Review

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Assisted Reproduction: Impact of Mitochondrion (Dys)function and Antioxidant Therapy

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Review

Assisted Reproduction: Impact of Mitochondrion (Dys)function and Antioxidant Therapy

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Simple Summary: One of the effects of the industrial development and environmental changes is the direct negative impact on mammal's fertility being mandatory to find strategies to mitigate this effect. The main causes of fertility decline and ways of trying to increase it are presented and discussed in this review. An especial approach focused on the role of mitochondria and oxidative stress in this problem and how natural and mitochondriotropic antioxidants can help to solve it was performed.

Abstract: In the last years, major changes in the biosystem related to the industrial development and environmental modifications have had a direct impact on human and animal fertility, as well as on biodiversity. It is widely demonstrated that all these changes impair in the reproductive function. Several studies have connected the increase of reactive oxygen species (ROS) generated in mitochondria to the recently identified decline of fertility due to various factors, including heat stress. The study of antioxidants and especially of mitochondria targeted antioxidants, has been addressed to identify more efficient and less toxic therapies that could circumvent the problem of infertility in mammals. These antioxidants can be obtained from natural compounds used in the diet and converted into more effective forms to mitochondria, which will be a much more natural therapy. The use of mitochondriotropic diet-based antioxidants in Assisted Reproductive Technologies (ART) may be an important way to circumvent the low fertility, allowing the conservation of biodiversity in animal species, including domestic breeds. This paper provides a concise review of the current state of the art on this topic, with a particular focus on antioxidants: Mitoquinone, AntiOxBEN$_2$, AntiOxCIN$_4$, Urolithin A and the most recent Piperine.

Keywords: fertility; reproduction; gamete; embryos; oxidative stress; antioxidants; mitochondria

1. Introduction

In recent years, pollution levels have increased significantly, largely as a consequence of industrialization during the 20th century. Agricultural fertilizers, manure, sewage, heavy metals, plastics, chemicals and industrial discharges are polluting coastal regions, oceans and continental ecosystems. Today, many of these compounds are found in the air, drinking water, soil, food, plants and in the bodies of animals and humans [1–4]. Poor air quality has become a global problem not limited to industrialized cities. All these changes and exposure to other specific environmental factors, such as temperature, climate, radiation and nutrition create an environment with increasing stress factors (Figure 1). These factors have a serious impact on the adaptation, reproduction and survival of living organisms by changing the ecosystem and biodiversity, with negative consequences, namely the growth of degenerative diseases and the decrease of fertility [5–7].

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Environmental pollution has revealed to impair fertility in all mammal species [2,8]. Many of these toxic compounds are endocrine disruptors or have endocrine active substances that can damage the reproductive performance, with important social and economic consequences [3,4,9,10].

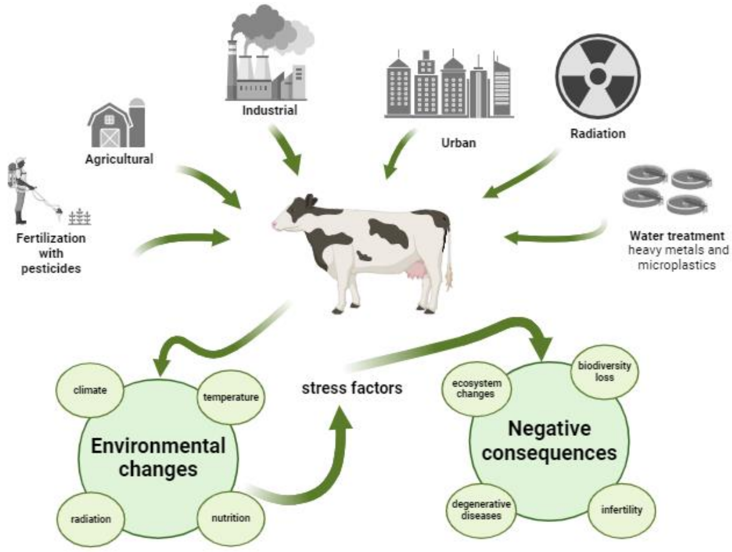

Figure 1. Impact of environmental stress on animal welfare and fertility, as well as on biodiversity (Created in *Biorender.com*).

The most frequent cause of infertility has been related to the low number and quality of germ cells, namely azoospermia and oligospermia in the male or ovulation failure caused by hormonal disorders or behavioral changes in the female. It can also be caused by problems in the pelvic cavities such as endometriosis, Sertoli cell disorders or obstruction of the genital tract [11–14]. Infertility can also be linked to other factors, not only genetic or pathological, but also issues related to the age of the parents, nutritional problems, the environment, stress and the use of certain drugs [1,4,8,15]. This issue, compounded by factors such as population growth, climate change, socio-economic shifts, economic globalization, and financial constraints, has underscored the urgency of conducting research and developing strategies to manage fertility and maintain biodiversity. These efforts include the preservation of endangered local breeds, which are critical for sustaining ecological balance and genetic diversity [6,16]. It is estimated that a considerable number of species will become extinct in a near future [5].

Environmental changes, often caused by human activity, have repercussions on reproductive effectiveness and in the maintenance of species and subspecies. Guillette & Edwards (2005) showed that alligators in a lake contaminated with pesticides with nitrogenous compounds suffered from reproductive problems. Problems were also found in rats exposed to methoxychlor pesticide [1]. The relationship between hormonal system dysregulation and environmental contaminants has been proven over time [1,17].

Likewise, heat stress, closely associated with climate change, can also affect reproductive effectiveness. Different studies have showed that oocytes collected during warmer weather were of lower quality than those collected in winter, especially in extreme heat conditions [9,18] and that spermatogenesis is also affected, leading to low fertilization rates and embryonic development [19]. According to Leroy et al. (2008) the fertility of dairy cows has been declining since the mid-80s. The low quality of oocytes and embryos has been described as the main reason for low birth rates and high embryonic death rates. Low fertility rates are also associated with high production and feeding and it has been described that balanced diets, with a good supply of protein and energy, are essential for good energy stability and a consequent improvement in gamete quality. For instance, diets rich in starch can improve energy status and, therefore, ovarian activity in the early postpartum period. However, oocyte and embryonic quality can suffer from these diets, and diets with a high protein content can raise ammonia and urea concentrations in the blood, leading to modifications in the female reproductive system [20,21].

It is, therefore, urgent to find solutions to mitigate the deleterious effects of environmental and other changes, on fertility and welfare of animals and humans. This review briefly summarizes the current problems associated with infertility, focused on bovines and other animals, mainly related to stress factors that lead to increased oxidative stress and possible therapeutic approaches based on antioxidants, whether they are of natural origin or manipulated to act on the mitochondria.

2. Technological Applications to Improve Fertility

The commercialization of bovine embryos is widespread globally, and despite the economic challenges posed by the COVID-19 pandemic, the embryo industry has continued to grow. A deep analysis of data on the international trade of embryos is particularly useful to understand that these technologies have been adopted in 1/3 of the countries, which represent more than half of the world's cattle population [22]. The commercial value of this activity for the meat and milk trade is directly linked to genetic quality and crossbreeding schemes, which can be obtained by using oocytes, semen and embryos from selected donors, as well as, through the implementation of reproductive management strategies to improve pregnancy rates. Another advantage is related to the number of "cheap" embryos that can be produced *in vitro* and used for research [23,24] or infertility treatments such as embryo transfer for repeat-breeding cows [24–26]. Although some limitations associated to the in vitro fertilization technique were detected specifically linked to the low rate of produced embryos and some associated problems in the offspring it is an excellent tool for solving infertility problems and implementing studies related to fertility/infertility [23,27,28].

A huge scientific and technological advance has resulted from the extensive research carried out on several studies including reproduction and epigenetic alterations [29,30], oxidative stress in fertilization [31–34], among others. It should also be noted that ART practiced on different livestock species is a very successful business worldwide and as such, plays an important role in the livestock farming development [35,36].

2.1. Assisted Reproductive Technologies (ART)

Artificial insemination (AI) is the oldest technique used in assisted reproduction for both animals and humans. Initially introduced for animals of zootechnical interest in the late 1930s, AI had a strong sanitary focus. However, its large-scale adoption was driven primarily by economic factors. Following World War II, the development of semen cryopreservation greatly facilitated its distribution and expanded its commercial potential, particularly in the dairy industry. Additionally, AI has been applied to address issues related to sexual dysfunction and infertility [37–39].

The development of ART has opened new possibilities for the preservation of germplasm and fertility [6]. However, significant challenges remain, including technical limitations in laboratory procedures and escalating costs. To address these issues, concerted and sustained investment in research is essential to enhance these technologies and ensure their accessibility on a global scale [27,40]. Despite great efforts to improve the quality of both oocytes and in vitro produced embryos, the quality of these blastocysts is still inferior to those produced in vivo. These differences can be seen in morphology, metabolism, gene expression, tolerance to cryopreservation and persist throughout the development [41–43].

ART cover all *in vivo* and in vitro treatments or procedures on oocytes and sperm or embryos to establish a pregnancy, and have been widespread throughout the world, even in developing countries [22,40]. These include, among others, superovulation treatments and IA, sexing, *In Vitro* Fertilization (IVF), Intra Cytoplasmic Sperm Injection (ICSI), embryo transfer, and cryopreservation of gametes and embryos [11,24,37]. IVF was developed for use in humans and cattle, allowing embryo transfer worldwide [44]. Nevertheless, to be successful, IVF requires rigorous control and manipulation of the reproductive cycle to select the greatest number of oocytes and spermatozoa, assessing their quality, as well as, the quality of the embryos [43,45]. The ICSI, which consists of the microinjection of a single sperm into the cytoplasm of the oocyte, is currently the most widely used

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method to overcome serious male infertility problems and when the IVF method is unsuccessful, making it the most widely used in human and equine ART [38,45,46]. In horses, ICSI can be used when the number or quality of sperm is low, making it possible to overcome infertility problems in these animals. Also, oocyte retrieval and ICSI make it possible to manage normally fertile mares and stallions, reproducing and storing embryos of high genetic quality, creating a highly competitive market worldwide [38,39]. However, ICSI raises questions about the health of the offspring as it goes against natural selection [47], unlike IVF, where insemination itself allows the union of the two gametes to be brought closer to natural. The great success of embryo transfer and even IVF rates in cattle has spread its use worldwide, while ICSI is rarely used in this species [35].

As mentioned, ART comprises several stages, which can include ovarian stimulation, semen collection and freezing, IVF or ICSI, and also embryo culture, occasional biopsy, and finally embryo transfer, which involves significant changes to the gametes and the embryonic environment. It is now known that the early stages of mammalian embryonic development are very sensitive to their microenvironment [48]. Only 70% of the oocytes fertilized in vitro reach cleavage after three days of culture, and of these only 30%-50% give rise to blastocysts, falling short of development in vivo, even after the technique has been improved over the years [31,44,48]. For instance, pregnancy rates following the transfer of bovine in vitro produced embryos are 10-30% lower than those of embryos developed in vivo [48–50].

Deterioration in the quality of oocytes is one of the factors associated with the failure of ART, since their quality is a determining factor of the potential for development of the embryo after fertilization. Ovulation asynchrony and aged oocytes have been reported to be detrimental to the success of AI and embryo production programs in mare, cow and sheep, implying significant economic losses [20,46,51]. Any disturbance in the follicular or other environment affects the maturation of the female gamete and reduces its quality. In vitro maturation models have even shown that some of these metabolic alterations reduce the suitability of the oocyte, with repercussions on the development and quality of the embryo. Therefore, a technique to ensure optimal oocyte maturation is needed, as studies indicate, that adverse conditions for oocyte growth and maturation can also jeopardize the health and performance of the offspring [29,48,51,52].

In order to replicate the conditions of the maternal reproductive system, in vitro embryo production systems techniques must recreate the in vivo environment, with correct regulation of oxygen (O_2) concentration and composition of culture media [53–55]. Several physicochemical factors affect oocytes and embryos, such as temperature control, maintenance of osmolality, pH and protection against oxidative stress and toxic substances [55,56]. De Munck et al. (2019), Leite (2017) and Amin et al (2014) showed a better efficacy of 5% O_2 during embryo culture. Also, for cattle oocyte maturation and IVF the temperature should be 38.8ºC, in atmospheric conditions saturated with humidity and with 5% carbon dioxide (CO_2)[53,57]. The culture media recreate the carbohydrate concentrations of the fallopian tubes and uterus by adding pyruvate, lactate, glucose, and proteins normally albumin, glutamine, and other amino acids [55,58,59]. Antioxidants have been reported to be also necessary [60,61]. Nevertheless, the culture conditions must be adapted to the stage of ART and species, existing protocols that differ between laboratories [6,43,57].

3. The Role of Mitochondria in Gametes Functionality

Environmental stressors, including heat stress, significantly affect the developmental competence of gametes. Furthermore, the mitochondrial response to these stressors has been identified as a major cause of reduced oocyte quality (Figure 2) and spermatozoa functionality [9,31,51,60,62]. Mitochondria are cellular organelles, responsible for several metabolic processes including oxidative phosphorylation (OXPHOS) and fatty acid metabolism, while also regulating cytosolic calcium concentration, the production of reactive oxygen species (ROS) and regulation of cell death pathways [63,64]. In addition to the nucleus, mitochondria are the only organelles in animal cells that contain their own DNA, known as mitochondrial DNA (mtDNA), which carries essential genetic information [44,65].

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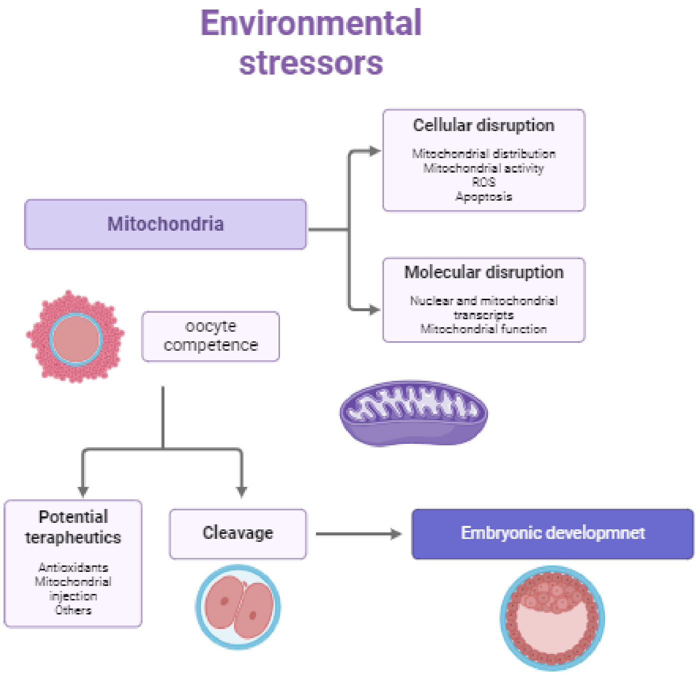

Figure 2. Effects of environmental stress factors on oocyte mitochondria. Stress induces cellular and molecular changes in the oocyte, which in turn can reduce the gamete's developmental competence. It has been shown that supplementation with mitochondrial agents, antioxidants [epigallocatechin gallate, melatonin], or injection of mitochondria into oocytes, attenuates these effects and improves competence for subsequent development (adapted from [9] and created in Biorender.com).

The mitochondria use the energy released by the oxidation of glucose and other sugars to synthesize adenosine triphosphate (ATP) from adenosine diphosphate (ADP). Electrons from nicotinamide adenine dinucleotide (NADH) or succinate produced in the mitochondrial matrix during the Krebs cycle are transferred to molecular oxygen by a series of protein complexes in the mitochondrial inner membrane (or in the cristae membranes), creating a transmembrane electrochemical gradient, which is used by the ATP synthase to produce ATP from ADP and inorganic phosphate [66]. This process is called OXPHOS and occurs under aerobic conditions. In addition to energy conversion, this process gives rise to the generation of ROS as by-products of electron transport chain (ETC) activity [67].

Within the oocyte, mitochondria are involved in ATP generation, calcium homeostasis, regulation of cytoplasmic redox status, signal transduction and apoptosis [9]. As oocyte maturation requires a large amount of ATP for continuous transcription and translation, the availability of the right number of functional mitochondria is essential. In addition, during oocyte maturation, the mtDNA copy number increases dramatically and the distribution of mitochondria changes significantly [44,65].There is therefore a correlation between oocyte quality and mtDNA copy number and ATP concentration [44,65,68].

In spermatozoa, mitochondria are also susceptible to loss of membrane potential and electron leakage during OXPHOS, reducing their energy production capacity [19,69–71]. Several studies have analyzed mitochondrial function in sperm cells establishing a positive correlation between sperm mitochondrial membrane potential (MMP) and other parameters required for sperm functionality such as motility, viability, capacitation status, acrosome, and chromatin integrity, suggesting that mitochondrial status reflects the quality of the sperm functionality [60,62]. Moreover, according to Jorge et al. (2024), differences among male sperm bioenergetic parameters can be used to predict spermatozoa functionality and developmental potential and therefore for the selection of bull breeders.

On the other hand, if IVF conditions are not optimal, there may be changes in the morphology of mitochondria and in the coding of proteins associated with their function, which can lead to a

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reduced ability to counteract ROS production, leading to oxidative stress [65]. Heat stress is closely associated with alterations in mitochondrial distribution and MMP, disrupting the expression of genes linked to mitochondrial function. This includes genes involved in the transcription and replication of mtDNA and those encoding OXPHOS complexes essential for ATP production [9].

Moreover, Soares et al (2020) have reported significant age-related alterations in bovine oocytes namely in the cytoplasmic volume, mitochondrial aggregation pattern, and mitochondria-produced H_2O_2 levels [72]. Aged oocytes have also presented reduced nuclear maturation progression, MMP, developmental competence, and altered gene expression levels [51]. Those unique characteristics make mitochondria appealing to study fertility/infertility and to test new therapeutic strategies aiming to improve the mitochondrial function of gametes and embryos.

4. Reactive Oxygen Species (ROS) in Gametes and Embryos

Oxidation-reduction reactions are a basic component of the systems of living beings and the free radicals, including oxygen ions and hydrogen peroxide that result from them, are also essential for nuclear maturation or sperm capacitation. These radicals are the result of cellular metabolism and enzymatic reactions that occur in the body and require a balance between the loss and gain of electrons [9,60,61,73,74]. As previously stated, during OXPHOS, a residual production of ROS occurs naturally as a consequence of electron transfer. Additional studies focusing on specific complexes embedded in the mitochondrial inner membrane have established that complexes I and III are the sites with the greatest capacity for generating ROS due to electron slippage [67,75] (Figure 3).

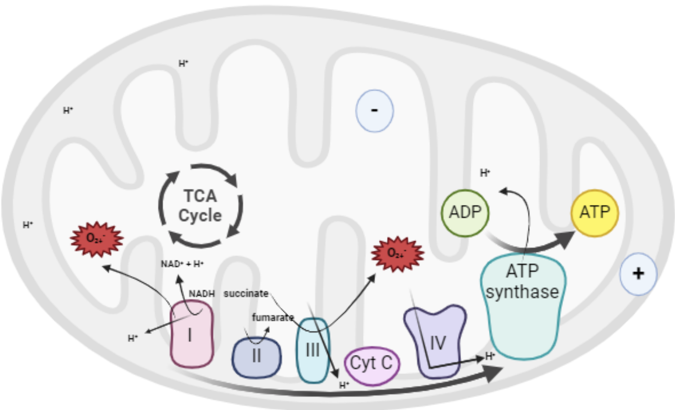

Figure 3. Respiratory chain in mitochondria - During aerobic respiration, electrons from NADH and succinate are transferred to molecular O_2 through a chain of redox enzymes (Complexes I-IV) embedded in the inner mitochondrial membrane, which is accompanied by pump protons across the inner membrane, generating an electrochemical gradient. This proton gradient is used for the mechanical work needed to produce ATP from ADP and inorganic phosphate (Created in Biorender.com).

ROS plays direct and indirect roles in a very wide range of physiological processes. However, when there is an imbalance in their homeostasis, i.e. an imbalance between oxidants and antioxidants in favor of the oxidants, leads to a disruption of redox signaling and control and/or molecular damage [60,61,73,74,76]. Oxidative stress and mitochondrial dysfunction have been associated with metabolic diseases and age.

The presence of excess ROS within the ovary and endometrium has significant physiological and pathological implications for conceiving [74]. In IVF there are various sources of ROS, including the cells themselves, such as oocytes and spermatozoa, as well as the composition of the culture media used. Likewise, the laboratory culture conditions practiced during maturation and insemination, namely excess light, temperature variation, and high oxygen tension, increase the production of ROS and can have detrimental effects on post-fertilization development and assisted reproduction results, as they have a detrimental effect on mitochondria, DNA, RNA and fertilization [31,33,61].

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The increase in ROS is well documented in oocytes exposed to heat stress or environmental toxicants [9,18] and in spermatozoa, causes damage to membrane lipids and decreased sperm motility. The exposure of DNA to both ROS and apoptosis enzymes during the cell division of spermatogenesis makes spermatozoa vulnerable, impairing not only fertilization but also subsequent embryonic development [19,70,77]. In addition, this increase in ROS production and consequent oxidative stress affects the quality of gametes, their environment, and ultimately, their interaction which can not only harm IVF success rates but, can also result in epigenetic and genetic changes in the embryo, resulting in transgenerational effects [31,61,78,79].

Marques et al. (2014) detected the production of mitochondria-specific ROS (mtROS) in sperm, using flow cytometry, with a mitochondria-specific superoxide fluorescent probe MitoSOX Red. In this study, they have shown that ejaculates were heterogeneous in mtROS production, with three detectable subpopulations, where the sperm subpopulation that produces the least amount of mtROS presented the most functional subset of male gametes, which were correlated with the largest number of live gametes and non-apoptotic sperm. In addition, this subpopulation was clearly more effective in samples that gave rise to pregnancies after assisted reproduction. Recently, Nakai et al. (2024) have shown that increased ROS production were associated with oocyte maturation inhibition, and demonstrated that the loss of a protein membrane (Mul1) was directly connected with the increasing of ROS concentrations in oocytes, resulting in an abnormal preimplantation embryogenesis. They reinforced that manipulating the mitochondrial ROS levels in oocytes may be a potential therapeutic approach to target infertility [80]

Several studies have therefore concluded that the use of antioxidants is an effective approach to mitigate oxidative stress and improve fertility [31,81,82]. Agarwal et al. (2005) state that infertility treatment strategies should focus on oral supplementation with antioxidants or, in the case of IVF, supplementation of the culture media with antioxidants to reduce oxidative stress.

5. Antioxidants in the Reproductive Medicine

Antioxidants are substances that inhibit oxidation by neutralizing free radicals found in the environment, reducing their effects on various diseases such as cancer, diabetes, degenerative diseases aging, and infertility [83]. Eukaryotic cells have antioxidant strategies and multifaceted mechanisms to maximize energy production while limiting the negative and toxic effects of ROS production [67]. There are several natural antioxidants in the body, including enzymatic antioxidants such as catalase, SOD, glutathione peroxidase [74,84]. In addition, non-enzymatic antioxidants such glutathione (GSH), vitamins C and E, hypotaurine, and taurine, among others provide maternal protection from oxidative stress to oocytes and embryos [41].

Excessive ROS production, if not counteracted by intrinsic defense mechanisms, will cause oxidative damage to cellular components, and, as mentioned above, can lead to DNA damage and cell death by necrosis or apoptosis [9,73,85–87]. Thus, antioxidant supplementation can be a strategy to improve fertility. Moreover, the use of antioxidants has been reported to be necessary for ART [60,61].

5.1. Endogenous Antioxidants

Reduced glutathione (GSH) is an endogenous tripeptide antioxidant (Figure 4) present in both male and female gametes that plays a very important role in fertility. GSH has a role in intracellular defense against oxidative stress and contributes to the regeneration of other antioxidants. [88,89]. A study by Ogata et al. (2022) has shown positive effects on the early formation of male pronuclei without negative effects on DNA integrity and cell number in blastocyst stage embryos. Although the effect was dependent on the bull and GSH concentration, semen supplementation has improved in vitro embryo production from frozen semen [88]. In this study, after GSH supplementation (0, 1, 5 and 10 mM), sperm quality varied between bulls. One bull had decreased total sperm motility, and two other bulls had decreased sperm DNA integrity. The GSH supplementation had positive effects on embryonic development in all three bulls. Two of them showed improved cleavage rates and blastocyst formation, while the other only showed a higher cleavage rate. Besides GSH, to counteract

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the harmful effects of ROS, many studies have included vitamin E in the semen extenders or embryo culture media to improve cryosurvival parameters, sperm cell viability, and embryo production [90–92]. Vitamin E is a well-established naturally-occurring lipid-soluble chain-breaking antioxidant that scavenges oxygen radicals within the membranes, acting in synergy with vitamin C, and protecting germlines [92,93].

Coenzyme Q10 (CoQ10) or ubiquinone (Figure 4), a free radical scavenger and a very important component of the mitochondrial, ETC has also been pointed out to impact male and female gametes [9,18,94]. CoQ10 antioxidant function (ubiquinol) works by inhibiting lipid peroxidation in vitro and in vivo. In addition to inhibiting lipid peroxidation without the mediation of vitamin E, ubiquinol can also amplify the antioxidant effect of this vitamin. It should be noted that ubiquinol is the only known fat-soluble antioxidant that can be synthesized in animal cells and regenerated through the mitochondrial electron transport system to its reduced antioxidant form. These characteristics, together with its high degree of hydrophobicity and its general occurrence in biological membranes and low-density lipoproteins, suggest a very important role for ubiquinol in cellular defense against oxidative damage [95,96].

Gendelman & Roth (2012) found that oocyte maturation medium supplemented with 50µM of CoQ10 induced changes in mitochondrial distribution within the oocytes and increased the proportion of polarized mitochondria. In addition, CoQ10 incorporation into oocytes induced changes in gene transcription involved in the mitochondrial ETC and a higher proportion of embryos that developed into blastocysts. However, CoQ10 did not improve oocyte development in winter and summer, which suggests that this antioxidant only contributes beneficially in phases with moderate climate damage, as there was an improvement in the fall [18]. Even so, these authors have suggested the widespread use of this antioxidant to increase the in vitro production of bovine embryos.

In 2017, Yang et al. tested the action of the natural antioxidant melatonin (10^{-9} M) (Figure 4) in IVF with positive results in the maturation of bovine oocytes and an increase in the number of produced embryos, concluding that melatonin improves the distribution of mitochondria and preserves the ATP production [41].

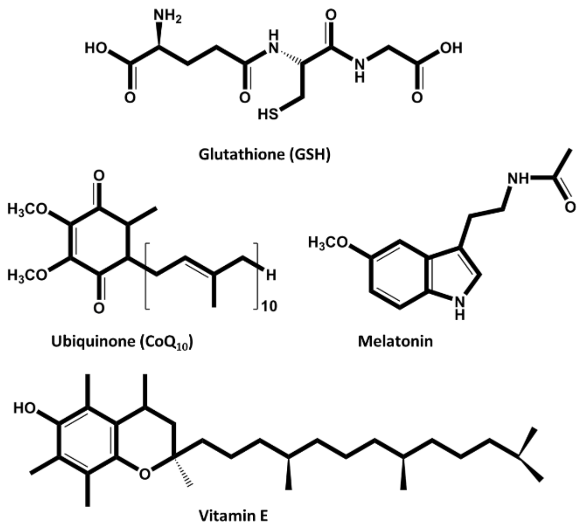

Figure 4. Chemical structures of endogenous antioxidants evaluated in ART context. Glutathione (GSH), Ubiquinone (CoQ_{10}), Melatonin, and Vitamin E have been tested in bovine germplasm to improve fertility.

5.2. Exogenous Antioxidants

Natural antioxidants derived from the diet have antioxidative characteristics with the potential to prevent diseases caused by oxidative stress [83]. In fact, antioxidants in fruit, vegetables, and drinks play an important role in mammalian health, such as preventing cancer and cardiovascular disease and reducing the incidence of different diseases [97]. The study of these natural compounds that are available in food/feed will contribute to increasing their use, rather than the use of artificial drugs.

5.2.1. Naturally-Occurring Antioxidants

Piperine is a simple alkaloid found in black pepper seeds (Figure 5), whose biological properties have been extensively studied at the pharmacological level and has shown great therapeutic potential as an antioxidant, anticancer, anti-inflammatory, antihypertensive, hepatoprotective, neuroprotective and in improving fertility. It also can alter gastrointestinal disorders and drug metabolizing enzymes [98,99].

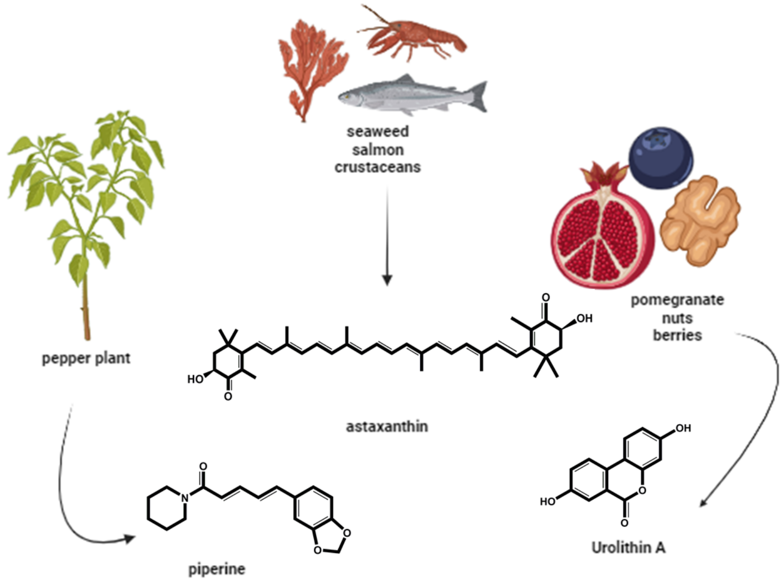

Figure 5. Exogenous naturally occurring antioxidants used to improve ART outcomes – Piperine, Astahantine and Urolithin A have been tested in animal germplasm to improve fertility (created in BioRender.com).

Piperine induces specific toxicity in cancer cells, but does not affect normal cells, and has also been shown to restore the function of aged cells around cancer cells [100]. In addition, piperine improves the efficacy of current cancer therapies and represents a good adjuvant to certain phytochemical compounds such as curcumin or resveratrol.

The use of piperine as a bioactive compound is still in its infancy, so studies on this compound are still essential for the formulation, improvement and discovery of new drugs. Recently our team has used piperine during bovine oocyte maturation. It was demonstrated that 1 and 10µM piperine improved the number of mature oocytes (metaphase II.) Also, higher MMP was attained with 1µM concentration compared to the control [101].

Polyphenols are secondary plant metabolites mostly involved in defense against oxidative stress and are found largely in fruits, vegetables, cereals and beverages present in the diet [102–104]. Over the last few years, several studies have found that foods rich in polyphenols protect against age-related diseases such as atherosclerosis, cardiovascular disease, cancer, arthritis, cataracts, osteoporosis, type 2 diabetes mellitus, hypertension, Alzheimer's disease, and diseases with mitochondrial etiology [102,105].

In 2024, Yang et al. showed the positive effect of the supplementation of the natural antioxidant astaxanthin (Figure 5) to porcine granulosa cells culture identifying positive variations in morphology, apoptosis, ROS levels, and the expression of apoptosis and anti-oxidation-related genes [106]. Astaxanthin also has ameliorated oxidative stress and reproductive outcomes after assisted reproduction [107].

Another example of an antioxidant of natural origin is Urolithin A (Figure 5), which is a metabolite resulting from the transformation by intestinal bacteria of ellagitannins, ellagic acid and polyphenols, found in foods such as pomegranates, berries and nuts. Several studies have shown that it plays an important role in preventing ageing and various diseases [51,108,109]. Fonseca et al. (2021) have tested the use of this antioxidant in the development of oocytes from old and young cows and concluded that supplementing this antioxidant in the oocyte maturation medium prevented oocyte aging and improved embryonic development. Later, Jorge et al. (2024) reinforced the potential therapeutic value of Urolithin A for addressing reproductive sub/infertility problems and improving ART outcomes. Their results have showed that Urolithin A has improved sperm motility quality, increasing ATP production while reducing oxidative stress levels in a dose-dependent manner.

5.2.2. Synthetic Mitochondriotropic Antioxidants

For several years, different approaches have been established to prevent oxidative damage to mitochondria, including the development of ETC inhibitors, OXPHOS, mitochondrial Ca^{2+} modulators, and mitochondriotropic antioxidants [86]. The pharmacological activities of new naturally derived and synthetic molecules have been extensively studied [83]. Currently, the prevention of mitochondrial oxidative damage through pharmacological solutions is recognized as an indispensable tool [51,102], to strengthen mitochondria's antioxidant power. Mitochondrial function can be improved through the use of antioxidants and various types of antioxidants have already been tested in animal and human oocytes with promising results [65], especially when targeted at the mitochondria [59,60,110].

Mitoquinone (MitoQ) is one of the most widely studied mitochondriotropic antioxidants. MitoQ has shown encouraging pre-clinical results in numerous studies on isolated mitochondria, cells, and tissues subjected to oxidative stress and death by apoptosis, as it can reproduce the role of the endogenous mitochondrial antioxidant CoQ10. This coenzyme is covalently linked to a triphenylphosphonium cation (TPP) by a 10-carbon alkyl chain (dTPP), a lipophilic spacer (Figure 6) that allows the molecule to cross mitochondrial membranes and considerably increases its antioxidant capacity. This antioxidant represents the first clinical attempt to deliver an antioxidant to the mitochondria. The results of the clinical trials with MitoQ were very important for understanding the relevance of a mitochondria-oriented approach [102,111].

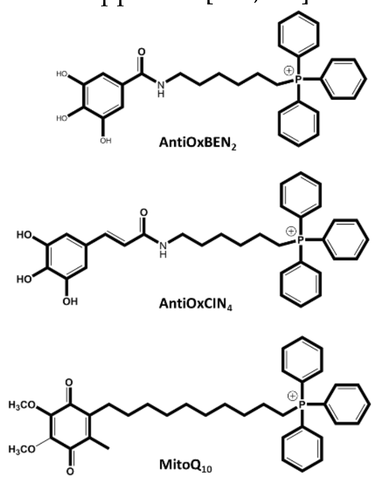

Figure 6. Chemical structures of synthetic mitochondriotropic antioxidants evaluated in ART context. AntiOxBEN2, AntiOxCIN4, and MitoQ10 have been tested in bovine cells to decreased ROS and increase the results in ART.

A study in bovine oocytes demonstrated that MitoQ (1µM) when supplemented to the culture medium improved mitochondrial function and enhanced embryo development. This effect was achieved by reducing mitochondrial ROS levels below the critical thresholds that trigger apoptosis [112]. Another study carried out during the maturation of rat oocytes also concluded that the addition of MitoQ (0, 0.01, 0.02 and 0.04µM) to IVF culture media improved oocyte fertilization and the subsequent development of blastocysts [113]. An increased GSH level and membrane potential of oocytes concomitant with reduced intracellular ROS concentrations were identified. Also, MitoQ supplementation (25, 50, and 100nM) to a stallion semen extender before cryopreservation showed the positive effect of this antioxidant on sperm mobility, especially at 25nM. The highest concentration of 200nM harmed the mobility and viability parameters of frozen-thawed semen. Nevertheless, none of the used concentrations affect the plasma membrane, acrosome, DNA integrity, MMP or intracellular ROS concentrations [114]. Conversely, the addition of MitoQ (0, 0.2, 2 and 20nM) to the semen extender before cryopreservation was unable to improve the post-thaw quality of bull sperm [115]. According to some studies, MitoQ supplementation has harmed the bioenergetic function of mitochondria and did not contribute to the inhibition of iron toxicity [102,116]

AntiOxBEN$_2$ is a mitochondria-targeted antioxidant derived from gallic acid, a hydroxybenzoic acid. AntiOxBEN$_2$ was synthesized by conjugating the antioxidant gallic acid with the lipophilic triphenylphosphonium cation (TPP+) (Figure 6) through a 6-carbon aliphatic chain, designed to specifically accumulate within the mitochondrial matrix [102,117,118]. Results in in vitro models show that AntiOxBEN2, unlike MitoQ10, proved capable of chelating ferrous iron upregulating antioxidant systems, and improving mitochondria function by activating the Nrf2/Keap1 pathway [119]. Teixeira et al. (2020) have studied the effect of mitochondriotropic antioxidant AntiOxBEN$_2$ on the prevention of oxidative stress in bovine oocytes and embryos by supplementing it to the oocyte maturation medium with concentrations of 10, 20, 50 and 100µM. AntiOxBEN$_2$ improved oocyte maturation and embryo production in a dose-dependent manner. These authors have suggested its future use at a concentration of 10µM during the oocyte maturation process. Later, Santos et al. (2022) supplemented culture media during IVF of bovine oocytes with AntiOxBEN$_2$, demonstrating its benefits in reducing ROS, increasing spermatozoa MMP and consequently sperm quality. When the sperm capacitation and the fertilization media were supplemented with a concentration of 1µM of AntiOxBEN$_2$, an improvement in embryo development, due to an increase of the number of cleaved embryos and blastocysts, was seen.

AntiOxCIN$_4$ is a mitochondriotropic antioxidant based on caffeic acid that has been described to prevent oxidative stress-related events through the activation of endogenous ROS-protective pathways in normal primary human fibroblasts (PHSF) and in PHSF from sporadic Parkinson's disease patients (Figure 6). AntiOxCIN$_4$ also increased cell stress resistance in human hepatoma-derived cells (HepG2) by activating the Nrf2-p62-Keap1 axis, leading to up-regulation of antioxidant defenses, triggering macroautophagy and/or mitochondrial autophagy (mitophagy) and mitochondrial biogenesis [116,120]. Additionally, AntiOxCIN$_4$ supplementation was shown to improve steatotic liver in a metabolic disease mice model [121]. Teixeira et al. (2020) have supplemented the oocyte maturation medium with concentrations of 10, 20, 50 and 100µM of AntiOxCIN$_4$, improving oocyte maturation in a dose-dependent manner. Recently, the supplementation of AntiOxCIN$_4$ to the capacitation and fertilization media, at concentrations of 0.1 and 1µM, was studied. It was found that the supplementation of 1µM AntiOxCIN$_4$ during the sperm capacitation process improved some of the functional characteristics of the spermatozoa and that both concentrations (0.1 and 1µM) have increased the number of good quality embryos. However, the study is still ongoing for a more robust approach [122].

Finally, AntiOxBEN$_2$ and AntiOxCIN$_4$ antioxidants at concentrations of 1, 2.5 and 10µM have also been tested in the embryo culture media to prevent oxidative stress. The concentration of 2.5µM improved the quality of the produced embryos. Based on these results, this concentration was used to further study the resistance of these embryos to the vitrification process. The data have shown that AntiOxCIN$_4$ and especially AntiOxBEN$_2$ had a beneficial effect on embryo development and cryopreservation survival, pointing to a possible therapy to prevent oxidative stress in ART [123,124].

6. Conclusions

This review has shown that infertility problems are increasing worldwide, both in humans and in animals. The reasons given range from human-induced environmental changes such as the industrial revolution of the 20th century, with its repercussions on pollution and all its surroundings, to climate change and lifestyles adopted, which encompass a large number of changes. Presently, ART procedures are imperative to overcome infertility problems, but also to maintain animal productivity and biodiversity. The study of natural antioxidants targeting mitochondria is essential, since mitochondria play a predominant role in the formation of ROS and oxidative stress. These compounds have been shown to present several different and positive roles, not only in terms of fertility, but also in the prevention and therapy of ageing, cancer, and neurodegenerative diseases, among others. Some studies have already been carried out with these antioxidants in ART, with promising results. However, this is still an area to be explored and more robust data are needed to consolidate the existing ones.

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