and semen extenders were discussed. Nevertheless, various methods for determining the level of ROS in germ cells have been briefly described. Still, it is crucial to develop standardized antioxidant supplement systems to improve overall IVP success. Further studies should explore the safety, efficacy, mechanism of action, and combination of different antioxidants to improve IVP outcomes.

Keywords: cryopreservation, free radicals, gametes, *in vitro* production, oxidative stress

INTRODUCTION

Assisted reproductive technology (ART) is the application of clinical or laboratory approaches to gametes (oocyte/sperm) or embryos for reproduction (Zegers-Hochschild et al., 2009; Scaravelli and Spoletini, 2015). The frequently used ART includes artificial insemination, IVM/*in vitro* fertilization (IVF) of oocytes, somatic cell nuclear transfer, intracytoplasmic sperm injection (ICSI), embryo transfer, and the cryopreservation of gametes and embryos (Gadea et al., 2020). In humans, ARTs represent a vital treatment option for infertile couples (Billari et al., 2007). It considers a standard assisted fertility preservation strategy for cancer patients (De Felice et al., 2018). Fertility preservation is also an important use of the *in vitro*-produced embryo for endangered species and economically valuable animals, such as the horse (Hinrichs, 2018; Herrick, 2019). The application of ART in livestock production have used to increase the yield of embryos from genetically superior females, positively impact agricultural

food production and production of transgenic animals and human pharmaceutical proteins, models for biomedical research, and source for xenotransplantation (Chen et al., 2022). However, despite advances in the field, the success rate of ART procedures remains unsatisfactory in many cases (Chambers et al., 2021) and requires further improvements. Nevertheless, these technologies can not realize their full potential without efficient *in vitro* production (IVP) systems. Among various causes, oxidative stress (OS) has been recognized to affect the IVP outcome (Guérin et al., 2001; Agarwal and Allamaneni, 2004).

OS in reproduction

OS is an imbalance between the reactive oxygen species (ROS) production and the total amount of antioxidants in favor of the oxidants (Pizzino et al., 2017). At low concentrations, ROS physiologically act as signaling molecules in several processes. In male reproduction, these redox mechanisms play an important role in the regulation of several functions, including spermatogenesis, chromatin condensation, sperm maturation during transport in the epididymis, sperm capacitation, acrosome reaction, and sperm-oocyte interactions (Fisher and Aitken, 1997; Bardaweel et al., 2018). In females, redox homeostasis is critical for folliculogenesis, implantation, and placentation (Sharma and Agarwal, 2004; Agarwal et al., 2005). On the other hand, higher levels of ROS can damage cellular lipids, cell membranes, organelles, and DNA, alter enzymatic function, and trigger apoptosis (Birben et al., 2012; Redza-Dutordoir and Averill-Bates, 2016). ROS-induced lipid peroxidation produces highly reactive and mutagenic products, such as malondialdehyde (MDA), an indirect molecular marker of OS (Marnett, 1999).

Source of ROS

Free radicals are unstable and highly reactive species that become stable by acquiring electrons from nucleic acids, proteins, lipids, carbohydrates, or any nearby molecule causing a cascade of series reactions resulting in cellular damage and disease. ROS are free radicals that possess one or more unpaired electrons. The most common forms of ROS are superoxide radical ($O_2 \cdot -$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) (Pierce et al., 2004; Halliwell and Gutteridge, 2015). Several factors could be responsible for increased ROS generation in an ART condition, leading to suboptimal outcomes. ROS can be produced intracellularly, from sperm, oocytes, and embryos. In addition, numerous external factors may induce OS in an ART setup (Fig. 1). In this regard, the impact of atmospheric oxygen levels on embryos has been emphasized (Yuan et al., 2003; Kitagawa et al., 2004; Corrêa et al., 2008). Indeed, most body tissues, including the fallopian tubes, function properly at oxygen concentrations of 4% to 10%. However, *in vitro* conditions require manipulations of oocytes and gametes during IVM and *in vitro* fertilization that generates OS (Torres-Osorio et al., 2019). OS also arises from embryo metabolism and embryo surroundings. The laboratory air, the gases used and the quality of culture media can also contribute to OS in an ART setting. In addition, the centrifugation process (force and duration), visible light, temperature, and humidity can trigger OS directly or indirectly (Guérin et al., 2001;

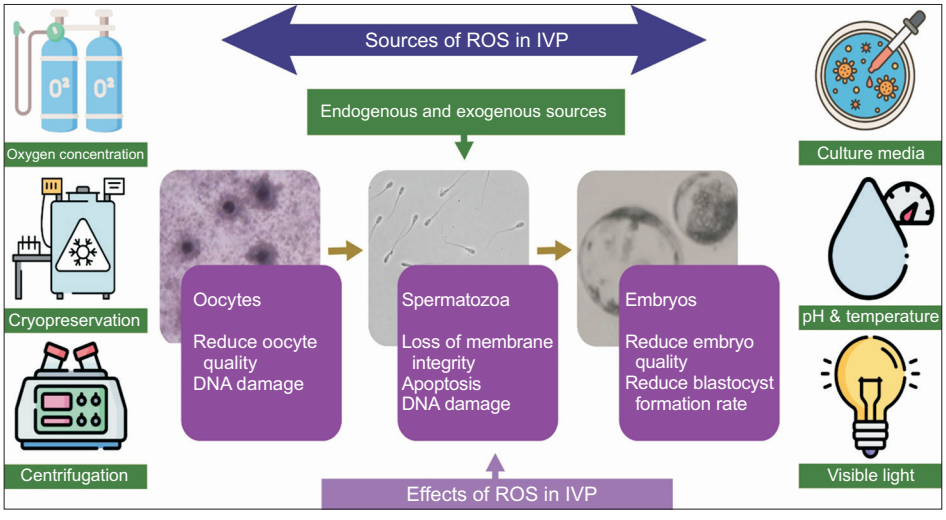

Fig. 1. Free radicals: Production and damage in IVP condition.

Agarwal et al., 2014a). Nevertheless, ROS produced during the freezing-thawing process of gametes or embryos, thus increasing the risk of ROS-induced cryo-damage (Bansal and Bilaspuri, 2010; Agarwal et al., 2022). These factors can act throughout the ART, from gametes preparation and fertilization to embryo development until the blastocyst stage. Hence, strategies to moderate the risk of OS in ART include optimization of the laboratory environment, sperm preparation techniques, embryo culture media, and cryopreservation procedures. Two major approaches have been employed to moderate the side effects of ROS during the IVP. First, oxygen concentration, especially in embryo culture, has been reduced up to 5%, and second, various antioxidant compounds have been used (Agarwal et al., 2014b; Sciorio and Smith, 2019).

Antioxidants

Generally, the antioxidant definition is based on activity rather than structure or mechanism. Halliwell (2007) defined antioxidants as "any substance that delays, prevents or removes oxidative damage to a target molecule". Similarly, Khlebnikov et al. (2007) demarcated antioxidants as "any substance that directly scavenges ROS or indirectly acts to upregulate antioxidant defenses or inhibit ROS production". In other words, antioxidants either help in ROS neutralization or make them harmless or counteract their production. In general, antioxidants could be classified as endogenous, like catalase (CAT), glutathione, and super oxide dismutase (SOD). Exogenous antioxidants: include different types of vitamins, amino acids, fatty acids, hormones, herbal plants, disaccharides, etc. (Ciani et al., 2021; Abdel-khalek et al., 2022) (Fig. 2).

Significance of antioxidants in IVP and OS

1) Antioxidant supplementation in *in-vitro* maturation (IVM) and *in-vitro* culture (IVC)

In vitro embryo culture is a lengthy process, during which the oocyte reaches the competence to be fertilized and undergo embryogenesis. However, it is still not widely used in clinical practice because of its underperformance compared to *in vivo* conditions. The influencing factors, such as the IVM system, culture medium, and oxidative stress, have a marked effect on the outcomes of IVC (Cao et al., 2020; Yang et al., 2021). Numerous studies have been conducted to optimize IVP media and moderate oxidative stress by adding antioxidants. Sovernigo et al. (2017) indicated that using quercetin, cysteamine, and vitamin C during IVM reduces oxidative stress either by decreasing ROS levels or increasing glutathione levels in bovine oocytes. Furthermore, antioxidants β-mercaptoethanol and vitamin E decreased the H_2O_2 content, suppressed oxidative damage, and as a consequence, reduced DNA fragmentation and improved the developmental ability in *in-vitro* cultured porcine embryos (Kitagawa et al., 2004).

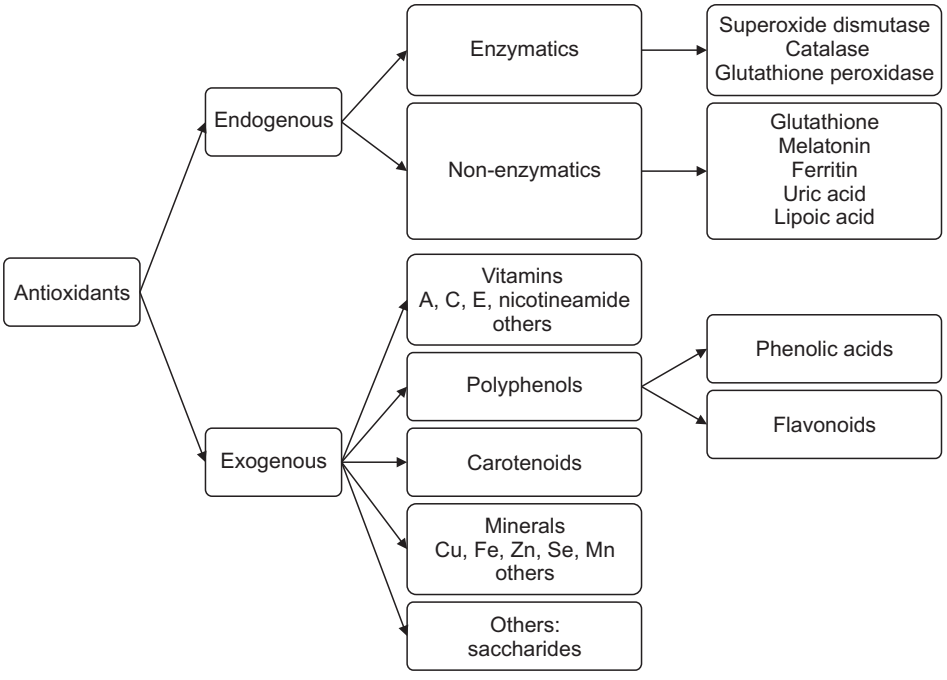

Fig. 2. Classification of antioxidants.

In bovine, the addition of cysteine improved the development of embryos, while N-acetyl-l-cysteine, CAT, and superoxide dismutase (SOD) had no positive effect on embryonic development (Ali et al., 2003). The former authors also indicated the addition of antioxidants during the IVF period reduced the subsequent rate of embryo development to the blastocyst stage, while antioxidants supplemented during IVM and IVC enhanced embryo development. Suggesting that type and the phase of antioxidants supplementation has a substantial effect on the outcome. On the other hand, the addition of high concentrations of antioxidants in IVP media reduced the blastocyst formation rate compared to treatment with low concentrations (Boquest et al., 1999; Kang et al., 2016), indicating that only the appropriate dose of an antioxidant can contribute to improving the quality of embryos.

2) Antioxidant supplementation in semen extender

Sperm cryopreservation is the most efficient approach for the long-term storage of semen. However, frozen-thawed (FT) semen exposes to physical and chemical stress; as a consequence, 40% to 50% of spermatozoa do not survive cryopreservation (Watson, 2000; Rath et al., 2009; O'Neill et al., 2019). High levels of ROS can cause sperm DNA fragmentation, either directly or indirectly through MDA. Increased sperm DNA fragmentation has correlated with low embryo quality, high abortion rates, and low live birth rates after IVF and ICSI (Aitken et al., 2016). Thus, the development and optimization of cryopreservation protocol are ultimately essential, because semen in liquid form is only useful for a few days (Knox, 2015; Yeste et al., 2017). Indeed, the potential for enhanced fertility of FT sperm through the use of antioxidants to protect against cell damage appears most promising method to advance this technology for practical application (Jovičić et al., 2020). In this regard, the inclusion of antioxidants such as glutathione (Hu et al., 2016), butylated hydroxytoluene (Roca et al., 2004), and tannins (Galeati et al., 2020) in the freezing media have had dramatic effects on protecting spermatozoa *in vitro*, and this influence remains when applied for insemination. Also, several researchers emphasized plant-derived antioxidants (lower cytotoxicity, economical, and frequently available) as excellent sources of natural antioxidants in preserving semen (Abdel-khalek et al., 2022). However, an optimum antioxidant level remains the fundamental factor in ameliorating sperm survival following the freeze-thaw process.

Methods for assessing the level of ROS in germ cells

Several OS biomarkers have been investigated in sperm, oocytes, and embryos. ROS is the initial marker and different other markers are available to measure the end product of ROS-induced damage on cellular components such as lipid peroxidation, proteins, and DNA damage (Tunc et al., 2010; Gosalvez et al., 2017; Robert et al., 2021). Additionally, enzymatic antioxidant activities can be measured using commercially available assay kits, which include SOD, glutathione peroxidase, and CAT (Elomda et al., 2018; Kurkowska et al., 2020).

A variety of techniques have been developed for this purpose including chemiluminescence (luminol and lucigenin), flow cytometry, and epifluorescence microscopy (MitoSOX Red, dihydroethidium, 4,5-diaminofluorescein diacetate, and 2',7'-dichlorodihydrofluorescein diacetate), and spectrophotometry (Nitro Blue tetrazolium) (Agarwal et al., 2004; Aitken et al., 2013; Gosalvez et al., 2017). In this sense, the fluorescence-based 2',7'-dichlorodihydrofluorescein diacetate staining method is used widely for detecting intracellular ROS in sperm (De Iuliis et al., 2006), cumulus-oocyte complexes (COCs) and embryos (Yang et al., 1998; Morado et al., 2009). In addition, Nitro Blue tetrazolium (NBT) is an electron acceptor that becomes reduced in the presence of ROS to form a blue-black compound, formazan. This simple histochemical staining method targets cells generating ROS (Sharma et al., 2013). Recently, developed NBT staining was introduced as an alternative method for detecting and quantifying intracellular ROS in oocytes, cumulus cells, and embryos (Javvaji et al., 2020).

CONCLUSION AND FUTURE PERSPECTIVES

This review briefly summarizes the effects of ROS and the role of antioxidant supplementation on gametes and preimplantation embryos for improving the efficiency of IVP outcomes. Studies show that the addition of antioxidants to culture media or sperm extender can mitigate the impact of ROS and improve IVP outcomes. Nevertheless, more studies are needed regarding various antioxidants' effectiveness on different species and standardizing their

optimal concentration and stage of supplementation.

Author Contributions: Conceptualization, A.A.; Investigation, A.A., Y.I., J.Y.; data curation, A.A., Y.I., J.Y.; writing—original draft preparation, A.A.; writing—review and editing, A.A., Y.I., J.Y.; supervision, Y.I., J.Y.; project administration, J.Y.; funding acquisition, J.Y.

Funding: This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2019R1A6A1A03033084).

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

Acknowledgements: We wish to express our gratitude to Prof. Joohyeong Lee for his valuable comments. We also acknowledge Mr. Seongju Lee and Mrs. Rana Osman for technical support.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

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