Antioxidants and Male Fertility: from Molecular Studies to Clinical Evidence

David Martin-Hidalgo 1,2,* , Julia Bragado 2 , Ana R. Batista 3 , Pedro F. Oliveira 4,5 and Marco G. Alves 2,*

1 Unit for Multidisciplinary Research in Biomedicine (UMIB), Laboratory of Cell Biology, Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto; pfbox@gmail.com
2 Research Group of Intracellular Signaling and Technology of Reproduction (SINTREP), Institute of Biotechnology in Agriculture and Livestock (INBIO G+C), University of Extremadura, Cáceres; jbragado@unex.es
3 Merck S.A.; Ana Rita Batista@merckgroup.com
4 i3S - Instituto de Investigação e Inovação em Saúde, University of Porto, Porto; zip code, Portugal
5 Faculty of Medicine, University of Porto, Porto; zip code, Portugal;
* Correspondence: davidmh@unex.es (D.M.-H.) and alvesmarc@gmail.com (M.G.A.)

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Abstract: Spermatozoa are physiologically exposed to reactive oxygen species (ROS) that play a pivotal role on several sperm functions through activation of different intracellular mechanisms involved in physiological functions such as sperm capacitation associated-events. However, ROS overproduction depletes sperm antioxidant system, which leads to a condition of oxidative stress (OS). Subfertile and infertile men are known to present higher amount of ROS in the reproductive tract which causes sperm DNA damage and deleterious effects in lower fertility and pregnancy rates. Thus, there is a growing number of couples seeking for-fertility treatment and assisted reproductive technologies (ART) due to OS-related problems in the male partner. Interestingly, although ART can be successfully used, it is also related with an increase in ROS production. This has led to a debate if antioxidants should be proposed as part of a fertility treatment in an attempt to decrease non-physiological elevated levels of ROS. However, the rationale behind oral antioxidants intake and positive effects on male reproduction outcome is only supported by few studies. In addition, it is unclear whether negative effects may arise from oral antioxidants intake. Although there are some contrasting reports, oral consumption of compounds with antioxidant activity appears to improve sperm parameters, such as motility and concentration, and decrease DNA damage, but there is not sufficient evidence that fertility rates and live birth really improve after antioxidants intake. Moreover, it depends on the type of antioxidants, treatment duration, and even the diagnostics of the men regarding its fertility, among other factors. Literature also suggests that the main advantage of antioxidant therapy is to extend sperm preservation to be used during ART. Herein, we discuss ROS production and its relevance in male fertility and antioxidant therapy with focus on molecular mechanisms and clinical evidence.

Keywords: assisted reproductive technologies; sperm ROS; pregnancy; infertility; antioxidants therapy; reproductive outcome

1. Introduction

The mammalian spermatozoon is a cell with a high demand for energy to perform its function. Spermatozoa obtain their energy by two main metabolic pathways: glycolysis that occurs in the principal
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piece of the flagellum and oxidative phosphorylation (OXPHOS) that takes place on mitochondria located at the midpiece of the flagellum [1]. Spermatzoa contain between 50 and 75 mitochondria [2] and as any other kind of cell that perform aerobic metabolism is associated with the production of free radicals named reactive oxygen species (ROS) that include the hydroxyl radicals (•OH), superoxide anion (•O2−), hydrogen peroxide (H2O2), and nitric oxide (NO). These ROS are highly reactive molecules due to the presence of an unpaired electron in their outer shell. In addition, they have a very short half-life in the range of nanoseconds to milliseconds. ROS are produced as a consequence of natural cell machinery and participate in the normal function of a cell. However, when ROS production overcomes cellular antioxidant defenses surpassing a physiological range, they cause deleterious effects due to oxidative stress (OS) that results in oxidation of lipids, proteins, carbohydrates, and nucleotides [3].

Men-Male subfertility and infertility has been associated with OS. Moreover, since infertile men have lower seminal plasma antioxidant capacity in comparison with fertile men, when higher levels of ROS occur, they led to an increase of lipid peroxidation (LPO) [4]. It is well described that when ROS overproduction occurs, it induces sperm DNA damage, although they have the potential to fertilize embryo development and fertility might be disturbed [5,6]. It is unclear how this is related with the fact that nowadays infertility is becoming a worldwide health problem, where one out of six couples are under fertility treatment and thus the use of assisted reproductive technologies (ART) to overcome this problem is growing exponentially. Nevertheless, ART itself is not harmless and is also associated with an increase of ROS production [7]. Although there is literature focused on the effects of consumption with antioxidant properties on sperm parameters, the purpose of this review is to discuss the efficiency of antioxidants intake as a dietary supplement as well as an additive through ART procedures to counteract excessive ROS production that leads to infertility. We will also focus on the molecular mechanisms of action of those compounds with antioxidant activity in the male reproductive system, mainly reviewing literature that relates antioxidant treatment with ART, clinical pregnancy, and live birth as final outcomes.

2. Sources of ROS in Spermatzoa

Several situations result in non-physiological levels of ROS overwhelming the natural scavenger systems (Figure 1). For example, lifestyle habits, such as alcohol consumption, smoking, exposure to toxicants, or pathologies such as obesity, varicocele, stress, and ageing have been associated with increased production of ROS in seminal plasma [8]. Presence of leucocytes in semen, as well as high percentage of spermatzoa with morphological anomalies [9] or immature spermatzoa with cytoplasmatic droplets containing high amount of enzymes are some examples associated to high ROS levels [9-12].

Currently, human infertility is a global health problem that has led to an exponential grown in the use of ART in the last years to overcome fertility problems. However, ART protocols imply sample centrifugation, light exposure, change of oxygen concentration, pH, or temperature, and the use of culture media with metals content that can produce hydroxyl radicals after Haber-Weiss and Fenton reactions take place (see explanation below). All of them related with ROS production [13]. Hence, optimization of ART protocols has been proposed to minimize artificial ROS production, for instance, by decreasing g-force during sperm selection by gradient [14], decreasing spermatzoa incubation time during in vitro fertilization (IVF), which in turn decreases the time where aberrant spermatzoa that produce more ROS are in contact with the oocyte, and as well as by decreasing sperm concentration or atmospheric oxygen concentration during embryo culture under in vitro conditions [7]. In order to reduce human leucocyte contamination on raw semen, paramagnetic bead technology (Dynabeads®) can be used. Thus, magnetic beads coated with leukocyte antigen CD45 decrease leukocyte contamination [15,16], doubling the percentage of spermatzoa–spermatozoa–oocyte penetration, as it was shown in a heterologous assay using hamster oocytes [17].
Mammalian spermatozoa are extraordinary cells able to survive in a different body from where they were created. They are very specialized cells having as the only purpose to deliver the paternal genome into the oocyte. However, after ejaculation, spermatozoa are incapable to fertilize oocytes as they should undergo through a complex process within the female reproductive tract collectively named capacitation, which allows spermatozoa to fertilize the oocyte [18,19]. Capacitation is a cascade of different cellular events that imply high production and consumption of energy. Although there is controversy on the preponderant metabolic pathways, glycolysis or OXPHOS, used by spermatozoa to generate energy in the form of ATP, it seems that there are sperm species preferences [1]. OXPHOS is the most efficient pathway, obtaining about 30 molecules of ATP by oxidizing one molecule of glucose, while during glycolysis, only two molecules of ATP are obtained by one molecule of glucose. It has been described that OXPHOS is the major source of ROS in spermatozoa [20]. Furthermore, ROS might play a bivalent role on sperm function. Mild ROS levels boost different intracellular events that culminate on oocyte fertilization, while higher ROS levels induce sperm DNA damage and embryo miscarriage [21,22]. In a comprehensive review, Ford summarized ROS physiological functions on sperm capacitation [23]. It is known that soluble adenylyl cyclase (sAC) is activated by bicarbonate and Ca$^{2+}$, converting ATP in cAMP, subsequently activating the PKA pathway that mediates the phosphorylation of protein in tyrosine residues, which is used as a hallmark of sperm capacitation, among others [24,25]. It has been proposed that ROS participate in the activation of the cAMP/PKA pathway by increasing cAMP levels, although the mechanism of cAMP production is still not clear in spermatozoa [23]. In other cells (adipocytes), it has been proposed that the mechanism of action is through inhibition of phosphodiesterase activity [26]. In human spermatozoa, it was proven that ROS mechanism of action is mediated by PKA [27]. Thus, the induction of tyrosine phosphorylation was suppressed by a PKA inhibitor (H89) and the responsiveness to progesterone (sperm-oocyte fusion) when spermatozoa were coincubated with NADPH proved it to be a ROS generator [27]. In a different study, capacitated human spermatozoa showed increased levels of cAMP that was mimicked in vitro by exposure of spermatozoa to superoxide anions (O$_{2}^-$) and superoxide dismutase (SOD) addition inhibited cAMP levels and the sperm acrosome reaction in a concentration-dependent manner [28]. These results were confirmed by others where superoxide anions...
increased cAMP concentration and capacitated spermatozoa produced H$_2$O$_2$ leading to an increase in protein tyrosine phosphorylation [29]. Nevertheless, when ROS production overcomes antioxidant defenses, detrimental effects on spermatozoa can be summarized as increased LPO and DNA damage and reduction of sperm motility, which are associated with lower fertilizing sperm capacity and fertility (Reviewed by [30]). Thus, ROS production homeostasis is pivotal for male reproductive potential as they mediate important functions of sperm, such as capacitation, but when ROS levels surpass these biological levels, they readily oxidize lipids and proteins at membranes and compromise sperm quality and fertilization capacity.

**Figure 2.** Proposed model of the bivalent role of reactive oxygen species (ROS) in sperm. (i) High levels of ROS concentration induced by different factors such as assisted reproductive technologies (ART), diseases, medical treatment, life style, etc., overwhelming the sperm antioxidant system induce plasma membrane lipid peroxidation and DNA damage. (ii) Physiological ROS level produced mainly by mitochondria induce production of high levels of cAMP by an undetermined mechanism, activating the PKA pathway and leading to tyrosine phosphorylation, a hallmark of sperm capacitation.

### 4. Mechanism of ROS Defense in Spermatozoa

Spermatozoa differentiation is achieved during spermiogenesis as they gradually lose their cytoplasm. Indeed, by the end of the process, the cytoplasm content is very small compared to other cells, where most of the space is occupied by DNA (sperm head). This special feature results in spermatozoa possessing low intracellular antioxidant activity consisting of superoxide dismutase (SOD), nuclear glutathione peroxidase (GPx), peroxiredoxin (PRDX), thioredoxin (TRX), and thioredoxin reductase (TRD) [31]. Therefore, sperm ROS scavenger activity basically depends on the antioxidant content of the seminal plasma, which is formed mainly by a trio of enzymes where SOD converts superoxide anion (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$), preventing the formation of hydroxyl radical that is an inductor of LPO. However, the H$_2$O$_2$ generated is a strong membrane oxidant that is rapidly eliminated either by catalase (CAT) or GPx activities, giving H$_2$O as a product. Finally, seminal plasma also contains non-enzymatic
antioxidant components such as α-tocopherol (vitamin E), ascorbic acid (vitamin C), pyruvate, urate, taurine, and hypotaurine [32].

It should be noted that most of the ART involves simply washing steps, meaning that all the natural antioxidant defenses contained in seminal plasma are removed. Likewise, this also happens after natural insemination. During ejaculation, spermatozoa are surrounded by antioxidant molecules coming from seminal plasma but once the ejaculate reaches the vagina, seminal plasma is diluted, leading in both cases to spermatozoa facing ROS insults. Although spermatozoa possess antioxidant scavenger systems, it seems that they are not strong enough when ROS levels exceed physiological levels and subsequently making spermatozoa a highly susceptible cell to OS.

5. Lipid Peroxidation

The sperm plasma membrane contains a high proportion of polyunsaturated fatty acid (PUFAs) to generate the fluidity needed in order to accomplish the membrane fusion events associated with fertilization. This high PUFAs content makes spermatozoa especially susceptible to suffer LPO [33,34]. The highly reactive hydroxyl radical (OH−) is an inductor of LPO produced through two consecutive reactions (Figure 3): the first is the Haber–Weiss reaction in which a ferric ion (Fe3+) in the presence of a superoxide radical (O2−) is reduced to ferrous ion (Fe2+), followed by Fenton reaction, where Fe2+ reacts with hydrogen peroxide (H2O2), forming Fe3+ and a hydroxyl radical (OH).

\[
\begin{align*}
\text{Fe}^{3+} + \cdot \text{O}_2^- & \rightarrow \text{Fe}^{2+} + \text{O}_2 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot \text{OH}
\end{align*}
\]

Total Reaction net:

\[
\cdot \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \cdot \text{OH}
\]

Figure 3. Haber–Weiss Reaction and Fenton reaction.

Secondary products are formed during LPO: malondialdehyde (MDA), propanol, hexanol, and 4-hydroxynonenal (4-HNE) [35], which being are these products highly reactive and may attack other nearby PUFAs, thus propagating a chain reaction with harmful effects over the cell that eventually disrupts membrane fluidity. These secondary products are used as lipid oxidative stress biomarkers.

Nowadays, cryopreservation is becoming an important issue for the subsequent success of ART in humans and also in the livestock sector. Although cryopreservation is routinely used, it is a tough procedure associated with deleterious effects on sperm function due to an increase of ROS production linked to LPO and thus an increase of membrane permeability [36–38]. In this context, the use of antioxidants as additives during cryopreservation/thawing procedure is a common strategy to counteract ROS-negative effects of ROS over sperm function.

6. Effects of Oral Antioxidants Intake on Male Reproductive Outcome

Currently, there is a growing trend of oral antioxidants intake to counteract high levels of ROS found in spermatozoa and seminal plasma of subfertile or infertile men. This hypothesis is supported by several works that describe an improvement of sperm parameters after oral antioxidants intake. Among those improvements, sperm concentration, motility, or decrease of DNA damaged are reported (Reviewed by [39]). However, only a few works have shown the effect of antioxidant therapy on fertility outcomes. Here,
we discuss the major findings of oral antioxidant intake in reproduction outcome and its endpoints, such as fertility and live birth (summarized in Table 1).
Table 1. Effects of oral antioxidants intake on infertile men’s reproductive outcome.

<table>
<thead>
<tr>
<th>Antioxidant type and daily dose</th>
<th>Period intervention (months)</th>
<th>ART</th>
<th>Relevant findings</th>
<th>Participants</th>
<th>Problem</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astaxantin (16 mg)</td>
<td>3</td>
<td>NI</td>
<td>↑ Pregnancy rate 54.5% (5/11) vs. 10.5 % (2/19) placebo group</td>
<td>30</td>
<td>Infertile</td>
<td>[48]</td>
</tr>
<tr>
<td>LC (1 g twice)</td>
<td>3</td>
<td>NI</td>
<td>↑ ROS levels</td>
<td>54</td>
<td>PVE</td>
<td>[43]</td>
</tr>
<tr>
<td>LAC (0.5 g twice)</td>
<td>3</td>
<td></td>
<td>↑ Pregnancy (11.7%) in patients with abacterial-PVE with normal values of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>leucocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsteroidal anti-inflammatory + carnitine (Carnitene, 2 g + Nicetile 1 g)</td>
<td>2 + 2</td>
<td></td>
<td>It didn’t improve pregnancy (0%) in those abacterial-PVE patients with high levels of leucocytes</td>
<td>98</td>
<td>PVE with</td>
<td>[44]</td>
</tr>
<tr>
<td>Carnitine (Carnitene, 2 g + Nicetile 1 g)</td>
<td>4</td>
<td></td>
<td>23.1% pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsteroidal anti-inflammatory</td>
<td>4</td>
<td></td>
<td>0% pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsteroidal anti-inflammatory + carnitine (Carnitene, 2 g + Nicetile 1 g)</td>
<td>4</td>
<td></td>
<td>6.2% pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ne Anti-inflammatory + carnitine (Carnitene, 2 g + Nicetile 1 g)</td>
<td>4</td>
<td></td>
<td>3.8% pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC (3 g), LAC (3 g), LC (2 g) + LAC (1 g)</td>
<td>6</td>
<td></td>
<td>↑ Total oxyradicals scavenging capacity of seminal fluid</td>
<td>60</td>
<td>Asthenozoospermic</td>
<td>[49]</td>
</tr>
<tr>
<td>LC (2 g), fumarate (725 mg), LAC (500 mg), Fructose (1000 mg), CoQ10 (20 mg), Folic acid (200 mg), Vitamin B12 (1.5 µg)</td>
<td>6</td>
<td></td>
<td>↑ Sperm motility and concentration, Pregnancy rate was not modified</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carnitine (Carnitene, 2 g + Nicetile 1 g)</td>
<td>6</td>
<td></td>
<td>23.1% pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsteroidal anti-inflammatory</td>
<td>4</td>
<td></td>
<td>0% pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsteroidal anti-inflammatory + carnitine (Carnitene, 2 g + Nicetile 1 g)</td>
<td>6</td>
<td></td>
<td>6.2% pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC fumarate (2 g), LAC (1 g)</td>
<td>3 - 4</td>
<td></td>
<td>↑ Sperm concentration, % of sperm motile or progressive motility as well as</td>
<td>173</td>
<td>Oligo- and/or asteno- and/or teratozoospermia</td>
<td>[46]</td>
</tr>
<tr>
<td>LC fumarate (2 g), LC (1 g), Acetyl-L- carnitine HCl (0.5 g), Fructose (1 g), Citric acid (50 mg), Vitamin C (90 mg), Zinc (10 mg), Folic acid (200 µg), Coenzyme Q10 (20 mg), Vitamin B12 (1.5 µg)</td>
<td>6</td>
<td></td>
<td>↑ Sperm concentration, % of sperm motile or progressive motility as well as</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Treated men achieved 29% pregnancy versus 17.9% in the placebo group</td>
<td>90</td>
<td>After performed a varicocelectomy</td>
<td>[50]</td>
</tr>
<tr>
<td>Vitamin E (600 mg)</td>
<td>3</td>
<td>IVF</td>
<td>Improvement of zona pellucida binding test</td>
<td>30</td>
<td>Infertile</td>
<td>[51]</td>
</tr>
</tbody>
</table>

For PEER REVIEW
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Study Design</th>
<th>Outcome</th>
<th>Improvement</th>
<th>Control Group</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E (300 mg)</td>
<td>3 NI</td>
<td>21%</td>
<td>Improved sperm motility and achieved pregnancy where 81.8% of pregnancies finished with a live birth</td>
<td>52 Atherospermic</td>
<td>[52]</td>
</tr>
<tr>
<td>Vitamin E (200 mg)</td>
<td>1 IVF</td>
<td>↓ Sperm LPO</td>
<td>Fertility rate: 19.2 ± 23.3 pre-treatment versus 29.1 ± 22.2 post-treatment</td>
<td>15 Normospermic infertile</td>
<td>[53]</td>
</tr>
<tr>
<td>Vitamin E (1 g) Vitamin D (1 g)</td>
<td>2 ICSI</td>
<td>↑ Fertility rate (6.9 vs 49.3%) ↑ Implantation rate (2.2 vs 19.2%) Equal embryo quality</td>
<td>38 Infertile men non responding to ICSI</td>
<td>[54]</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (400 IU), Selenium (200 µg)</td>
<td></td>
<td></td>
<td></td>
<td>690 Infertile</td>
<td>[55]</td>
</tr>
<tr>
<td>Vitamin E (400 IU), Vitamin C (100 mg), Lycopene (6 mg), Zinc (25 mg), Selenium (20 µg), Folate (0.5 mg), Garlic (1000 mg)</td>
<td>3 IVF-ICSI</td>
<td>Double pregnancy rate (63.9 vs 37.5%), Double implantation rate (46.2 vs 24%), Double viable pregnancy rate (38.5 vs 16%)</td>
<td>60 Infertile men with ↑ levels of DNA fragmentation and poor motility and membrane integrity</td>
<td>[56]</td>
<td></td>
</tr>
<tr>
<td>Zinc sulphate (220 mg)</td>
<td>4 NI</td>
<td>21.4% (3/14) of patients achieved pregnancy Zinc levels were increased in seminal plasma</td>
<td>14 Human</td>
<td>[57]</td>
<td></td>
</tr>
<tr>
<td>Zinc sulphate (900 mg)</td>
<td>3 NI</td>
<td>Improved pregnancy (22.2%) vs placebo (4.3%) Zinc levels were not modified on seminal plasma</td>
<td>100 Atherospermic</td>
<td>[58]</td>
<td></td>
</tr>
</tbody>
</table>

Carnitines are synthesized by the organism and found in seminal plasma at higher concentration than in spermatozoa. The L-carnitine (LC) isomer is the bioactive form [40] with a pivotal role in mitochondrial β-oxidation, acting as a shuttle of the activated long-chain fatty acids into the mitochondria [41] where t-acyl-carnitine (LAC) is an acyl derivative of LC. Long-chain fatty acids provide energy to mature spermatozoa (with positive effects on sperm motility) and also during maturation and the spermatogenic process [42]. Oral intake of LC (1 g twice/day) and LAC (0.5 g twice/day) for three months reduced ROS levels in spermatozoa and improved pregnancy (11.7%) in patients with abacterial prostate-vesiculo-epididymitis (PVE) with normal values of leucocytes, but it did not improve pregnancy at all (0%) in those PVE patients with high levels of leucocytes [43]. A year later, the same group tested patients diagnosed with abacterial PVE concomitant with high levels of leucocytes and showed that pretreatment for two months with a nonsteroidal anti-inflammatory followed by two months of carnitine oral intake achieved 23.1% pregnancy in comparison with the four-months carnitine intake group (0%), nonsteroidal anti-inflammatory group (6.2%), and the group receiving four months of nonsteroidal anti-inflammatory compounds and carnitines (3.8%) [44]. In another study, was discriminated the effect of daily intake of LC (3 g), LAC (3 g), or a combination of LC (2 g) and LAC (1 g) and the placebo men control was discriminated during over six months and results were followed up two months after intervention in idiopathic asthenozoospermic men (n = 60) [45]. Treated men improved their total oxyradicals scavenging capacity of seminal fluid [45]. Overall, LAC or the combination of LAC + LC treatment had better improvement of sperm motility and concentration. Nevertheless, those patients with lower basal values of sperm motility had higher probability to respond to the treatment but pregnancy rate was not improved by any treatment in comparison with placebo control group [45].

Recently, co-administration of LC fumarate (2 g), LAC (1 g), and clomiphene citrate (50 mg) concurrently with vitamins and minerals in patients with idiopathic oligo- and/or asteno- and/or teratozoospermia (n = 173) enhanced sperm concentration specially in those patients with multiple impairment semen parameters (oligoasthenoteraatozoospermic patients), but did not improve the morphology, progressive sperm motility neither pregnancy rates in comparison with control group [46]. A meta-analysis concerning carnitine used as an oral antioxidant therapy concluded that this molecule might be effective for improving pregnancy rates regarding the limits of patients inclusion criteria and the lower number of men evaluated in each study [47].

6.2. Vitamins

The interest of vitamin E and its use as antioxidant is due to its protective activity against ROS which subsequently decreases LFO, and therefore exerts positive effects on sperm functions such as for example increase of sperm concentration and motility [59]. However, its effects in fertility are less clear. For example, in a small clinical trial (n = 30), oral administration of vitamin E (300 mg twice daily) for three months raised the levels of vitamin E in blood serum, although human seminal plasma levels were not modified, questioning its possible effects on reproductive parameters [50]. Nevertheless, in this clinical trial, vitamin E treatment achieved an improvement of the zona pellucida binding test without any other improvement described, including ROS level [50]. Similarly, 15 normospermic infertile men after one month of daily consumption of 200 mg of vitamin E improved their fertilization rate (19.3 ± 23.3 pretreatment versus 29.1 ± 22.2 post-treatment) after IVF.

Those results were associated with lower sperm LFO levels in comparison with pre-intervention values [52]. In another work, oral administration of vitamin E (100 mg thrice daily) on to patients with asthenospermia (n = 52) established a three different groups of men according to the results: i) men without improvement of their sperm motility (40%); ii) men that improved their sperm motility but did not achieve pregnancy (39%); iii) men that improved their motility and achieved pregnancy (21%), and where the of which 81.8% of pregnancy pregnancies finished in live birth, while the placebo control group did not achieve any pregnancy [52]. Later, daily intake of a
combination of vitamin E and C (1 mg of each component) for 2-3 months in patients where intracytoplasmic sperm injection (ICSI) had previously failed was studied (n = 38). The results showed two different subpopulations: (i) those where the antioxidant treatment decreased the percentage of sperm DNA damage (n = 29) and (ii) those where the treatment did not affect this parameter (n = 9) [54]. The most interesting result was observed in the responsive group that after ICSI, the pregnancy rate (6.9% vs. 4.9%) and implantation rate (2.2% vs. 1.2%) were improved compared with the pretreatment group, although no differences were found in embryo quality [54]. The following results were obtained from a non-placebo-controlled and non-double-blind design trial, where a daily intake of a combination of selenium (200 µg) and vitamin E (400 UI) followed for 3.5 months only in infertile men (n = 690) achieved 10.8% of spontaneous pregnancy [55].

Several studies have been performed looking for a possible sum of beneficial effects from a combination of multiple compounds with antioxidant activity having in common vitamins as components. For example, a formulation using a mix of several compounds with antioxidant activity (vitamin C, vitamin E, carnitine, folic acid, lycopene, selenium, and zinc) was evaluated using a mouse Gpx5 knockout (KO) subjected to a second stress: scrotal heat (KO + SH) (42 °C for 30 min) [59]. Although the exact ingestion quantity of this antioxidant combination could not be determined, their effects include the reversion of sperm DNA oxidation induced in KO + SH animals and protection of seminiferous tubules organization. The most striking results showed that animals supplemented with animals + KO versus the non-supplemented animals + KO had double the fertilization rate (73.7% vs. 35.2%) and reduce-fetus reabsorption was halved (8.9 vs. 17.8%) obtained by natural mating [59]. In another trial, human infertility patients with oligo- and/or astheno- and/or teratozoospermia with or without varicocele (n = 104) using a combo combination of antioxidants formulation (vitamin C 90 mg, vitamin B12 1.5 µg, LC 1mg, fumarate 725 mg, LAC 500 mg, fructose 1000 mg, CoQ10 20 mg, zinc 10 mg, and folic acid 200 µg) were studied for 4-6 months. The results showed that those individuals from the treated group, regardless of whether they suffered from varicocele or not, presented an improved sperm concentration, progressive and total sperm motility [49]. Moreover, after treatment, 22.2% (10/45) of supplemented patients achieved pregnancy, while in those from the control group, only 4.1% (2/49) of the couples were pregnant [49]. A close analysis of the men from the supplemented group revealed that only 4.8% (1/21) of patients suffering varicocele improved after treatment, while the non-varicocele group achieved 37.5% (9/24) pregnancy [49]. A different group studied the effect of a commercial multi-antioxidant supplement (vitamin E 400 IU, vitamin C 100 mg, lycopene 6 mg, zinc 25 mg, selenium 26 µg, folate 0.5 mg, garlic 1000 mg) for 3 months on 60 men with high levels of DNA fragmentation and poor sperm motility and membrane integrity [56]. The treatment achieved doubled pregnancy rate (63.9 vs. 37.5%), implantation rate (46.2 vs. 24%), and viable pregnancy rate (38.5 vs. 16%) versus the placebo group without any modification of any sperm parameters, fertilization, or embryo quality rates [56]. However, this work has been criticized later because of the experimental design used, particularly the low number of individuals in the trial, unequal distribution of individuals between the placebo (n = 16) and treatment groups (n = 36) and the suitability of the statistical analysis used [60].

Contradictory results were found when men were supplemented with different oral antioxidants after varicocelectomy. Oral intake of vitamin E (300 mg twice/day) during for 12 months (n = 40) improved the sperm parameters, as of sperm concentration and the percentage of motile spermatozoa, although these data were not significant compared with control [61]. Recently, a multiple antioxidant combo was tested (L-carnitine fumarate 1 g, acetyl-L-carnitine HCl 0.5 g, fructose 1 g, citric acid 50 mg, vitamin C 90 mg, zinc 10 mg, folic acid 200 µg, selenium 50 µg, coenzyme Q10 20 mg, and vitamin B12 1.5 µg) after varicocelectomy (n = 90) during for 6-12 months [50]. Surgery improved the following sperm parameters: sperm concentration, percentage of motile spermatozoa or progressive motility and also spermatozoa with normal morphology, although these improvements were significantly better in men treated with the combo. Moreover, treated men achieved 29% pregnancy versus 17.9% in the placebo group [50].
6.3. Zinc

Zinc is a metalloprotein cofactor for DNA transcription and protein synthesis. Moreover, zinc is necessary for the maintenance of spermatogenesis and optimal function of the testis, prostate, and epididymis [62], in addition to their antioxidant properties preventing LPO [63]. A trial using zinc sulphate as an antioxidant therapy administrated orally (250 mg twice daily) for 3 months reported an improvement in the reproductive outcome of asthenozoospermic men (n = 100), particularly in seminal parameters of concentration, motility, and sperm membrane integrity (hyposmotic swelling test). It was also noticed a decrease of antisperm antibodies on seminal plasma without modification of zinc levels on seminal plasma [58]. Pregnancies were also improved in couples where men had undergone treatment when compared with placebo, 22.5% (11/49) versus 4.3% (2/48), respectively [58]. In another trial with only 14 patients and no control group, sperm parameters were improved after zinc treatment (220 mg daily for 4 months) and 21.4% (3/14) of patients achieved pregnancy and increase zinc levels on seminal plasma [57]. Although beneficial evidence has been found on reproductive outcome after zinc intake, the lower number of studies and subjects under treatment without a proper control does not allow a further discussion of the possible positive effects of zinc intake on reproduction outcome.

6.4. Natural Compounds—Traditional Medicine

Natural compounds have been used traditionally to treat diseases. For instance, beneficial effects on reproductive outcome have been reported using products derived from tea (Camelia sinensis (L.)), which is the second most consumed beverage after water [64]. For example, an in vitro experiment using green tea extract or epigallocatechin-3-gallate (EGCG) added to human spermatozoa media improved sperm capacitation hallmarks such as tyrosine phosphorylation and cholesterol efflux, through the estrogen receptor pathway [65]. EGCG has been shown to have beneficial effects when extreme stresses are applied to male mouse mice [66,67]. Interestingly, adverse effects induced by artificial testicular hyperthermia were ameliorated by oral administration of green tea extract [66]. Positive effects were visible after 28 days of heat stress induction, improving sperm concentration, percentage of motile and progressive spermatozoa, as well as sperm membrane integrity [66]. Another example of the beneficial effects of EGCG were described when intraperitoneal administration (50 mg/kg) protected against testicular injury induced by ionizing radiation in rats [67]. Thus, treated animals restored testicular function having an improvement in the number of pups by litter reducing LPO (TBARs) and protein carbonyl levels [67]. EGCG’s mechanism of action is through the mitogen-activated protein kinase/BCL2 family/caspase 3 pathway [67]. In another work, the combination of two different tea extracts, white and green, where evaluated as additives to improve an ART sperm of rats stored at room temperature. The authors found that there were doubled levels of epigallocatechin (EGC) and EGCG in white tea in comparison with green tea [68], highlighting that there is a variability associated with the type of tea extract used. Moreover, although both extracts had positive effects, the white tea extract had better ferric reducing antioxidant power than the green tea extract per the control. The beneficial effects were proportional to the concentration used, being with 1 mg/mL of white tea extract the best concentration tested for improving sperm survival and decreasing LPO levels after 72 hours of storage at room temperature [68]. Encouraged by the antioxidant attributes and effects of the extract on sperm parameters of white tea, the same group explored the oral administration potential of the extract to improve prediabetic type II (PreDM) male reproduction features known to be decreased due to oxidative stress [69]. PreDM is characterized by mild hyperglycemia, glucose intolerance, and insulin resistance and has been related with infertility or subfertility problems in the males [70]. Consequently, using rat as an animal model, drinking white tea counteracted the negative effects of PreDM on the male reproductive tract. For example, white tea consumption improved testicular antioxidant power and consequently decreased lipid peroxidation and protein oxidation [69]. Ingestion of white tea also restored sperm motility and...
restored sperm showing morpho-anomalies to normal levels, improving sperm vitality in comparison with PreDM animals not treated as well as increasing sperm concentration when compared with control animals [69].

7. Antioxidants as a Tool to Improve Male ART Outcomes

Human infertility already affects one of six couples worldwide [71] and male factors contribute to 20–30% of infertility [72]. Infertile men have been related to having higher ROS levels than fertile men. To counteract these fertility problems, different ART have been developed, mainly IVF and ICSI. In both cases, gametes are extracted from the body and incubated in in vitro conditions and, after a while, an embryo is transferred into the uterus. It should be noted that due to legislation and ethical issues, it is easier to perform experiments in animal models than in humans to test antioxidant effects on different ART. The interest in the use of antioxidants to improve sperm parameters is not new. As early as 1943, in a study focused on sperm metabolism and oxygen consumption, MacLeod showed that sperm produce hydrogen peroxide, which has deleterious effects on sperm motility, and it could be counterbalanced by addition of catalase to the media, being one the first publications showing interest in antioxidant effects in in vitro sperm functions [73]. Later, some authors followed the same rationale and tried to adapt MacLeod’s hypothesis to different ART, such as cryopreservation, IVF, and ICSI.

Sperm conservation for long periods of time in liquid nitrogen (cryopreservation) is designed to keep viable sperm viable for an indefinite period of time. From a practical point of view, cryopreservation is a tool to manage male infertility. To be done, for example, before chemotherapy, radiotherapy, vasectomy, or exposure to toxicants, or just to have time enough to screen donors for infectious agents, such as the human immunodeficiency or hepatitis B viruses [74]. On the other hand, from the animal industry point of view, the use of cryopreservation aims to maximize the number of services (inseminations) that can be performed from a simple ejaculated sample that ensures the high quality of genetical material preserved, or allows allowing the transportation of this genetical material to distant places. Cryopreservation is also of special interest to preserve endangered species. However, cryopreservation is not a harmless technique, inducing DNA and LPO damage in sperm and others adverse effects [75]. Moreover, cryopreservation, like other ART, involves centrifugation, which is associated with production of ROS [14] and removing removal of seminal plasma where is located which contains the main sperm antioxidant scavenger systems.

For all the aforementioned, antioxidant supplementation to cryopreservation media has been proposed as a way to overcome ROS production and OS status in spermatozoa (summarized in Table 2). For example, supplementation with a synthetic phenolic antioxidant, butylated hydroxytoluene (BHT), during boar sperm cryopreservation improved post-thawing sperm survival, decreased MDA levels at the concentration of 0.4 mM BHT, and embryo development was improved (28.8% vs. 15.8%) without modification of embryo cleave percentage in comparison to the control [76]. Later, it was described that 1 mM BHT improved antioxidant sperm activity, pregnancy rate (86.7±6.9% vs. 63.6%), the number of gilts farrowing (86.7±6.9% vs. 45.4%), and the number of piglets born (10.8±1.6 vs. 8.2±2.2) after performing intrauterine artificial insemination (IUI) using cryopreserved sperm versus control [77]. Subsequently, in a multi-test where in which four different compounds with antioxidant activity (BHT 2 mM, ascorbic acid 8.5 mg/mL, hypotaurine 10 mM, and cysteine 5 mM) were added during goat sperm cryopreservation, procedure describing that they effectively decreased LPO was decreased but only ascorbic acid and BHT significantly improved fertility in comparison with control 42.85%, 35.71% and 26.38% respectively, after performing an artificial insemination (AI) [78].
Table 2. Antioxidants used as additives to different ART extenders and their reproduction outcomes.

<table>
<thead>
<tr>
<th>Antioxidant Type and Dose</th>
<th>Administration</th>
<th>Procedure</th>
<th>Principal Results Found</th>
<th>Stress</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHT 0.4 mM</td>
<td>In vitro</td>
<td>IVF</td>
<td>↑ Sperm MDA levels at the concentration</td>
<td>Cryopreservation</td>
<td>Boar</td>
<td>[76]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Embryo develop: 26.8% treated vs. 15.8% control</td>
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</tr>
<tr>
<td>BHT 1 mM BHT</td>
<td>In vitro</td>
<td>IUI</td>
<td>↑ Pregnancy rate (86.7 vs. 65.6%)</td>
<td>Cryopreservation</td>
<td>Boar</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ n° of gilts farrowing (86.7 vs. 45.4%)</td>
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<td></td>
<td></td>
<td></td>
<td>↑ n° of piglets born (10.8 ± 4.6 vs. 8.2 ± 2.2)</td>
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<td></td>
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<tr>
<td>BHT (2 mM), Ascorbic acid (8.5 mg/mL), Cysteine (5 mM), Hypotaurine (10 mM)</td>
<td>In vitro</td>
<td>AI</td>
<td>↑ Sperm LPO</td>
<td>Cryopreservation</td>
<td>Goat</td>
<td>[78]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>↑ Fertility: ascorbic acid (42.85%), BHT (35.71%), control (26.38%)</td>
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<tr>
<td>Caffeine (1.15 mM), β-mercaptoethanol (20 µM)</td>
<td>In vitro</td>
<td>AI</td>
<td>No effect on pregnancy rate</td>
<td>Cryopreservation</td>
<td>Boar</td>
<td>[80]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>↑ n° of piglets born (86.7 vs. 45.4%)</td>
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<tr>
<td>CAT (200 IU/mL)</td>
<td>In vitro</td>
<td>AI</td>
<td>↓ 2 pronucleus zygote (25.5% control vs. 13.2% treated)</td>
<td>Cryopreservation</td>
<td>Ram</td>
<td>[81]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ Cleaved embryos: 7.6% treated vs. 16.7% control</td>
<td></td>
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</tr>
<tr>
<td>Carnitine, Folic acid, Lycopene, Selenium, Vitamin C, Vitamin E, Zinc</td>
<td>Oral</td>
<td>NI</td>
<td>Duplicate fertilization rate (73.7 vs. 35.2%)</td>
<td>Cryopreservation</td>
<td>Mouse</td>
<td>[59]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Halved litter realabsorption (9 vs. 18%)</td>
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<td></td>
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<tr>
<td>Cysteine (2 mM)</td>
<td>In vitro</td>
<td>IUI</td>
<td>↑ Sperm total motility ↓ acrosome abnormalities</td>
<td>Cryopreservation</td>
<td>Bull</td>
<td>[82]</td>
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<tr>
<td></td>
<td></td>
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<td>↑ % acrosome membrane damaged</td>
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<td></td>
<td></td>
<td></td>
<td>↑ Sperm viability rates</td>
<td>Cryopreservation</td>
<td>Boar</td>
<td>[83]</td>
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<td></td>
<td></td>
<td></td>
<td>↑ % of total motile and progressive motile spermatozoa</td>
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<tr>
<td>Cysteine (10 mM), Rosemary extract (Rosmarinus officinalis), or a combination of both</td>
<td>In vitro</td>
<td>IUI</td>
<td>No improvement of antioxidants features</td>
<td>Cryopreservation</td>
<td>Bull</td>
<td>[84]</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>No differences on non-return rate was found after IUI</td>
<td></td>
<td></td>
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<tr>
<td>Cysteamine (5 mM), Lycopene (100 µg/mL)</td>
<td>In vitro</td>
<td>IUI</td>
<td>No differences on non-return rate</td>
<td>Cryopreservation</td>
<td>Bull</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No differences on non-return rate</td>
<td></td>
<td></td>
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<tr>
<td>ECGG (50 mg/kg)</td>
<td>Intraperitoneal</td>
<td></td>
<td>Restore testicular function</td>
<td>Cryopreservation</td>
<td>Bull</td>
<td>[86]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>↓ LPO and protein carbonyl levels</td>
<td></td>
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<td></td>
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<td></td>
<td>↑ Number of pups by litter</td>
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<tr>
<td>GSH (0.5 and 1.5 mM) GSH 0.5 mM + SOD 100 U/mL</td>
<td>In vitro</td>
<td>IUI</td>
<td>Equal non-return rates</td>
<td>Cryopreservation</td>
<td>Bull</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ % of total motile and progressive motile spermatozoa</td>
<td></td>
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<tr>
<td>Melatonin (1 mM)</td>
<td>In vitro</td>
<td>IVF</td>
<td>↑ Sperm viability rates</td>
<td>Cryopreservation</td>
<td>Ram</td>
<td>[87]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ % of total motile and progressive motile spermatozoa</td>
<td></td>
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</tbody>
</table>
**DNA integrity**

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>In vitro</th>
<th>IVF</th>
<th>Duplicate fertilization rate and embryo development</th>
<th>Cryopreservation</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin (50 to 5000 µM)</td>
<td>In vitro</td>
<td>IVF</td>
<td>Decrease ROS</td>
<td>Thawing + H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Bull</td>
</tr>
<tr>
<td>NAC (1–10 mM)</td>
<td>In vitro</td>
<td>ICSI</td>
<td>ICSI outcome wasn’t modified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metformin (50 to 5000 µM), NAC (1–10 mM), α-Lipoic Acid (5 µM)</td>
<td>In vitro</td>
<td>IVF</td>
<td>Accelerated embryo development and blastocysts</td>
<td></td>
<td></td>
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<tr>
<td>Taurine (2 mM)</td>
<td>In vitro</td>
<td>IUI</td>
<td>↓ GSH and SOD levels but ↑ five-fold CAT levels</td>
<td></td>
<td></td>
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<tr>
<td>Zinc chloride (10 µg/mL), D-aspartic acid (500 µg/mL), Coenzyme Q10 (40 µg/mL)</td>
<td>In vitro</td>
<td>IVF</td>
<td>↑ % of total spermatozoa motile and progressive motility, ↑ 8-cells blastocyst: 51.4% treatment vs. 37.1% control</td>
<td></td>
<td></td>
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</tbody>
</table>

IVF: in vitro fertilization; AI: Artificial Insemination; NI: natural insemination; IUI: intrauterine insemination; TE: trophectoderm; ICM: inner cell mass; BHT: butylated hydroxytoluene; CAT: catalase; GSH: reduced glutathione; NAC: N-acetyl-L-cysteine; LAC: L-α-carnitine; EGCG: epigallocatechin-3-gallate.
The importance and the use of the amino acid cysteine in the fight against ROS deleterious impacts on the cell is due to the fact it is a limiting substrate for glutathione synthesis [79]. Cysteine (2 mM) and taurine (2 mM) (a cysteine derived) antioxidant properties were controversial when they were used during the cryopreservation procedure of bull spermatozoa [82]. For example, taurine decreased GSH and SOD levels, while CAT levels were five times higher than control, but MDA levels were also higher. However, cysteine increased SOD and CAT levels without an effect in MDA levels [82]. The non-return rate was not modified when IUI were performed by neither of the compounds. However, a non-significant (p > 0.05) tendency of improvement was observed in cysteine-cysteine-treated straws 74.54% (41/55) compared to control 57.14% (28/49) [82]. Similar results were obtained when a higher concentration of cysteine (5 mM) and trehalose (25 mM) were added again to bull cryopreservation media. Thus, the antioxidants features of these compounds were not proved neither MDA nor antioxidant GPx levels were enhanced [84]. Furthermore, no improvement on the non-return rate was found after IUI [84]. Similarly, using cysteamine (5 μM), a decarboxylated derivative of cysteine and lycopene (500 μg/ml) during bull sperm cryopreservation, no differences were found in the non-return rate [85]. In other study, the authors used N-acetyl-l-cysteine (NAC), an acetylated cysteine residue which has been shown to effectively reduce ROS formation when H2O2 stress were used in thawed bull spermatozoa [89]. However, neither sperm DNA was not improved nor the number of blastocysts were not improved after performing an ICSI using spermatozoa cryopreserved in the presence of NAC [89]. Nevertheless, in an IVF study on mouse mice using fresh spermatozoa, where gametes and embryos were stressed by incubation under 20% oxygen atmosphere (over physiological levels on oviduct and uterine from 2–8%) [92], a combination of substances with antioxidant activity were tested (LAC 10 μM, NAC 10 μM, α-Lipoic Acid 5 μM) in either IVF media, embryo culture media, or both. Treated samples had lower intracellular levels of H2O2, accelerated embryo development, and significantly increased trophectoderm (TE) cell numbers, inner cell mass (ICM), and total cell numbers [90]. All these effects were exacerbated when the antioxidant combo were added during the whole process—sperm capacitation and fertilization process following embryo development [90].

Positive effects were also described when thawed bull spermatozoa were supplemented with an antioxidant combination (zinc chloride 10 μg/mL, D-aspartic acid 500 μg/mL, and coenzyme Q10 40 μg/mL), obtaining a better percentage of total sperm motile and progressive motility and a decrease of DNA fragmentation through the sperm incubation [91]. Moreover, antioxidant supplementation improved embryo development, although no differences were found in the cleave percentage, the number of blastocysts that arrived reached the blastocyst stage, and the percentages were 37.1% in the control versus 51.7% in those generated by the treated group [91].

Following the rationale of MacLeod [73], adding antioxidant enzymes to counteract the adverse effects of ROS on spermatozoa, it was followed to use them to improve sperm cryopreservation procedure. For example, the use of enzymes with antioxidant properties were added to bull cryopreservation media—0.5 and 1.0 mM of reduced glutathione (GSH) or a combination of 0.5 mM of GSH and 100 μM of SOD—used to perform a total of 5,127 IUI but did not modified modify the non-return rates [86]. In another study, the use of enzyme CAT (200 IU/mL) was used to cryopreserve ram (Capra pyrenaica) epididymal spermatozoa obtained postmortem [81]. At this concentration, no differences were found in sperm parameters but negative effects were described on fertility capacity, obtaining lower pronucleus zygotes (25.5% control vs. 13.2% treated) and cleaved embryos were obtained on-from treated samples after performed IVF (16.7% control vs. 7.6% treated) [81].

Natural compounds with antioxidant activity have also been tested in ART. Thus, metformin, a biguanide isolated from Galea officinalis used worldwide as a treatment for diabetes type II [93], was recently added to the cryopreservation sperm media of chicken due to its antioxidant properties, among other properties [94]. In other species, cryopreserved mouse spermatozoa treated with metformin displayed better motility, sperm viability, doubling-doubled fertilization rate and embryo development, and halved DNA fragmented-fragmentation embryo rate [88]. These
promising results in terms of supplementation of cryopreservation media with metformin appeared to be related with to the activation of 5’AMP-activated protein kinase (AMPK). However, recently, negative results have been described when metformin (1 and 10 mM) was used to improve boar sperm preservation at 17°C, decreasing sperm motility associated to a decrease of mitochondrial potential [95]. In an in vitro study performed in human spermatozoa kept at physiological temperature, metformin (10 mM) induced a reduction of sperm motility, where metformin the mechanism of action was associated to with PKA pathway inhibition [96]. Nevertheless, in any of these works was tested if metformin used as supplement in sperm extender media improved fertility chances. Following with the studies performed using natural compound, beetro spermatozoa were co-incubated during cryopreservation with rosemary extract (Rosmarinus officinalis) or cysteine (10 mM) or a combination of both [83]. Although both compounds enhance some sperm properties, the most noticeable effects were found by rosemary compound, enhancing total sperm motility, and progressive motility and in preventing acrosome membrane damaged after 3-three hours post-thawing in comparison to control [83]. Rosemary-treated spermatozoa yielded better cleave percentages without affecting blastocyst formation rate after performing an IVF study [83].

Melatonin (MLT) is a hormone endogenously synthesized mainly by the pineal gland and it has been detected in human seminal fluid [97] and melatonin receptors have been described in sperm of several species [98]. MLT’s antioxidant properties was tested in cryopreserved human spermatozoon [99]. MLT increased the expression of the antioxidant-related genes Nrf2 as well as its downstream genes SOD2, CAT, Hsp-1, and GSTM1, enhancing antioxidant capacity by also increasing GSH, GSH-Px, SOD and CAT activities leading to a lower ROS levels and LPO [99]. By another hand, MLT (1 µM) used during boar semen extension preservation at 17°C only showed a modest membrane protective effect [100]. By contrast, cryopreserved ram sperm supplemented with MLT achieved higher viability rates, higher percentages of total motile and progressive motile spermatozoa, and higher DNA integrity [87]. However, after IVF, only faster first embryonic division without any other embryo output difference was observed in those samples supplemented with MLT [87].

Yamaguchi, et al. [101] showed that thawed boar thawed spermatozoa supplemented with caffeine improved fertility [101]. Later, the same authors tested a combination of caffeine (1.15 mM) with the antioxidant compound β-mercaptoethanol (50 µM) but pregnancy rate was not modified (20 vs. 21% control and treatment respectively) after perform AI. However, astonishing results were found on the litter size where treated samples (10.0 ± 1.0) was almost doubled the data from control samples (5.7 ± 1.5, p < 0.07) [80]. The obtention of almost two-fold piglets by litter entails great economic advantages.

8. Antioxidants as a Therapy to Improve Reproduction Outcome in a Nutshell

Sperm produce ROS as consequence of its high aerobic metabolism. ROS production at non-physiological levels overwhelm cellular scavenger systems and results in deleterious effects, such as lipid and protein peroxidation and DNA damage. Notably, infertile men are known to possess pathological ROS levels, leading to sperm DNA fragmentation and lower ART outcome [30]. Thus, to deal with ROS overproduction and their deleterious effects at cellular levels in the male reproductive system, different strategies have been tested: (i) antioxidant oral consumption and (ii) antioxidants used as additives to the media during ART.

Literature concerning the use of compounds with antioxidant activity and the improvement of sperm function is extensive, for example by increasing sperm concentration, decreasing morphological abnormalities or improving motility [see review (102)]. Nevertheless, others have found negative results [103]-[104], questioning the real beneficial impact of antioxidant prescription and arguing that there are no clear evidences supporting that oral prescription of antioxidants is reflected on antioxidant seminal activity [105] or even that the over exposure to antioxidants can be risky leading to other pathologies as for example prostate cancer [106]. Others have found that administration of high doses of antioxidants have harmful effects on health [107,108], producing the paradoxical
To establish.

A—o establish.

ation of seminal plasma and their effect on sperm parameters and

References

Conflicts of Interest: The authors declare no conflict of interest.


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