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REVIEW AND HYPOTHESIS

Oxidative versus reductive stress: a delicate balance for sperm integrity

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ABSTRACT

Despite the long-standing notion of "oxidative stress," as the main mediator of many diseases including male infertility induced by increased reactive oxygen species (ROS), recent evidence suggests that ROS levels are also increased by "reductive stress," due to over-accumulation of reductants. Damaging mechanisms, like guanidine oxidation followed by DNA fragmentation, could be observed following reductive stress. Excessive accumulation of the reductants may arise from excess dietary supplementation over driving the one-carbon cycle and transsulfuration pathway, overproduction of NADPH through the pentose phosphate pathway (PPP), elevated levels of GSH leading to impaired mitochondrial oxidation, or as a result NADH accumulation. In addition, lower availability of oxidized reductants like NAD^+, oxidized glutathione (GSSG), and oxidized thioredoxins (Trx-S2) induce electron leakage leading to the formation of hydrogen peroxide (H_2O_2). In addition, a lower level of NAD^+ impairs poly (ADP-ribose) polymerase (PARP)-regulated DNA repair essential for proper chromatin integrity of sperm. Because of the limited studies regarding the possible involvement of reductive stress, antioxidant therapy remains a central approach in the treatment of male infertility. This review put forward the concept of reductive stress and highlights the potential role played by reductive vs oxidative stress at pre-and post-testicular levels and considering dietary supplementation.

Abbreviations: ART: Assisted reproductive technology; OS: Oxidative stress; ROS: Reactive oxygen species; 8-OHdG: 8-hydroxy-deoxyguanosine; $OH^{\bullet}$: Hydroxyl radical; $O2^{\bullet}$-: Superoxide anion; H2O2: Hydrogen peroxide; ETC: Electron transport chain; NOX4: NADPH-dependent oxidase 4; α-KGDH: α-ketoglutarate dehydrogenase; SOD: Superoxide dismutases; GPx: Glutathione peroxidases; Trx: Thioredoxins; Prx: Peroxiredoxins; GSH: Reduced glutathione; GSSG: Oxidized glutathione; NAD^+/NADH: Nicotinamide adenine dinucleotide; $NADP^+$/NADPH: phosphorylated nicotinamide adenine dinucleotide; TCA: tricarboxylic acid; GR: Glutathione reductase; Hcy: Homocysteine; PPP: Pentose phosphate pathway; OXPHOS: Oxidative phosphorylation; NAC: N-acetyl cysteine; FMN: Flavin mononucleotide; CoQ: Coenzyme Q; HIF-1α: Hypoxia-inducible factor 1α; TDP1: Tyrosyl-DNA phosphodiesterase 1; PARP: Poly (ADP-ribose) polymerase; ALC: Acetyl-L-carnitine

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Introduction

Despite the fact that DNA accounts for half of the embryonic genome, routine semen analyses such as concentration, morphology, and motility are mainly used to assess sperm quality for assisted reproductive technology (ART). However, sperm function tests and assessment of sperm DNA integrity are now being considered new, highly relevant tests to predict normal fertilization, pregnancy, and even embryo growth (Deng et al. 2019; World Health Organization 2021). Chromatin is compacted by a group of proteins known as protamines to protect sperm DNA from potentially damaging conditions during transport through the male and female reproductive tracts (Henkel and Franken 2011). However, poor protamination leaves sperm DNA at risk of oxidative stress-mediated DNA damage (Simon et al. 2017; Mohammadi et al. 2020).

Sperm DNA fragmentation is characterized by single- and double-strand breaks in the sperm genome. These breaks negatively affect the sperm's potential to fertilize the oocyte competently and are associated with reduced fertilization rates, embryo quality, pregnancy rates as well as increased mutation in offspring as a consequence of incomplete or faulty repair

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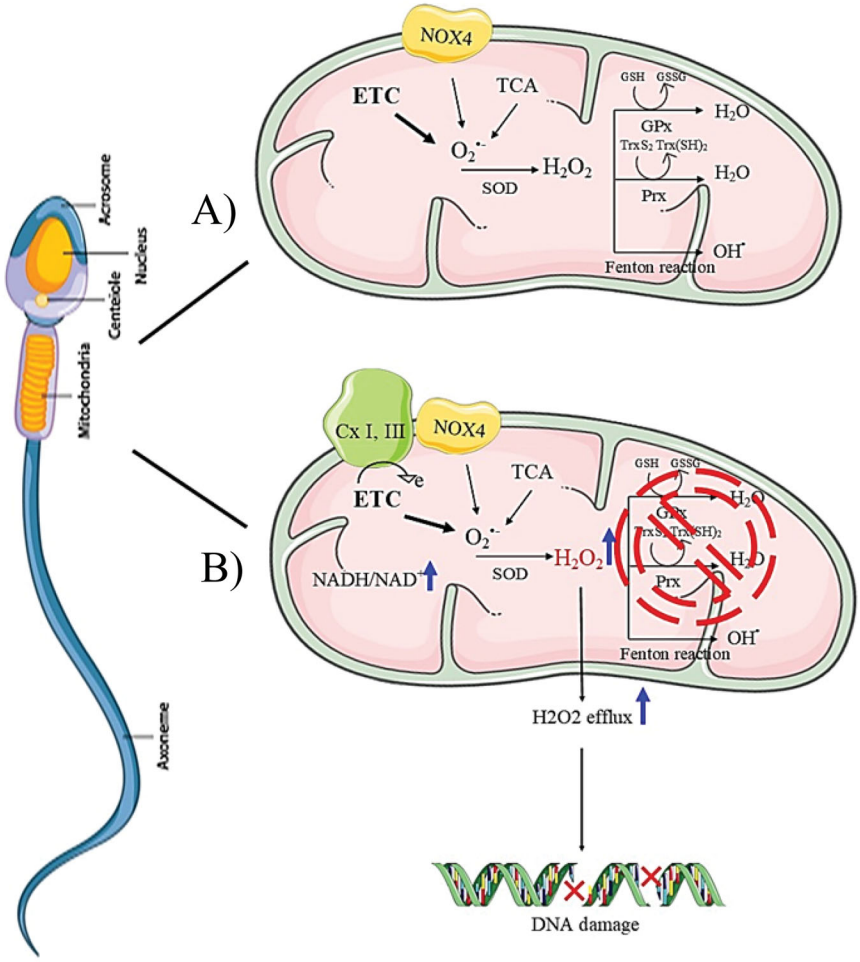

Figure 1. ROS generation in sperm mitochondria. A) $O_2^{\bullet-}$ as a primary ROS is generated from O_2 through NOX4, TCA cycle enzyme, and the electron transport chain in mitochondria. SOD enzymes catalyze the conversion of $O_2^{\bullet-}$ to H_2O_2. To protect mitochondria from H_2O_2-mediated toxicity, GPx and Prx reduce and convert H_2O_2 to H_2O using their cofactors; GSH and Trx. B) However various stress conditions can dysfunctional mitochondria protein complexes and inhibit electron transport. Under this state, incomplete oxidation of reductive respiratory equivalents like NADH and the limited supply of electron acceptors like GSSG or Trx lead to electron pressure in mitochondria and induce ROS leakage from protein complexes (especially complexes I and III). NOX4: NADPH-dependent oxidase 4; TCA: tricarboxylic acid cycle; SOD: superoxide dismutase; GPx: glutathione peroxidase; Prx: peroxiredoxins; GSH: reduced glutathione; Trx: thioredoxin.

mechanisms in mature male gametes (Casanovas et al. 2019; Aitken and Bakos 2021; Agarwal et al. 2022). It became clear that oxidative stress (OS), owing to the excessive level of spontaneous reactive oxygen species (ROS) generation, plays a significant role in the etiology of sperm DNA damage (Hosen et al. 2015). Based on the extent of oxidative stress, various types of DNA modifications occur in sperm chromatin, from oxidized residues (8-hydroxy-deoxyguanosine (8-OHdG) due to the DNA bases oxidation) to DNA strand breaks (single or double breaks at both low and high oxidative stress intensities) (Drevet and Aitken 2020; Sadeghi et al. 2021).

Hydroxyl radical ($OH^{\bullet}$) produced by different mechanisms, like the Fenton reaction (Figure 1), is one of the most potent ROS damaging essential biomolecules (proteins, membrane lipids, and DNA) in cells. Hydroxyl radicals attach to both nucleus and mitochondrial DNA bases and create an extensive array of oxidation products that negatively affect the DNA template integrity and increase the mutation rate (Valavanidis et al. 2009). 8-OHdG produced by $OH^{\bullet}$ reaction with guanine base is the most common DNA lesion in cells as they can be easily created and are highly mutagenic given the frequent faulty repair of oxidized bases (Bisht et al. 2017). Unrepaired 8-OHdG in paternal DNA may be transferred to the next generation and, may impact the embryo developmental process if within genes or regulatory regions (Drevet and Aitken 2020). A recent study reported an increase in the accumulation of germline mutations in mice across generations owing to defective 8-OHdG

excision repair and transmission to progeny, resulting in de novo mutation and mutation-mediated diseases (Ohno et al. 2014). On the other hand, apart from being shared in many conditions associated with male infertility, cytosine methylation can also be impacted and causes improper DNA methylation in embryos due to adjacent oxidized guanine base on CpG islands. Such epigenetic alteration in the embryo can contribute to altered gene expression, genomic instability, and vulnerability to disease in offspring (Franco et al. 2008; Wu and Ni 2015; Drevet and Aitken 2020; De Luca et al. 2021).

Based on the fact that oxidative stress is likely the most frequent cause of sperm DNA fragmentation in infertile patients (Lewis et al. 2013), antioxidants are considered the first line of therapy before any assisted reproduction treatment. In the past several decades, many studies have been conducted to prove the positive influences of different oral antioxidants on the improvement of fertility potential (Ross et al. 2010; Gharagozloo and Aitken 2011; Buhling et al. 2019; Smits et al. 2019; Agarwal et al. 2022). However, DNA fragmentation does not uniquely arise from DNA oxidation. Various intrinsic factors can induce sperm DNA fragmentation, including abortive apoptosis, defect in chromatin packaging, or the unrepaired transient DNA double-strand breaks during spermiogenesis (Henkel and Franken 2011; Grégoire et al. 2016, 2018; Esteves et al. 2021). Given a potential impact on the redox balance, oral antioxidants administered to patients with a high level of DNA fragmentation, not induced by oxidative stress, may have deleterious consequences as outlined below.

Cellular redox balance

Cellular redox is defined as a balance between pro-oxidants and antioxidants. As previously reported in the literature, cellular pro-oxidants comprise ROS, generated in the form of superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) from the electron transport chain (ETC), or mitochondrial enzymes such as NADPH-dependent oxidase 4 (NOX4) and α-ketoglutarate dehydrogenase (α-KGDH) (Figure 1) (Handy and Loscalzo 2012; Xiao and Loscalzo 2020). That is why cells contain a broad range of antioxidants, including enzymatic antioxidants (superoxide dismutases (SOD1-3), catalase, glutathione peroxidases (GPx1-8), thioredoxins (Trx1-2), and peroxiredoxins (Prx1-6) and non-enzymatic antioxidants (GSH, α-tocopherol, Folic acid, Bilirubin, uric acid, Carotenoids, ascorbate, etc.) to neutralize cellular oxidants (O'Flaherty 2014; Cheng and Ko 2019; Xiao and Loscalzo 2020; Rashki Ghaleno et al. 2021). However, cellular functions such as cell signaling, proliferation, and differentiation require a steady-state level of cellular pro-oxidant under physiological conditions. Hence, any impairment in pro-oxidants and antioxidants status leads to either oxidative or reductive stress known as redox stress (Pérez-Torres et al. 2017).

As outlined above, excessive ROS production and/or reduced enzymatic and non-enzymatic antioxidants result in oxidative stress and further damaging consequences on proteins, lipids, and DNA. However, the current review intends to emphasize the much less covered aspects of redox stress named "reductive stress" in which an elevated cytosolic GSH/GSSG and NAD(P)H/NAD(P)$^+$ ratio both lead to an increase in cellular reducing equivalents (specifically NADH, NADPH, and GSH). Such a situation can be as harmful as oxidative stress and known to be involved in a variety of disease conditions (Xiao and Loscalzo 2020).

ROS in the male gamete

As local oxidation is needed for some basic cellular processes, the physiological amount of ROS as a second messenger is an essential component in cells (Xiao and Loscalzo 2020). For instance, the physiological level of ROS regulates signals involved in embryo development, apoptosis activation, and disulfide bond formation and is essential for proper protein folding in the endoplasmic reticulum. Therefore, excessive ROS quenching would be expected to interfere with proper cellular function (Drevet and Aitken 2020). Considering the negative effects of decreased physiological ROS on cell functionality, excessive antioxidant intake can have the same consequences. This is supported by a previous observation where ascorbic acid was found to impair disulfide bridge formation and consequently damage protein folding (Giustarini et al. 2008). Similarly, daily intake of antioxidants combination such as vitamins C and E, β-carotene, zinc, and selenium caused elevated DNA decondensation and pregnancy failure in IVF and ICSI patients, which can be linked with the vitamin C potential for breaking disulfide bonds (Ménézo et al. 2007). It has been reported that excessive SOD supplementation in media leads to impairment in spermatozoa capacitation in vitro through inhibition of local oxidation (Lamirande et al. 1998; Cheng and Ko 2019). Moreover, recent studies provide evidence that high antioxidant levels trigger redox-sensitive transcription

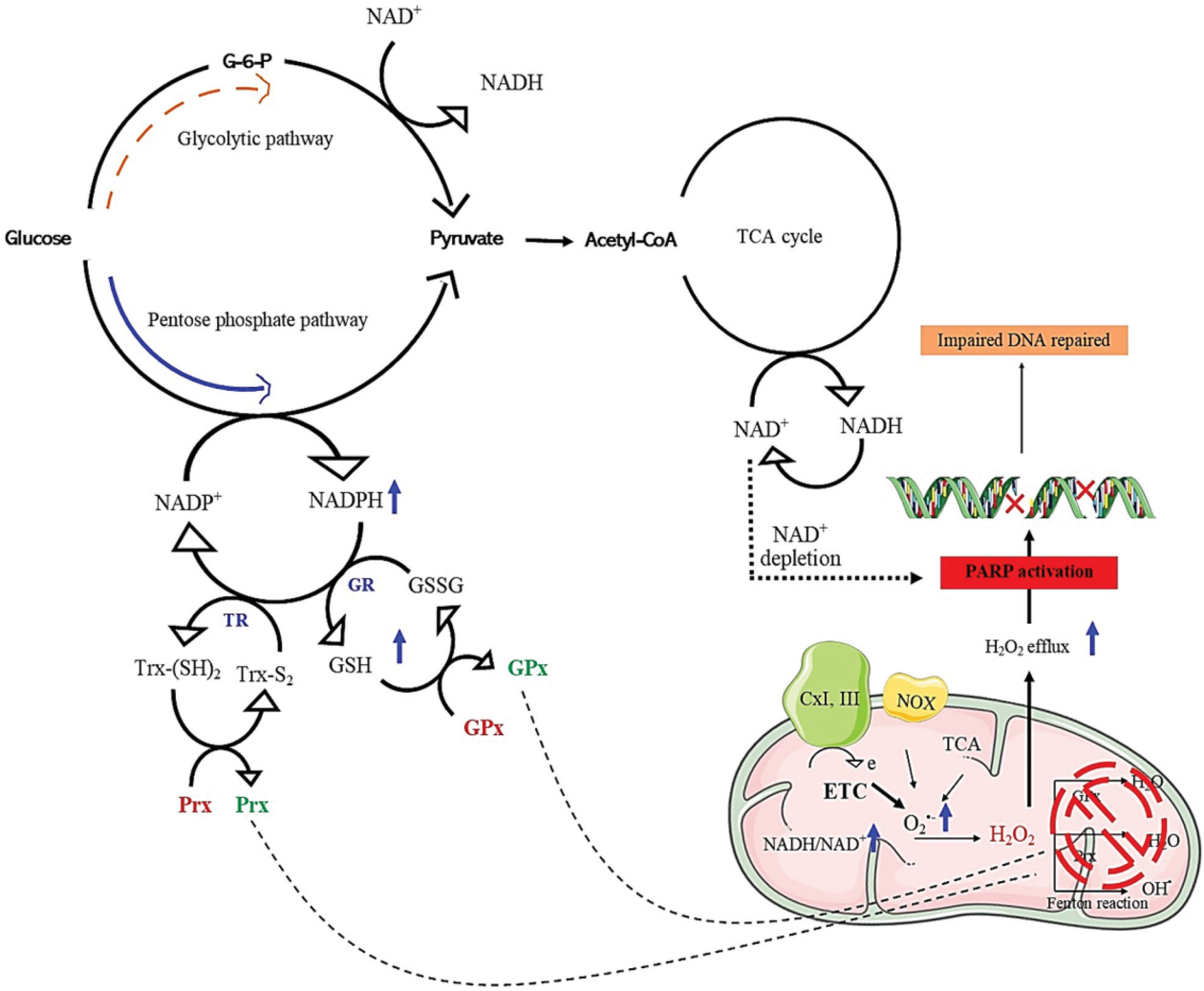

Figure 2. Reactions of cell metabolism during mitochondrial respiratory dysfunction. Under stress conditions, an elevated NADH/NAD^+ ratio causes O_2 reduction into $O_2^{\bullet-}$ and then H_2O_2. Accumulated NADH due to either intensive TCA cycle activity or defects in mitochondrial complexes leading to ROS production inactivating some key enzymes involved in glycolysis switching the energy production from glycolysis to PPP to promote NADPH production against ROS. Elevated reduced equivalents such as NAD(P)H and GSH (over PPP or transsulfuration pathway) and lack of oxidized reductants as electron acceptors in mitochondria worsen the reductive state and lead to extra H_2O_2 efflux from mitochondria. ROS generated by mitochondria causes DNA damage (single- and double-strand breaks) which can activate the PARP system to trigger DNA repair. However, the activity of PARP -DNA repair response depends on NAD as its substrate. The excessive PARP activity due to a high amount of genotoxic factors like H_2O_2 and also the decreased efficiency of glycolysis leads to frequent cellular NAD^+ depletion and altered DNA repair. NADH/NAD^+: Nicotinamide adenine dinucleotide (NAD^+)/reduced NAD^+ (NADH); $O_2^{\bullet-}$: Superoxide anion; H_2O_2: Hydrogen peroxide; TCA cycle: tricarboxylic acid cycle; PPP: pentose phosphate pathway; GSH: glutathione; ROS: Reactive oxygen species; PARP: Poly (ADP-ribose) polymerase.

factors activity and gene expression patterns leading to developmental retardation and malformations (Henkel et al. 2019; Harvey et al. 2002).

ROS generation following any cellular injury is a protective or defensive function to protect the cell against further damage, this protection would be restricted with a high dose of antioxidants (Cheng and Ko 2019). In the male reproductive system, $O_2^{\bullet-}$, $OH^{\bullet}$, and H_2O_2 are the ROS that significantly contributes to the regular function and structure of sperm cells over their transfer from the testis to the oocyte (Ford 2004). Hence, lack of ROS relative to reducing equivalents in the form of redox couples (GSH/GSSG, NAD(P)H/$NAD(P)^+$) would drive the sperm cell towards "reductive stress" as described below and negatively affects its functions (Korge et al. 2015). A surprising observation is that the increased NADH/NAD^+ ratio in reductive stress itself causes excessive ROS production in mitochondria and overflows the H_2O_2 into the cytoplasm, which can eventually create DNA lesions in human spermatozoa (Figure 1) (Murphy 2009; Liang et al. 2018) How reductive stress

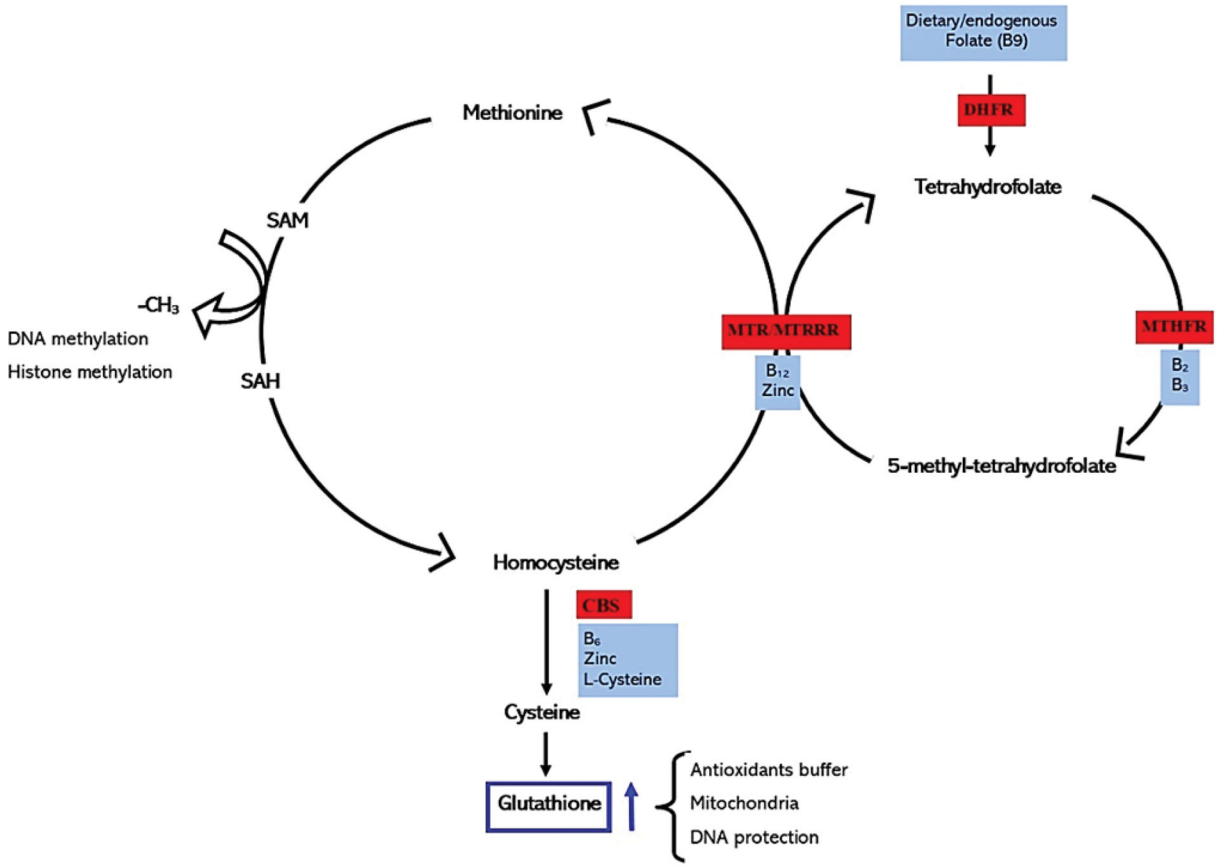

Figure 3. One-carbon metabolism. The blue box displays the components obtained from the diet. Vitamin B6, zinc, and cysteines allow homocysteine transsulfuration to GSH. High levels of cysteine and cofactors increase GSH production. GSH: glutathione.

promotes the excessive production of ROS is a subject of further investigation.

The cellular reductive stress hypothesis

As outlined above, $O_2^{\bullet-}$ is one of the most critical early types of ROS produced by the respiratory chain and/or tricarboxylic acid (TCA) cycle that is converted to the more stable H_2O_2 form in the mitochondria. H_2O_2 can in turn harm the cell function while generating $OH^{\bullet}$ through the Fenton reaction (Angelova and Abramov 2018). Some enzymatic antioxidants, including catalase, GPx, and Prx, convert H_2O_2 into H_2O. Although catalase has a low affinity for H_2O_2, GPx as the main ROS scavenger depends on reduced glutathione (GSH) in cells that can reduce H_2O_2 into H_2O. In this reaction, glutathione reductase (GR), which uses NADPH as an electron donor, catalyzes the conversion of GSSG to GSH (Xiao and Loscalzo 2020). That is the reason why the cellular redox status is closely associated with the presence of NADPH (Figure 2). Notably, GSH production itself depends on cysteine, a sulfur-containing amino acid, produced through the methionine cycle followed by the transsulfuration pathway in which high amounts of methionine cause homocysteine (Hcy) breakdown and additional GSH biosynthesis (Zhu et al. 2019). As depicted in Figure 3, the methionine cycle and transsulfuration pathway, two main parts of the one-carbon cycle, require essential substrates or cofactors, including folate (vitamin B9), other B vitamins (B2, B6, and B12), methionine, and cysteine (Clare et al. 2019). Considering that many vitamin supplements contain relatively high concentrations of such components, there is a possibility that taking a high dietary dose of such supplements drives the one-carbon cycle and subsequently the transsulfuration pathway into forming cysteine and, accordingly, excessive production of GSH (Mates et al. 2012) which can be responsible for cellular redox imbalance. As GR requires NADPH to renew GSH from the oxidized form (GSSG), increased NADPH consumption stimulates the activity of the pentose phosphate pathway (PPP) to produce more NADPH to sustain cellular reducing ability (Mullarky and Cantley 2015). Apart from the GSH-induced NADPH accumulation, various investigations have shown that increased GSH in defective mitochondria leads to impaired oxidation of NADH (Zhang et al.

2012; Ma et al. 2020) and consequently induces reductive stress. Considering the role of mitochondrial complex I, as the primary enzyme for NADH recycling and providing electrons for ATP production through oxidative phosphorylation (OXPHOS) (Yan 2014), and the contribution of mitochondrial dysfunction to ROS damage, it also is likely that any defect in mitochondrial complex I function accelerates NADH accumulation and reductive stress in the gamete potentially leading to male infertility (Agarwal et al. 2020). This can also be associated with motility defects, varicocele, and idiopathic male infertility factors (Park and Pang 2021),

Considering that GSH and NADPH accumulation contribute to NADH metabolism (Oka et al. 2012; Yan 2014; Ma et al. 2020) and the excessive accumulation of GSH and/or NAD(P)H are closely connected with reductive stress (Xiao and Loscalzo 2020), overuse of antioxidant without any prior knowledge of cellular redox state might explain inconsistencies about the beneficial influence of antioxidant supplementation and provide justification as to why detrimental effects of antioxidants were observed in some reports (Miller et al. 2005; Henkel et al. 2019).

Considering the aforementioned, an increase in GSH and NADPH appears as a protective mechanism induced by oxidative stress, which is followed by mitochondrial and cellular dysfunction in case of aberrant upregulation of GSH (Ma et al. 2020). Similar observations were made by Singh et al. (2015), where antioxidant treatment using N-acetyl cysteine (NAC) (one mM for one hour) was shown to cause reductive stress in rats myoblasts by raising the $NADH/NAD^+$ ratio, mitochondrial H_2O_2 levels, and free radical leakage altogether. In addition, it has been demonstrated that NAC, as a potent antioxidant, directly enhances intracellular cysteine levels, resulting in increased GSH (Tenório et al. 2021). But how reductive stress would lead to an increase in ROS remains paradoxical. A potential explanation is given in the following.

In mitochondria, the electron transport chain is a set of four protein complexes linked to redox processes to produce ATP through the OXPHOS process. ATP production through this system requires NADH and $FADH_2$, two main glycolysis and citric acid cycle products in mitochondria. Electrons from NADH enter the respiratory chain in which complex I and then the Flavin mononucleotide (FMN) cofactor receives the electrons and transfers them to Coenzyme Q (CoQ) (Ahmad et al. 2020). So the reduced FMN level is supposed to be adjusted by the $NADH/NAD^+$ ratio in intact mitochondria. However different factors such as GSH accumulation, mutation, hypoxia, loss of cytochrome c, or lesser need for ATP can inhibit the respiratory chain leading to increased $NADH/NAD^+$ ratio and resulting in $O_2^{\bullet -}$ formation which has a direct role in generating additional ROS such as H_2O_2 and $OH^{\bullet}$ (Murphy and Smith 2007; Ma et al. 2020). In addition to ROS production through respiratory activity, low access of two main ROS scavenging NADPH oxidases, GPx and Prx, to their oxidized reductants like GSSG and Trx induces electron leakage to generate H_2O_2 (Caroppo and Dattilo 2022). A recent study by Swain et al. (2020) on sperm samples of varicocele patients, reported that improper oxidation of reductive respiratory equivalents, like NADH, hydroquinone, ubiquinone, and respiratory cytochromes could induce electron pressure in mitochondria and result in electron leakage through complex I and complex III as a result of OXPHOS pathway dysfunction (Figure 2). So, the reason for high ROS production and oxidative stress in sperm cells of patients with varicocele can be related to chronic reductive stress followed by suppression of OXPHOS in mitochondria (Swain et al. 2020). In this regard, Swain et al. (2020) proposed that dilated pampiniform plexus and impaired draining of blood from the testis results in reduced oxygen supply to the testis and subsequently testicular hypoxia. Under hypoxia, hypoxia-inducible factor 1α (HIF-1α) signaling is activated to increase intracellular cysteine levels and GSH synthesis, which amplifies the reductive condition (Lu et al. 2015; Stegen et al. 2016; Swain et al. 2020; Xiao and Loscalzo 2020).

Generally, during reductive stress, despite the high power of antioxidants for ROS scavenging due to GSH/GSSG and $NADPH/NADP^+$ elevated ratio, excessive ROS production originating from reduced $NADH/NAD^+$ pool cannot be neutralized. The reason for this apparent saturation is the limited oxidized reductants (GSSG or Trx) required for enzymatic antioxidants like GPx and Prx which leads to ROS (H_2O_2) spillover, matrix oxidation, protein aggregation, and DNA damage. In other words, ROS production is restored in a highly reduced state to the point that it overcomes ROS scavenging potential (Figure 2) (Cortassa et al. 2014; Korge et al. 2015).

The antioxidant paradox

Twenty years ago, Halliwell first discussed the theory of the antioxidant paradox (Halliwell 2000), which stated that giving a hefty dose of antioxidants without

consideration of patient status might have harmful effects on cellular injury and disease through impairment of proper oxidative mechanisms (Cheng and Ko 2019). As outlined above, this can explain the discrepancy in the efficacy of dietary antioxidants on fertility potential improvement reported in the literature (Silver et al. 2005; Steiner et al. 2020; Sabetian et al. 2021). Although the reports on the role of reductive stress in different cells and organs are on the rise (Rajasekaran 2020) and have established the consequences of ROS deficiency or reductive stress—from disturbing insulin signaling and glucose homeostasis (McClung et al. 2004) to triggering cardiomyopathy, obesity (Rajasekaran 2020), and diabetes (Wu et al. 2016), as well as increasing mortality (Bjelakovic et al. 2007)—published papers regarding this critical point and its consequences in the male reproductive tract are still limited. A search through the Scopus database up to May 2021 on the association between both oxidative or reductive stress and male infertility separately led to a total of 3493 studies. Surprisingly only 17 of these reports discussed the role of reductive stress in male infertility. This was underscored by Symeonidis et al. (2021) in a meta-analysis study regarding the effect of beneficial and detrimental effects of antioxidants on different aspects of male fertility. They suggested that although the logical justification for antioxidant therapy is based on the reduction of oxidative stress, this does not take into consideration the delicate redox balance for homeostasis and potentially deleterious effects on sperm physiology.

Reductive stress and sperm DNA damage

The differentiation of spermatids into spermatozoa (spermiogenesis) is accompanied by the chromatin remodeling process where nucleosome-bound DNA is replaced by protamines to create the large doughnut-shaped and highly condensed chromatin structure. However, this process proceeds gradually and incorporates histone modifications, and notably, a transient endogenous surge of DNA strand breaks presumably to help eliminate DNA supercoils and facilitate histone elimination (Rathke et al. 2014; Grégoire et al. 2018). However, the results of the comet test revealed that accumulated DSBs during spermatid elongation are repaired for the most part and vanish in the following steps of the sperm differentiation process (Marcon and Boissonneault 2004; Laberge and Boissonneault 2005; Leduc et al. 2011; Ahmed et al. 2015). Various experimental models suggest that topoisomerase II is responsible for cleaving sperm DNA and double-strand breaks induction, which is needed for histone replacement by protamines over spermiogenesis (Shaman et al. 2006; Har-Vardi et al. 2007; Rathke et al. 2014). Labeling of the free 3'OH group strongly suggests that the transient double-strand breaks are induced enzymatically and can trigger a damage response as γH2AX foci are detected in the whole population of spermatids (Leduc et al. 2008; Akematsu et al. 2017). Following the DSB induction, other DNA repair components such as tyrosyl-DNA phosphodiesterase 1 (TDP1), DNA polymerase, and Poly (ADP-ribose) polymerase (PARP), essential in TOP2B-complexes removal from DNA and DNA breaks repair, have been identified in elongating spermatids (Rathke et al. 2014; Mourrain and Boissonneault 2021). PARP, a nuclear enzyme, utilizes NAD^+ as a substrate of ADP-ribose moieties to form linear or branched homopolymers in target proteins. The NAD^+ -dependent addition of pADP-ribose adducts targets proteins essential for the DNA damage repair mechanism (Patel et al. 2020). One may infer that under reductive stress induced by increased GSH/GSSG and NAD(P)H/$NAD(P)^+$ ratio, altered PARP activity may severely impact DSBs repair activity. So, considering the association of PARP activity and NAD^+ availability, one hypothesis is that antioxidants-mediated reductive stress would be expected to interfere with PARP function regulating DSBs repair mechanism. As outlined in Figures 2 and 3, receiving a high dose of the supplement required for transsulfuration pathways such as Vitamin B6, zinc, and cysteines can lead to excessive GSH production that is followed by respiratory chain dysfunction in mitochondria resulting in NADH accumulation (Mates et al. 2012; Clare et al. 2019; Ma et al. 2020). Increased reductant equivalents such as NAD(P)H and GSH and lack of oxidized reductants as electron acceptors in mitochondria deteriorate the reductive state and drive extra H_2O_2 efflux from mitochondria (Murphy 2009). Under this circumstance, ROS generated by sperm mitochondria would cause DNA breaks which activate the PARP system to trigger DNA repair. However, PARP activity may not be optimal in this reductive context as it is dependent on cellular NAD^+ content for activity. This is supported by the observation that dietary flavonoids and flavones such as quercetin, with well-known antioxidant properties, can act as PARP inhibitors in lung epithelial cells. In addition, it has been recently shown that ascorbate acts as the PARP inhibitor in an experimental model of ovarian cancer (Caroppo and Dattilo 2022).

Hence, oral antioxidant pretreatment, which is mainly considered the first-line therapy for couples undergoing assisted reproductive technologies (Joseph et al. 2020) can potentially reduce NAD^+ and negatively impact the repair of transient DNA breaks during histone-protamine replacement (Caroppo and Dattilo 2022). The risk of oocyte fertilization with damaged DNA in the form of strand breaks is therefore of logical concern and justifies further investigations.

Reductive stress and male infertility

In the 1990s, the benefits of antioxidants to treat some diseases linked to oxidative stress were put forward but conveyed with the false assumption that antioxidants are beneficial regardless of the intake dose. This misconception has changed gradually because of increasing awareness of the critical physiological function of ROS in cellular physiology. However, oral intake of the wrong composition or overdosage of antioxidants is still a typical practice (Henkel et al. 2019; Tesarik 2021). Vessey et al. (2021) conducted a study to assess the potential benefits of oral administration of two commercially- available antioxidants namely L-carnitine and acetyl-L-carnitine (ALC) in two groups of infertile men either with an elevated or normal seminal plasma level of ROS. Interestingly, they reported that sperm function improvement was exclusively observed in patients with increased seminal ROS.

Several lines of evidence indicate that antioxidant therapy can counteract the excessive ROS production and oxidative stress in varicocele-mediated male infertility (Gual-Frau et al. 2015; Gamidov et al. 2017; Abbasi et al. 2020). In this regard, a systematic review and meta-analysis were conducted in order to compare the previous research findings on antioxidant therapy in varicocele patients. The study revealed no significant differences in pregnancy rate and conflicting results regarding sperm morphology, concentration, and motility, as well as DNA fragmentation in two groups of individuals with operated or non-operated varicocele after antioxidants treatment versus no treatment. Their study demonstrates that antioxidants therapy without previous screening for oxidative stress status in varicocele patients does not improve sperm function and, consequently, pregnancy rate (Pyrgidis et al. 2021), which once again emphasizes the fact that assessment of the redox status is recommended prior to antioxidant treatment.

These observations raise concerns as to whether or not, experimental models of varicocele are truly representative of human varicocele since antioxidant therapy generally leads to improvement in sperm parameters retrieved from the epididymis in rats. This apparent discrepancy may be explained as follows.

First, the expression or activity of enzymatic reducing agents like; catalase, SOD, and GPx, may vary from testicular cells to spermatozoa retrieved from different parts of the epididymis (head, caput, and tail), which harbor different redox states during this transit (Cheng and Ko 2019, Caroppo and Dattilo 2022). In humans, the assessment of these enzymes is carried out on ejaculated sperm while in the animal model, these assessments are performed on sperm retrieved from the epididymis. A redox difference between sperm retrieved from epididymis and ejaculate may be related to the impact of the seminal plasma that is enriched in antioxidants (Cheng and Ko 2019). Also, in contrast to the transcriptionally and translationally active testicular cells (Maciel et al. 2019), sperm quiescence is expected to provide less stability to the redox potential.

These notions require further investigations to confirm if a differential redox state exists between testicular, epididymal, and ejaculate sperm (see also below paragraph).

Second, antioxidants may induce their beneficial effect in the testis in different cells especially in round spermatids when the repair capacity of the differentiating haploid cells is optimal. Therefore, reductive stress in sperm may be a secondary event to the beneficial effect of antioxidants at the testicular level.

Third, after spermiation, spermatozoa need to remain quiescent as they await the process of ejaculation to become reactivated and oxidative phosphorylation must be reduced. To achieve this goal, the oxidative state may shift toward the "reductive state" or a "paused state." Over-supplementation may facilitate such a shift toward a reductive state.

Fourth, based on the proposed evidence, excessive ROS production in mitochondria arises more as a consequence of reductive stress, not oxidative stress (Caroppo and Dattilo 2022). Therefore, if this condition is acquired through "reductive stress," supplementation of antioxidants may aggravate this process.

Fifth, an alternative notion is that reductive stress may be considered to be part of a normal sperm physiological process. Based on the literature, sperm achieves its ATP requirement through glycolysis rather than oxidative phosphorylation, which is especially needed for sperm to acquire hyperactivated motility patterns, beneficial for sperm to pass through the cumulus cell layers surrounding the mature oocyte and then penetrate to the oocyte.

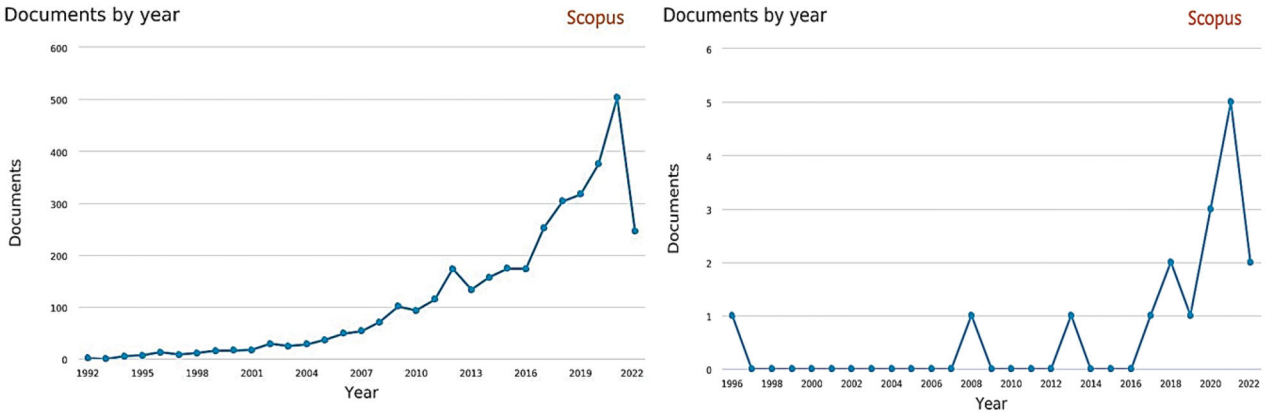

Figure 4. Published studies regarding the role of oxidative stress and male infertility compared with reductive stress. Left plot, oxidative stress in male infertility using the following keywords: ("Oxidative stress") AND ("male infertility" OR "sperm dysfunction" OR "semen quality" OR "sperm quality" OR "sperm DNA fragmentation") Right plot, reducing stress in male infertility using the following keywords: ("Reductive stress") AND ("male infertility" OR "sperm dysfunction" OR "semen quality" OR "sperm quality" OR "sperm DNA fragmentation") up to May 2022 (https://www.scopus.com).

In such cases, antioxidant therapy would rather help this normal physiological function. Under such circumstances, H_2O_2 is reduced by GPX present in semen. Lack of GPX would be expected to induce DNA damage.

Regarding this apparent paradox of "oxidative vs reductive stress" as being the mediator for the increased ROS in sperm of infertile individuals, especially those with varicocele, one more consideration should be given to the type of antioxidants prescribed. For example, vitamin E, astaxanthin, and β-carotene may prevent lipid peroxidation but may not have a direct effect on NADPH/$NADP^+$ or GSH/GSSG ratios, both being main mediators of reductive stress (Xiao and Loscalzo 2020). On the other hand, antioxidants like N-acetylcysteine and alpha-lipoic acid may boost GSH/GSSG ratio leading to reductive stress. If this is the case, why should such a treatment improve sperm quality of semen parameters and DNA integrity in individuals with male infertility? Could their beneficial effect mainly be exerted at the testicular level rather than in the epididymis or ejaculated sperm, which may better account for the improvement in sperm count? In addition, most of the complex supplements are related to dietary one-carbon supplementation, which has a paramount effect on the folic acid-methionine cycle involved in gene regulation and the trans-sulfuration pathway involved in the synthesis of GSH (Mohammadi et al. 2018; Shaygannia et al. 2018). Therefore, one-carbon supplementation may play an important role in acquiring a highly compact nucleus in late spermatogenesis, especially during histone/protamine replacement and passage through epididymis. During this passage -SH groups are converted to –S-S-, which results in GSH production, and thereby the ratio of GSH/GSSG increases eventually leading to reductive stress which may be considered a secondary event. These altered ratios may become aggravated by antioxidant therapy and result in reductive stress status.

In conclusion, this review causes one to consider emerging evidences that in addition to oxidative stress, reductive stress may also be involved in the production of an elevated level of ROS (Figure 4). Limited studies show that reductive stress may be involved in varicocele and over-supplementation of antioxidants and may aggravate ROS production. We are, however, at the early stages of understanding this new concept in male infertility. Potential mechanisms, involved in inducing reductive stress are presented but call for caution as this might be related to compartmentalization of the male reproductive tract and related to the physiological needs of sperm. Research is needed to test if the over-supplementation of antioxidants could lead to reductive stress and excess ROS production and delineate or revise clinical approaches to minimize its effect or permit remediation.

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Authors' contributions

Wrote the manuscript: NS; assisted with writing manuscript: GB, MT, MHN. All authors reviewed the manuscript, provided comments and suggestions, and finally approved the manuscript.

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