Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta Morphologica et Anthropologica, 30 (1-2)
Sofia ● 2023

Spermatozoa under Oxidative Stress: Risk or Benefit?

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Modern exploration of the etiology of many pathological processes focuses on oxidative stress as the culprit. This is especially studied in the context of reproduction namely spermatozoa and male infertility. With this review we aim to descriptively summarize the contemporary notion that reproductive inability in the male can stem, among other things, from oxidative stress and its interplay with other pathologies. Discussing the generation of free radicals, their sources and impact on the cellular morphology and physiology of sperm cells and their fertilizing capacity a detailed picture of current literature is provided. Approaches for identification and evaluation of oxidative stress is also considered in term of male reproduction and fertility.

Key words: oxidative stress, sperm, ROS, RNS, infertility

Free radicals and oxidative stress

Oxidative stress stems from an impaired balance between reactive molecules and cellular antioxidant systems. Most often oxidative damage is caused by free radicals – reactive oxygen (ROS) or nitrogen species (RNS) although other groups of radical molecules have also been studied. Free radicals can carry a positive, negative or a neutral charge depending on how they entered the reactive state – through gain or loss of an electron [56]:

$$A - 1e^- \rightarrow A^{\bullet +};$$
$$B + 1e^- \rightarrow B^{\bullet -}.$$

They are capable to react with proteins, membranes, and devastatingly with DNA [46].

Among the most extensively studied free radicals are ROS. They are a product in the course of aerobic metabolism and the flow of electrons during cellular respiration. Examples of reactive oxygen species are the superoxide anion ($O_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) (**Fig. 1**). They are generated when electrons flee

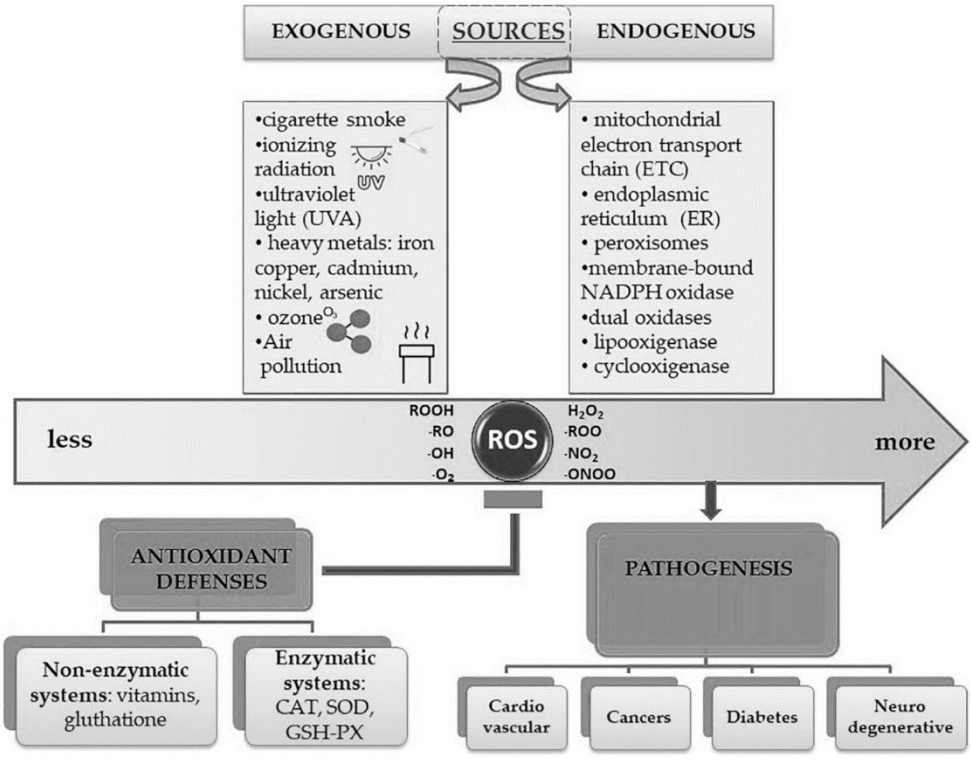

Fig. 1 Systematic comparison between the types of free radicals and their origin, followed by antioxidant defenses and pathologies [48].

the electron transport chain, under conditions of hypoxia or they can be generated by enzymes such as xanthine oxidase, NADPH oxidase. Apart from the mitochondrion they are abundant in the endoplasmic reticulum [13].

Another major group of damaging radicals are RNS. Here the reactive molecules are derived from nitrogen, and they are intrinsically connected to ROS. For instance, the reaction between the superoxide anion and nitric oxide (NO) gives one of the most typical representatives peroxynitrite ($ONOO^{\bullet}$) which can later undergo additional reactions to produce nitrogen dioxide ($NO_2^{\bullet}$) and nitric radicals. Similar to ROS, RNS can attack proteins, lipids and DNA which is described as nitrosative stress. This type of stress can be detrimental because it interferes with NO which has crucial role in the proper function of blood vessels including testicular blood flow.

Of course, these molecules are not accidentally generated in the cell – they serve a purpose. Free radicals are observed in multiple life forms such as animals, plants, aerobic bacteria, and fungi [45]. They can be utilized by the immune system where macrophages can produce them when infections are present in order to eliminate the pathogen [14]. Additionally, free radicals can exhibit anti-cancer properties and some chemotherapy drugs use this property [28]. They also have the capacity to affect the expression of certain genes and even induce epigenetic modifications [53].

When there is an overabundance of free radicals without their particular need cellular structures are in danger. Failing to address this in the long-term can cause premature aging, neurodegenerative diseases, cancer, infertility [8, 11, 40, 44]. Cells have the capacity to reduce the amount of free radicals. Part of its defense system are the enzymes catalase, superoxide dismutase (SOD), and glutathione peroxidase. These enzymes overlook the breakdown of H_2O_2, dismutation of $O_2^{\bullet}$, and protect lipids respectfully. Non-enzymatic antioxidants have been identified such as vitamin C, E, glutathione, and various exogenous molecules [38]. It can be deduced that ROS/RNS pose a threat to the cell when antioxidant defense systems are incapable of eliminating them.

Oxidative stress in sperm

Three main parts are structurally differentiated in sperm cells: the head, midpiece, and tail, each with specific features and functions. The sperm head is composed of the nucleus, acrosome, and postacrosomal region. The midpiece of spermatozoa is a unique region containing mitochondrial helix that provides energy necessary for the movement of the tail and hence for sperm motility. The sperm cell tail is a long, slender structure that pushes sperm towards the egg (**Fig. 2**) [31]. The structure and function of human spermatozoa are critical for successful fertilization and abnormalities in their structure leads to infertility.

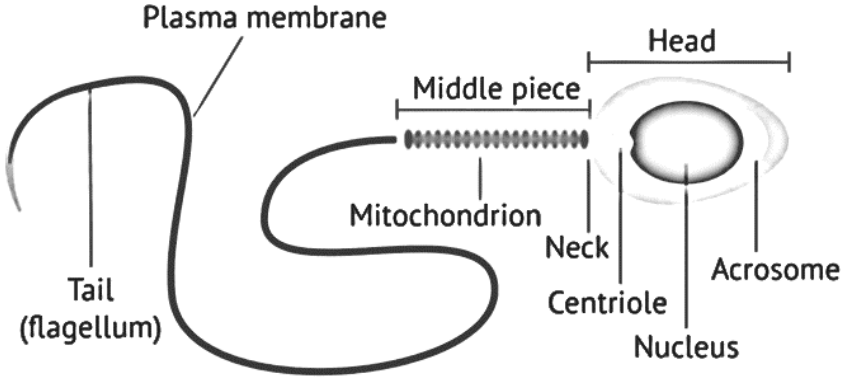

Fig. 2. Simple structure of typical human mature sperm [14].

While free radicals can be located throughout the sperm cell, they are mostly found in the midpiece region, specifically in the mitochondria which are the place where the cellular respiration cascade is located. Oxygen is utilized to produce energy (ATP – adenosine triphosphate) and inevitably this generates ROS as a byproduct, damaging the cells, in particular the mitochondria. Additionally, the midpiece of sperm cell is also where the majority of the cell's antioxidant defense systems operate, including enzymes such as SOD, catalase, and glutathione peroxidase as well as non-enzymatic antioxidants.

It is known that the volume of the mitochondria plays a key role in the availability of ATP hence the sperm performance. Measuring the oxygen consumption can provide an understanding of its metabolic state. It seems, increased oxygen consumption equals an abundance of ROS/RNS. This however, is not inherently bad as some authors reported that the most fertile ejaculates may have high amounts of free radicals [24]. According to other investigations significantly higher levels of ROS are found in the semen of infertile patients as compared to fertile men. Therefore, low and controlled (physiological levels) concentrations of ROS may play an important role in normal physiological processes in sperm such as capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion in order to ensure appropriate fertilization [4]. This may point to a beneficial aspect of free radicals as a product of intense mitochondrial activity.

Oxidative and nitrosative stress can exert their detrimental effects far beyond the mitochondria in the midpiece. Both types of stresses can react with plasma membrane which is exceptionally rich in polyunsaturated fatty acids [17]. High levels of ROS in sperm can cause loss of up to 60% of the fatty acids, hence affecting membrane fluidity, membrane-bound receptors and enzymes that are associated with abnormal fertilization [6]. Lipid peroxidation (LPO) is an autocatalytic process involving three main steps resulting in formation of end products, namely nonreactive malondialdehyde and 4-hydroxynonenal (4-HNE), which are disastrous for the genome and proteome as they can cause double-stranded DNA breaks [58]. Ultimately, the process of fertilization is affected negatively because a damaged membrane is incapable of proper sperm-oocyte fusion [27]. Parallel to impaired fusion, the motility of sperm can also be affected which makes passage through the cervical mucus impossible. One suggested mechanism is that ROS/RNS can inhibit the proper phosphorylation of axonemal proteins and render sperm immobile. This process can be reversed by treating ejaculate with antioxidants [7].

Despite its tight packing and relative resistance to oxidative stress DNA in spermatozoa is still considered a susceptible target. During the process of spermiogenesis, chromatin undergoes a series of modifications where histones are replaced with transitional proteins and finally with protamines. They condense DNA to form the principal unit of sperm chromatin called toroid which undergo further tightening by disulfide cross-linking to make DNA extraordinarily resistant to oxidative stress [61]. However, if the compactization is not optimal, the ROS can attack and cause base-free sites, deletions, frame-shift mutations, DNA cross-links, and chromosomal rearrangements. Damaged DNA was found in human testicular, epididymal and ejaculated spermatozoa [34]. Single or double stranded DNA breaks can be visualized by TUNEL and Comet Assay and they mark differences in reproductive capacity between fertile and infertile men [61]. Reparation of double stranded breaks is notoriously hard in the Y chromosome and this responsibility falls upon the oocyte [16]. Prolonged damage to DNA could result in meiotic sex chromosome inactivation (MSCI) where transcription is notably suppressed [37]. Y-bearing spermatogonia can be a target of mutations in the euchromatic Y region (Yq11), known as the azoospermia factor, resulting in complete absence of spermatozoa and infertility [50]. In case of unsubstantial DNA damage, spermatozoa are capable for self-repair mechanism and eventually maintain their fertilizing ability. However, oocytes are also capable of repairing damaged sperm [7]. DNA damage contributes to sperm apoptosis, poor fertilization rate, and high frequency of miscarriage and offspring morbidity. In

humans, 80% of DNA damage is of paternal origin and it is suggested to be a result of unsuccessful apoptosis [4].

Apoptosis is crucial for having healthy sperm and it is the quality control regulator in spermatogenesis, in particular germ cell to Sertoli cell ratio. Two main mechanisms are known to be responsible for initiation of germ cell apoptosis – Fas membrane mechanism of Fas ligand (FasL) and Fas receptor (FasR) and mitochondrial mechanism of Bcl-2 family. Similar to developing germ cells, spermatozoa can undergo apoptosis and they express apoptotic markers such Fas, Bcl-xl and p53. Sertoli cells express FasL, which after binding FasR on the germ cell membrane initiates apoptosis [25, 42] and that mechanism is probably involved in Sertoli cell control of germ cell/spermatozoa number. In transgenic models the overexpression of Bcl-2 or Bcl-xl leads to an abundance of spermatogonia and infertility and knockout of Bcl-xl causes a lack of spermatogonia [36, 43]. Knockout of p53 results in increased number of defective sperm due to suppression of germ cell apoptosis. A balance between pro and anti-apoptotic events is crucial for maintenance of adequate number of spermatozoa. The presence of ROS/RNS in semen due to the various stressor factors, cause mitochondrial damage and DNA fragmentation of sperm. Injured mitochondria leak cytochrome C, caspase 3, and 9 in seminal plasma that along with the broken DNA results in apoptosis [52, 57].

Sources of oxidative stress in sperm

Reactive oxygen species originate from various endogenous and exogenous sources. Endogenous free radicals generated by enzymes are just as harmful as exogenous ones. However, if an individual maintains a healthy lifestyle their generation and neutralization should be at equilibrium. Environmental stressors pose a much larger threat due to their potential to induce oxidative and nitrosative stress. In the course of modern daily life, the presence of an exogenous stressor is more frequent than others and alcohol is the most notable. According to the World Health Organization (WHO) 15+ year old Europeans consume at least 10 liters of pure alcohol per year and alcohol consumption has been increasing [62]. Ethanol metabolism has detrimental effect on whole organism and particularly on the male reproductive system. Excessive alcohol consumption is associated with reduced percentage of normal sperm in men with asthenozoospermia. Acetaldehyde, a product of ethanol metabolism interferes negatively with proteins, lipids and DNA generating oxidative and genotoxic stress that results in double stranded DNA breaks [4, 22].

Another distinct and documented inducer of free radicals in seminal fluid is cigarette smoke containing more than 4000 chemical compounds for some of which have shown to cause an imbalance between antioxidant system and ROS in semen of smokers. Decreased levels of antioxidant such as vitamin E and C was found in seminal plasma in smokers. Apart from being a complete carcinogen cigarette smoke worsens almost every aspect of semen such as DNA integrity, sperm motility, sperm count associated with increased seminal leukocyte concentration and levels of ROS [39].

Chemicals can become into contact with an organism both industrially and domestically. Exposure to well known toxicants such as heavy metal ions – mercury, cadmium, lead, and manganese is a serious threat leading to reduced sperm quality,

sperm volume and count associated with increased presence of reactive species [29]. Other exogenous factors such as industrial endocrine disrupting chemicals, can also contribute to an increased level of reactive species, in particular generation of ROS coupled with reduction of glutathione and induction of germ cell apoptosis [33]. Endocrine disruptors are widely spread throughout the environment and there are multiple ways for human exposure and penetration into the body. They are present in everyday items such as plastic containers, self-care products, water, fabrics, and food through pesticides, etc. In particular, phthalates were shown to affect spermatogenesis and induce DNA damage. It should be taken into account that concomitant sources of ROS are possible such as smoking, alcohol consumption and intake of multiple disruptors [54, 55].

Radiation, ionizing and non-ionizing, represents an emerging threat to male fertility and oxidative stress generation appears to be one of its damaging mechanism of action. Humans are not usually exposed to high levels of ionizing radiation (IR) in their everyday life and such incidences are often occupational or in a clinical setting. IR includes gamma rays and X-rays and it attacks primarily DNA which leads to two types of damage [12]. Direct action on the structure of DNA is resulting in damage of the sugar backbone or nitrogenous bases or double-stranded DNA breakage [59]. Indirectly, extortionately high levels of ROS, mainly hydroxyl radical are generated in the cytoplasm by water radiolysis resulting in impaired lysosomes and membrane damage [18]. Even at low doses, IR can cause reduced sperm count and sperm motility [60]. Mitotic dividing germ cells (spermatogonia) are the most vulnerable germ cell type, whereas primary spermatocytes during meiosis are somewhat protected as in that time frame proteins facilitating DNA repair are present and highly active [15].

Non-ionizing radiation (NIR) is equivalently studied for its negative effect on spermatozoa. Modern life introduces microwaves like radio-frequency electromagnetic radiation (RF-EMF) by laptops, cell phones, microwave ovens, security scanners at levels hardly found in nature. Free radicals appear to be the culprit behind the damaging effect of nonionizing radiation [26]. In an organism NIR can exercise a thermal effect where its energy is converted to heat or a non-thermal effect where electric and magnetic fields can alter skin and cell membrane permeability. *In vitro* exposure of human spermatozoa to electromagnetic radiation can worsen sperm motility, viability and sperm concentration depending on the duration of exposure and these changes are associated with elevated ROS production in the mitochondria and DNA fragmentation [32]. Additionally, kept in proximity to Wi-Fi connected laptops men can exhibit lower sperm count [35]. The thermal effect can raise the temperature in the scrotum and testes causing raised levels of ROS and induce germ cell apoptosis [49].

ROS found in seminal plasma originates from exogenous sources (mentioned above) as well as various endogenous ones. Leukocytes - mainly neutrophils as well as macrophages and defective spermatozoa are considered the main endogenous sources of ROS [4]. Abnormal sperm morphology manifested by excessive residual cytoplasm around midpiece is associated with high amount of ROS affecting sperm motility. It is well known that sperm, while in the testes are protected from immune system via the blood-testis barrier. Once they enter epididymis and move along the duct, the sperm are protected by antioxidant enzymes secreted by the epididymal epithelium into the lumen. Once ejaculation occurs, while located in the urethra sperm might come into contact with activated phagocytic leukocytes producing free radicals as

a result of an infection. Inflammatory process affecting prostate or seminal vesicles such as prostatitis can trigger peroxidase-positive leucocytes and they can produce exorbitant level of ROS. This condition is described as leukocytospermia and often requires pharmacotherapy [23]. In *in vitro* fertilization (IVF) procedures application of antioxidants in washing suspension is important to maintain sperm functional in case of leukocyte contamination [21].

As a result of such inflammation an increase in proinflammatory cytokines, such as interleukin (IL)-8 occurs in tandem with a decrease in the enzymatic antioxidant SOD that leads to production of high levels of ROS. Correlation between impaired sperm function and seminal plasma with elevated levels of ROS, TNF-α (Tumor Necrosis Factor), IL-6 and IL-8 was found to result in an increased LPO of sperm membrane [3].

Increase in free radicals is involved in varicocele - one of the major factors contributing to male infertility. This pathology is characterized by abnormally high venous dilation in the testes and around the spermatic cord responsible for blood pooling and hence local heat excess causing oxidative stress on sperm. The level of ROS is positively correlated with the grade of varicocele. In addition, in men with varicocele positive relationship between ROS and germ cell apoptosis was revealed and both are negatively correlated with sperm concentration [49].

Evaluation and preventions of oxidative stress

To evaluate oxidative stress in the male reproductive system first it needs to be identified and then quantified. Reactive oxygen species was suggested to account for 30% – 80% of pathology in infertile men [2]. The main tool for diagnosis of different cases of male infertility is routine semen analysis based on sperm count/concentration, sperm motility, viability and morphology. Numerous studies point toward a negative correlation between ROS and semen parameters although there is still lack of evidence of an interdependence between increased ROS levels and pregnancy outcomes [2, 9, 10, 19]. A reduction in semen parameters is more frequently found in men with oxidative stress (OS) and asthenozoospermia is suggested as a surrogate marker for OS. Another marker is hyperviscosity of seminal plasma associated with increased levels of MDA and impaired antioxidant status. Urobacteria infections that affect prostate and seminal vesicles can also contribute to increased seminal plasma viscosity and an increase in ROS production. The presence of a large number of round cells imply possible oxidative stress caused by leukocytospermia or immature spermatozoa. To distinguish leukocytes from germ cells a peroxidase test is required, CD45 (leukocyte common antigen) immunostaining or measurement of seminal elastase. Visualization of excessive residual cytoplasm in abnormal sperm is indicative for high levels of ROS.

A significant number of methodologies to measure the ROS levels in semen can be applied however few of them are clinically relevant taking into consideration cost and patient convenience. Direct assay of oxidative stress is applied to assess the amount of oxidation in sperm membrane by measurement of MDA via the thiobarbituric acid assay. This is one of the oldest and most widely used method demonstrating that MDA is associated with decreased sperm motility and sperm-oocyte fusion. For indirect measurement of OS a chemiluminescence method is applied by incubating semen plasma with luminol in luminometer [1]. The number of free radicals produced

is measured as relative light units/s/10^6 sperm. This method allows measurement of both intracellular and extracellular ROS. Based on chemiluminescence, attempts have been made to determine a reference range of ROS in human sperm in the context of different pathologies. They present a quick and easy approach that can be incorporated in standard laboratory practice [30].

Apart from being cost consuming, luminol can also be used for indirect measurement of the total antioxidant capacity of the seminal plasma and for analyzing the balance between ROS and the antioxidant protection of sperm. An assay that has gained popularity due to its cost-effectiveness is nitroblue tetrazolium (NBT) that measures intracellular ROS concentration. This is a light microscopy method that provides information on the source(s) of ROS and accurately predicts whether ROS have been produced by spermatozoa or leukocytes. Therefore, application of relevant tests for measurement of ROS has clinical importance as they can help to identify subgroups of infertile patients suffering from oxidative stress that may be treated with antioxidant supplementation.

Having in mind innate OS prevention mechanisms, it should be expected that spermatozoa can be protected from OS by the endogenous antioxidants present in the seminal plasma including the enzymes catalase and SOD as well as non-enzymatic compounds – vitamins C and E and carotenoids. Spermatozoa also contain the antioxidants lactoferrin and coenzyme Q10 [5]. Another mechanism to prevent OS involved prostasomes that are extracellular vesicles secreted by the prostate. Fusion of prostasomes with the sperm plasma membrane is required for regulation of different aspects of sperm function, such as motility and capacitation. The presence of prostasomes in the seminal plasma results in a decreased ability of neutrophils to produce ROS.

Should one decide to take action against free radicals in the body, effort should obviously be directed to lifestyle changes such as reducing alcohol consumption, cessation of smoking, balanced diet, exercise, and reducing exogenous sources of oxidative stress in general. Advances in pharmacology, nutraceuticals, and aging research have pinpointed numerous molecules that work to quench free radicals. For example, supplementation with antioxidants can be considered as a precaution from oxidative stress. Based on their mechanism of action they can be divided into two types: (1) preventive antioxidants which prevent the formation of ROS – metal chelators or binding proteins (lactoferrin and transferrin); (2) scavenging antioxidants which remove ROS that is already present - vitamins C and E [51]. Another example is application of approved drug metformin [20], quercetin, lycopene, various flavonoids and many others [41]. Within IVF techniques protection of semen is considered within the window between taking a sample and fertilization. During the stage of liquid cooled storage of sperm in the range 4°C – 25°C and the subsequent freezing both enzymatic and non-enzymatic antioxidants can be added such as melatonin, vitamin E, catalase [51].

Conclusion

Based on the depth of current scientific biomedical research in terms of oxidative stress in sperm cells it's reasonable to consider that oxidative stress causes harm rather than benefit. Despite beneficial biological function in both germ and somatic cells, the free

radicals remain a damaging stressor. The prognosis however, is positive since effort to prevent oxidative damage and its concomitant conditions consolidates finance and intellectual work in this direction. Knowing that education and prevention are cheaper and easier than diagnosis and treatment, a campaign to acknowledge the problem is an approach that can be implemented while waiting for the pharmaceutical industry to solve the problem. In cases where oxidative stress is a byproduct of exposure to environmental pollutants, their source should be limited. In addition, changes in lifestyle and use of balanced diet is another measure to manage oxidative stress. Despite the well documented sources of ROS and their effects maintenance of a balance between reactive species and antioxidant systems should be the goal in the strategy to protect sperm function and male fertility.

***Acknowledgements*:** This work was supported by the Bulgarian Ministry of Education and Science under the National Research Programme "Young scientists and postdoctoral students-2" approved by DCM 206/07.04.2022.

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