

## CAUSES, MECHANISMS, CONSEQUENCES AND WAYS TO CORRECT SPERM OXIDATIVE STRESS IN THE ANDROLOGY LABORATORY SETTING

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## UZROCI, MEHANIZMI, POSLEDICE I NAČINI ZA KORIGOVANJE OKSIDATIVNOG STRESA SPERMATOZOIDA U USLOVIMA ANDROLOŠKE LABORATORIJE

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### ABSTRACT

Male infertility, responsible for approximately 50% of infertility cases in couples, is a growing concern, with oxidative stress (OS) playing a key role in its etiology. OS in spermatozoa, characterized by an imbalance between reactive oxygen species (ROS) and antioxidants, leads to significant damage, including Deoxyribonucleic acid (DNA) fragmentation, reduced motility, and increased morphological defects. This review explores the causes, mechanisms, and consequences of sperm OS and identifies ways to address it within the andrology laboratory. Detecting and mitigating ROS levels is crucial for improving sperm quality, especially in assisted reproductive technology (ART) settings. It discusses endogenous and exogenous factors contributing to OS, such as systemic diseases, lifestyle choices, and cryopreservation processes. Notably, elevated ROS levels correlate with conditions like asthenozoospermia and teratozoospermia. Recent advancements in proteomics offer promising insights into the biomarkers of OS, which can aid in better diagnostic and therapeutic strategies. This review synthesizes current research on the causes, mechanisms, and consequences of sperm OS, highlighting diagnostic methods such as chemiluminescence assays and MiOXSYS (Male Infertility Oxidative System) for measuring ROS. Furthermore, methods for assessing OS, including flow cytometry, and redox potential systems, are examined. The review highlights the potential of antioxidant supplementation in mitigating sperm oxidative damage, particularly during cryopreservation. Various natural extracts, including those from sea cucumbers, cactus, and green tea, have shown promising effects in reducing ROS production and DNA fragmentation, thereby improving sperm quality. These findings underscore the importance of OS management in enhancing male fertility and improving ART outcomes.

**Key words:** spermatozoa; infertility, male; reproductive techniques, assisted; antioxidants.

### INTRODUCTION

In 50% of infertile couples, male factor is the main cause of infertility (1). The overall incidence of male infertility is about 15% of the general population (2). It is evident that there is an annual decline in the concentration of spermatozoa in the ejaculate, a phenomenon that is

### SAŽETAK

Muški infertilitet, odgovoran za približno 50% slučajeva neplodnosti kod parova, predstavlja sve veću brigu, pri čemu oksidativni stres (OS) ima ključnu ulogu u njegovoj etiologiji. OS u spermatozoidima, koji karakteriše disbalans između reaktivnih vrsta kiseonika (ROS) i antioksidanata, dovodi do značajnih oštećenja, uključujući fragmentaciju dezoksiribonukleinske kiseline (DNK), smanjenu pokretljivost i povećane morfološke defekte. U radu se istražuju uzroci, mehanizmi i posledice OS spermatozoida i identifikuju načini za njegovo rešavanje u androloškoj laboratoriji; detekcija i ublažavanje nivoa ROS-a ključni su za poboljšanje kvaliteta sperme, posebno u uslovima asistiranе reproduktivne tehnologije (ART). Razmatraju se endogeni i egzogeni faktori koji doprinose OS-u, kao što su sistemske bolesti, životni stil i procesi krioprezervacije. Značajno je da su povišeni nivoi ROS-a u korelaciji sa stanjima kao što su astenozoospermija i teratozoospermija. Nedavni napredak postignut u proteomici nudi obećavajuće uvide u biomarkere OS-a, što može pomoći u osmišljavanju boljih dijagnostičkih i terapijskih strategija. U radu se sintetizuju trenutna istraživanja o uzrocima, mehanizmima i posledicama OS-a spermatozoida i naglašavaju dijagnostičke metode kao što su hemiluminescentni testovi i MiOXSYS (sistem za oksidativni stres kod muške neplodnosti) za merenje ROS-a. Osim toga, ispitane su metode za procenu OS-a, uključujući protočnu citometriju i sisteme redoks potencijala. Naglašava se potencijal suplementacije antioksidantima u ublažavanju oksidativnog oštećenja sperme, posebno tokom krioprezervacije. Razni prirodni ekstrakti, uključujući one iz morskih krastavaca, kaktusa i zelenog čaja, pokazali su obećavajuće rezultate u smanjenju proizvodnje ROS-a i fragmentacije DNK, čime se poboljšava kvalitet sperme. Ovi nalazi naglašavaju važnost upravljanja OS-om za poboljšanje muške plodnosti i unapređenje ishoda ART-a.

**Ključne reči:** spermatozoidi; neplodnost, muška; asistiranе reproduktivne tehnologije; antioksidansi.

concomitant with the proportion of couples seeking Assisted Reproductive Technology (ART). This underscores the imperative to enhance the precision of male fertility assessment and to identify the underlying causes of infertility. Mammalian sperm are highly vulnerable to OS, which reduces their function, survival, and motility, and increases the number of abnormal sperm

(3). One potential cause of Deoxyribonucleic acid (DNA) fragmentation is OS (4). It has been observed that infertile men, who have reduced chances of conception naturally, frequently exhibit elevated levels of sperm DNA fragmentation (5). Currently, there is an extremely active search for techniques that can identify the causes of OS in the andrology laboratory. In this way, these techniques can increase the fertilizing capacity of the sperm.

The aim of this review is to summarize the current research findings investigating the effects of sperm OS on sperm competence and how to correct OS at the laboratory stage.

## MATERIALS AND METHODS

A comprehensive literature search was conducted utilising the following search engines: PubMed, eLIBRARY, Web of Science, and Google Scholar. The search was conducted for the keywords specified above, from 2014 to 2024. For the purposes of this descriptive review, publications were selected on the basis of their providing evidence-based experimental and clinical data on the effects of OS on spermatozoa. The search was conducted from April to December 2024 using combinations of relevant keywords.

### OXIDATIVE STRESS AND REACTIVE OXYGEN SPECIES

In 30-40% of infertile men, there is an increased content of ROS in seminal plasma. ROS – defined as free radicals or pro-oxidants - are molecules in which the oxygen atom carries an unpaired electron in the outer orbit. These molecules possess a high degree of reactivity, which can result in damage to proteins, nucleic acids, and lipids found in biological cell membranes. The primary source of ROS is enzymatic redox reactions; however, such molecules can also be spontaneously formed as a consequence of electron leakage. It is noteworthy that ROS in spermatozoa is implicated in physiological mechanisms, including binding to the oocyte. It has been demonstrated that low levels of membrane lipid peroxidation enhance the ability of spermatozoa to interact with the zona pellucida (6). Consequently, minimal amounts of ROS are required for optimal sperm metabolism; however, elevated levels have been observed to be deleterious to spermatogenesis (7). For protection against free radicals in the body there is an antioxidant system consisting of a set of mechanisms realized at the level of cells, tissues, organs and systems and aimed at preserving and maintaining homeostasis in the body. The equilibrium between these two systems in a state of physiological stability keeps peroxidation at a certain low level, prevents the development of the oxidative process and characterizes the antioxidant status of the organism

(3). An imbalance in these systems can be caused by an increase in the production of ROS, which in turn can lead to a state of OS (8).

### *Causes of origin of oxidative stress*

A wide range of lifestyle factors, such as excessive alcohol consumption, smoking, radiation, obesity, as well as infections and systemic diseases, can increase OS levels in the body and lead to infertility (9). Let us review the most significant causes of OS of endogenous and exogenous origin (Figure 1).

### *Endogenous causes*

#### *Systemic diseases*

Leukocytes, particularly neutrophils and macrophages, and dysfunctional spermatozoa are the major endogenous sources of ROS production in human semen. Infections and inflammation cause a massive (100-fold) increase in ROS release from leukocytes, significantly increasing OS (7). Infections of the genitourinary system and inflammation of the male genital tract can also increase the production of ROS through leukocyte activation, contributing to the development of sperm DNA fragmentation. High levels of ROS and resulting oxidative damage to sperm DNA and membranes have been linked in some studies to increased seminal leukocytes and the pro-inflammatory cytokines IL-8 and TNF- $\alpha$  (10). In several studies, leukocytospermia has been found in male patients with infertility (11).

A varicocele is a varicose vein in the scrotum. Men with varicocele-related infertility show higher levels of ROS, such as nitric oxide and lipid peroxidation byproducts, in their semen (12). Varicocele is considered a chronic condition characterised by heat stress in the genitalia. In conditions of hypoxia, the electron transport chains of the mitochondria are blocked, resulting in the generation of excess ROS and an increase in the formation of HIF-1 $\alpha$ . HIF-1 $\alpha$  is a protein that plays a regulatory role in cellular adaptation to hypoxia. Consequently, the convergence of heat exposure, local inflammation, hypoxia and venous stasis, all of which are associated with varicocele, has been demonstrated to result in increased production of ROS in target tissues (13).

### *Incomplete spermatogenesis*

During spermatogenesis, the process of spermatozoa formation, it has been observed that spermatozoa with excess cytoplasmic residues may be released. Such spermatozoa are termed immature spermatozoa. The excess cytoplasm contains glucose-6-phosphate dehydrogenase, which controls the production of beta

nicotinamide adenine dinucleotide phosphate (NADPH). This, in turn, is oxidised by NADPH oxidase located on the sperm membrane, resulting in the formation of ROS (14, 15). Thus, the membrane and mitochondria of immature spermatozoa are a source of ROS in sperm (16).

### ***Tumor***

When comparing the DNA fragmentation level in the spermatozoa of patients with cancer before chemotherapy treatment, with the DNA fragmentation level of healthy fertile men in the study by Smith et al. (17) indicated that the presence of cancer other than non-Hodgkin's lymphoma has no effect on sperm DNA fragmentation levels. Another team of scientists (18) reported that patients with testicular cancer have significantly higher DNA fragmentation levels than healthy fertile men.

### ***Exogenous causes***

#### ***Smoking***

Tobacco smoke contains a multitude of ROS and nitrogen, which have been demonstrated to induce OS. It is important to note that there are two variants of the oxidative response to smoking: "direct" and "indirect" ROS. "Direct" oxidative damage is attributed to the presence of ROS in the gas and tar phases of cigarette smoke. In the gas phase, ROS are formed when tobacco is burned and are inhaled with the smoke stream. The resin phase contains the polymeric complex quinone/hydroquinone, which has redox properties and, in the smoker's lungs, reduces molecular oxygen, subsequently forming free radicals - H<sub>2</sub>O<sub>2</sub> and -OH. "Indirect" ROS are formed by other toxins, including phenolic compounds, quinones and heavy metals. Hydroquinone and catechol, entering into redox reactions, form O<sub>2</sub><sup>-</sup>. Not only does the liquid in e-cigarettes contain nicotine, but the toxic aldehydes and ketones that are formed when this liquid is heated cause OS. Acrolein and formaldehyde react with proteins and DNA, causing endoplasmic reticulum stress, mitochondrial damage, and OS (19). Tobacco smokers were found to have decreased semen volume, sperm concentration, decreased sperm viability and motility, and impaired sperm morphology (20).

#### ***Alcohol***

Alcohol consumption increases the white blood cell count in semen (21). Chronic alcohol consumption compromises the integrity of the blood-testicular barrier. This leads to the penetration of immune cells into the seminal tubules and results in leukocytospermia (22). Steatosis in liver results in the increased peroxisomal activity which contributes to the OS formation (23). In addition to a direct increase in ROS production, in vitro

studies have shown that acetaldehyde causes intracellular redox imbalance by increasing stress levels and decreasing the concentration of the antioxidant glutathione (24). Acetaldehyde also reduces the enzymatic activity of superoxide dismutase, which is a potent endogenous antioxidant (25).

### ***Functional analogues of estrogens***

Changes in the functioning of mitochondria are associated with the leakage of electrons and, consequently, the disruption of their transfer to cytochrome oxidase. The etiology of this pathological condition of mitochondria is multifactorial. One such example is the use of artificially produced functional analogues of estrogens, including gossypol and parabens, which are found in food products such as sauces and cosmetic products including shampoos, hair conditioners, and shaving gels. Estrogens and similar phenolic compounds are believed to boost mitochondrial redox activity, causing electron leakage and ROS production within the mitochondria, thereby significantly harming sperm function. Using bisphenol A, parabens and estradiol as examples, estrogen-like compounds have been shown to reduce testosterone levels and cause OS in the testes (26–28).

### ***Cryopreservation of sperm samples***

Cryopreservation is the most effective method of long-term sperm preservation. However, it has been demonstrated that the freezing-thawing process results in a significant decline in sperm fertility, with a reported decrease of 34% (29). The increase in their level is the basis for gamete damage under in vitro conditions. Cryoprotectants, such as dimethylsulfoxide, are a source of ROS. Dimethylsulfoxide stimulates the release of excessive amounts of calcium from the endoplasmic reticulum into the cytoplasm. Mitochondria then absorb this calcium, which leads to the formation of activated oxygen species in mitochondria. ROS levels are particularly increased by freezing if the semen sample is contaminated with leucocytes. Removal of leucocytes from semen samples or treatment with antioxidants before cryopreservation can improve sperm viability and function (30).

### ***Effects of oxidative stress***

#### ***Changes in the cytophysiological properties of spermatozoa***

Damage to sperm cell structure and function can result from the oxidation of polyunsaturated fatty acids in sperm membranes (31). Malondialdehyde reacts with the N-terminal amino acids of proteins and the amino groups of

phospholipids. As a result, cross-links form within molecules, leading to a decrease in protein enzymatic activity and deformation of structural and channel-forming proteins. Consequently, membrane permeability increases, resulting in disruptions to the cell's morphofunctionality (32). The disruption of ion pumps and channels, causing impaired ion transport, results in decreased sperm motility (33). Reduced levels of the ion proteins VDAC2 and ATP1A4 were found in the sperm of men with asthenozoospermia (low sperm motility) (34). Lipid peroxidation produces 4-hydroxynonenal (4HNE). 4HNE binds to flagellar proteins of the spermatozoon, disrupting the motility apparatus. 4HNE also binds to mitochondrial proteins responsible for exchanging adenosine diphosphate (ADP) and adenosine triphosphate (ATP), disrupting this process. It also interacts with the electron transport chain, potentially inducing mitochondrial electron leakage, which disrupts the mitochondrial membrane potential in spermatozoa (35). Thus, as a result of lipid peroxidation induced by ROS, bioenergetic processes are disrupted, leading to impaired sperm functionality (36). Membrane damage and functional impairment of spermatozoa directly affect fertilization success and the outcome of ART programs (37).

### ***Increased DNA fragmentation***

DNA fragmentation refers to damage to the DNA molecule, which disrupts fertilization processes and normal embryo development. DNA fragmentation can lead to a reduced ability of a couple to conceive naturally, a lower pregnancy and birth rate following intrauterine insemination, decreased fertilization and implantation rates, reduced pregnancy success, poorer embryo quality after in vitro fertilization, an increased risk of miscarriage, and a lower number of live births. Both single-strand and double-strand breaks characterize DNA fragmentation. In the case of single-strand breaks, the complementary strand can serve as a template for replication. Single-strand breaks occur physiologically under the influence of abortive topoisomerase activity. Most breaks caused by topoisomerase are ligated (covalently bound to phosphate) by DNA ligase and do not pose a significant threat to genome stability (38). The most common types of damage include base and sugar modifications, as well as single-strand breaks resulting from oxidation, alkylation, deamination, and spontaneous hydrolysis (39). OS, lipid peroxidation, and protein modifications can also contribute to the formation of single-strand DNA breaks. Oxidation of paternal DNA bases, particularly guanine and adenosine residues, which are highly susceptible to oxidative damage, serves as a key trigger for DNA fragmentation in spermatozoa. This poses a threat to the integrity of the embryo's genetic material, regardless of

sperm DNA fragmentation. Despite the clinical significance of this issue, single-strand breaks caused by oxidative DNA base modifications in sperm have not attracted much attention from clinicians due to the lack of simple, reliable, rapid, and standardized methods for assessing this type of paternal genome damage (40). Studies suggest that single-strand DNA breaks in sperm do not substantially impact embryo development or implantation success. Conversely, some research indicates a link between higher numbers of single-strand DNA breaks in sperm and lower natural pregnancy rates (39).

Double-strand DNA breaks are considered the most hazardous to genomic integrity, as failed repair mechanisms can lead to genetic rearrangements. They result from single-strand breaks endogenously during DNA replication, namely replication fork disassembly, or due to increased free radical levels. External factors like ionizing radiation, genotoxic chemicals, and radiomimetic agents can also cause double-strand DNA breaks. Double-strand DNA breaks in sperm are linked to slower embryonic development, lower implantation rates, and recurrent miscarriage in couples where the woman's fertility is not a factor (41). Increased levels of DNA fragmentation can lead to recurrent pregnancy loss (42). After the fusion of the sperm nucleus with the damaged DNA and the oocyte, an embryo is formed that may have poor developmental prospects, fail to implant in the endometrium, or even result in miscarriage in the late first trimester (43). Normally, the oocyte possesses its DNA repair mechanism, which is activated after fertilization and can compensate for sperm DNA damage affecting less than 8% of its genome. This DNA repair system in oocytes must function properly, as early stages of zygote development involve chromatin remodeling (44). Sperm's lack of DNA repair mechanisms means that unrepaired damage in the zygote risks creating new mutations passed on to every cell of the resulting organism and its offspring (45).

### ***Mitochondrial dysfunction***

The development, maturation, and functioning of spermatozoa require high energy expenditure, and these processes depend on the morphology and functionality of mitochondria. Dysfunction of mitochondria is associated with asthenozoospermia. ATP synthesis is based on aerobic respiration and the electron transport chain, so ROS generation in mitochondria must be tightly regulated. In the absence of or defects in such regulation, excessive ROS levels can lead to mitochondrial damage. Mitochondrial DNA (mtDNA) is highly susceptible to oxidative damage from ROS. Mitochondria have limited repair capacity, so high ROS levels lead to increased mtDNA mutations. These mutations, caused by free radicals such as hydroxyl radicals (which damage bases

and chromosomes), can harm or kill cells. MtDNA damage is especially problematic because mutations accumulate over time, and damaged mitochondrial genes, which code for essential proteins, disrupt vital mitochondrial functions (46). The buildup of mtDNA point mutations and deletions in sperm causes mitochondrial structural damage and respiratory dysfunction, leading to impaired sperm function (pathospermia) (47). When the extent of mtDNA damage exceeds the repair capacity, subsequent changes activate stress-response genes, leading to cell division blockage and apoptosis (48). Defects in mitochondrial respiratory activity impact spermatogenesis, especially during meiosis. Mitochondrial dysfunction reduces energy production and causes meiotic arrest during spermatogenesis, which enhances apoptosis and manifests as oligozoospermia (49).

### ***Biological markers of sperm oxidative stress***

Currently, proteomics is the only method to identify biological markers. Proteomic analysis of semen and the protein profile expressed in spermatozoa can provide a basis for studying male infertility using biomarker analysis. If this technology can be used to analyze redox reactions, protein targets of OS can be identified and potential sites for further manipulation and therapy can be identified. Proteomic profiles associated with OS have been identified in varicocele, with important differences between infertile men with unilateral and bilateral varicocele. Specific markers of bilateral lesions include TEK3 and TCP11, involved in sperm motility and binding to the pellucid zone (50). A crucial step in sperm maturation is the phosphorylation of flagellar proteins required for motility. Calcium ions and cyclic adenosine monophosphate act as synergists to trigger a cascade of flagellar protein phosphorylation reactions. By comparing spermatozoa of men with asthenozoospermia and normozoospermia, as well as spermatozoa with different motility in patients with normozoospermia, markers of disorders leading to male infertility were identified (51). This marker is a non-receptor type-14 tyrosine phosphatase protein involved in mitochondrial function (52). As well as the protein DJ-1, which protects against the effects of OS (53). A correlation between elevated COX2 levels and defects in spermatogenesis in human testicular biopsy specimens was found (52). This research found a significant excess of normal COX2 expression in the head, middle part and tail of spermatozoa in men with leukocytospermia. COX2 may be a reliable indicator of male infertility. Furthermore, studies have revealed differing levels of proteins within a shared signaling pathway (including TGF- $\beta$ , MYC, MYCN, and TP53) in infertile men with normal sperm counts (54). Biomarkers of oxidation of DNA, proteins and lipids are 8-OHdG,

malondialdehyde and carbonyl moiety in proteins. Elevated content of these biomarkers in seminal plasma was found in infertile patients (55). In research by A. Nasiri et al. the correlation of the levels of sirtuin proteins SIRT1 and SIRT3 with OS and DNA fragmentation in men with asthenoteratozoospermia was evaluated. The results revealed that the levels of these proteins are negatively correlated with OS and DNA fragmentation in semen. Their low levels may lead to increased OS, DNA fragmentation and lipid peroxidation, ultimately resulting in immobile and immature spermatozoa (asthenoteratozoospermia) (56).

In summary, proteomic study of ejaculate can be a complementary method to evaluate the cytophysiologic fertilization ability of spermatozoa. This represents an important area for the evaluation of male infertility of unclear genesis. However, there are limitations to the use of this method, including, but not limited to, the poorly studied techniques, as well as their high cost, indicating that they are difficult to implement in routine laboratory practice.

### ***Methods for determining reactive oxygen species levels in andrology laboratory settings***

The level of ROS in semen sample can be determined using various diagnostic tests. Different kinds of disorders, such as asthenozoospermia, teratozoospermia, viscosopathy, leukocytospermia and necrospermia are displaying high levels of ROS (57). However, it should be noted that no clinical guidelines clearly indicate the necessity of these types of testing (58). This is primarily due to the limited availability of large-scale multicentre clinical trials, the absence of standardisation in testing methodologies, and the inability to make meaningful comparisons between study groups. It is noteworthy that the composition of patient groups with male infertility has been determined by disparate criteria, further complicating the interpretation of results (59). According to the recommendations of the World Health Organization (WHO) (2021), European Society of Human Reproduction and Embryology (ESHRE) (2023) and European Association of Urology (EAU) (2024), the utilisation of diagnostic tests for the determination of ROS levels is currently deemed to be appropriate solely within the context of research. This is primarily due to the current unavailability of such tests and the paucity of evidence supporting their benefits (60–62). To date, the assessment of OS has been proposed to be conducted through several methodologies. These include the determination of radical production in the native ejaculate, the assessment of ROS production by spermatozoa themselves, and the analysis of the antioxidant capacity of seminal plasma (63). OS can also be assessed using chemiluminescence assays or flow cytometry. When chemiluminescence is used with

lucigenin or luminol in the presence of free oxygen radicals, these substances produce a luminescence that is recorded by a chemiluminometer. However, this method is very sensitive, and the result is influenced by many factors: quality of sperm liquefaction, time of analysis, use of repeated centrifugation, pH (64). One such alternative assessment system is MiOXSYS (Male Infertility Oxidative System), which analyses the quantification of redox potential. This is based on the galvanostatic measurement of the presence of electrons in semen. The static redox potential is described as an integral measure of the balance between the total activity of oxidants (i.e. ROS) and the total activity of reducing agents (i.e. antioxidants - free thiols, ascorbate,  $\alpha$ -tocopherol,  $\beta$ -carotene, uric acid, etc.). This system is an inexpensive method compared to chemiluminescence and flow cytometry, which require expensive computerised equipment (65). In medical institutions where the performance of ROS tests is not feasible, the presence of OS is deduced from a compilation of laboratory indications, encompassing diminished sperm motility, teratozoospermia, elevated numbers of round cells within the ejaculate, augmented sperm viscosity, and compromised blastocyst development in the absence of overt female factor infertility (63, 66).

## CORRECTION OF SPERMATOZOA OXIDATIVE STRESS

### *Antioxidant supplements*

At present, the primary method of preserving a man's reproductive status is cryopreservation, which, in an ART laboratory setting, is the most critical stage for the generation of ROS and, consequently, OS in spermatozoa. As a result, there is an active search for solutions to eliminate the impact of ROS on sperm quality, reduce OS levels, and minimize DNA fragmentation at this stage. One such solution could be the use of antioxidant supplements in cryopreservation media. Antioxidant supplements are defined as those that significantly slow down or prevent substrate oxidation, meaning they suppress ROS formation, positively influence gene expression, and maintain cellular redox homeostasis (67). In this section, we review non-enzymatic antioxidants represented by biologically active substances of animal and plant origin used at the stage of human sperm cryopreservation (Table 1).

### *Holothuria parva extract*

Referred to as sea cucumbers, belonging to the class Holothuroidea, they are marine invertebrates inhabiting both benthic areas and deep seas around the world. The therapeutic antioxidant properties of sea cucumber species

*Holothuria parva* may be due to the presence of bioactive substances such as chondroitin sulfates, glycosaminoglycans, sulfated polysaccharides, glycosides and sulfates, glycosphingolipids, essential fatty acids. The effect of *Holothuria parva* intestinal extract on ROS in patients with normal spermogram values conforming to WHO (2010) standard criteria was investigated. Addition of 250  $\mu$ g/ml of this extract to the freezing medium had a positive effect on sperm motility. Increasing the concentration of the extract to 500  $\mu$ g/ml reduced sperm DNA damage and ROS production (29).

### *Extract of Opuntia ficus-indica (Opuntia ficus-indica) and Opuntia dillenii (Opuntia dillenii)*

The plant species of the Cactaceae family *Opuntia ficus-indica* contains several types of antioxidants. As a result, it was concluded that the addition of *Opuntia ficus-indica* extracts and resveratrol to the cryopreservation medium slightly but statistically significantly reduced sperm DNA fragmentation in the population without affecting sperm movement parameters and sperm viability (68). A study by Contino M et al. investigated phenolic compounds and antioxidant capacity of fruit extracts of *Opuntia dillenii* and *Opuntia ficus-indica* yellow and red varieties. The results showed that *Opuntia dillenii* had higher phenolic content and antioxidant power than *Opuntia ficus-indica* and were higher in red than in yellow varieties. In addition, samples treated with *Opuntia ficus-indica* extract of red varieties and *Opuntia dillenii* extract showed a reduction in OS compared to the control group (69).

### *Extract of Capparis spinosa L*

It is a perennial desert herb of the family Capparidaceae, distributed mainly in arid and semi-arid areas of tropical and subtropical regions of the world. The results of the study revealed the positive effect of *C. spinosa* leaf extract in vitro on human sperm function. The antioxidant effect of the leaf extract was six times higher than that of the fruit. When the concentrations of 30 and 45 mg/L were used, the motility in the groups using *C. spinosa* extract was significantly higher than the control group. The use of leaf extract may be useful in reducing the DNA fragmentation level of spermatozoa, when 45 mg/L concentration was used (70).

### *Tribulus terrestris extract*

An annual herbaceous plant of the Zygophyllaceae family. According to a study, the addition of 40 and 50  $\mu$ g/ml of *Tribulus terrestris* aqueous extract to human ejaculate improves sperm motility. The exact mechanism by which *Tribulus terrestris* aqueous extract improves

Table 1. Evaluation of the effect of non-enzymatic antioxidant additives on the properties of human sperm at the cryopreservation stage.

| Name                 | Sperm motility | ROS levels in the ejaculate | Morphological parameters of semen | Sperm concentration | DNA fragmentation |
|----------------------|----------------|-----------------------------|-----------------------------------|---------------------|-------------------|
| Holothuria parva     | +              | +                           | ?                                 | ?                   | +                 |
| Opuntia ficus-indica | ?              | ?                           | ?                                 | ?                   | +                 |
| Opuntia dillenii     | ?              | +                           | ?                                 | ?                   | ?                 |
| Capparis spinosa L.  | ?              | +                           | ?                                 | ?                   | +                 |
| Tribulus terrestris  | +              | +                           | ?                                 | ?                   | ?                 |
| Origanum vulgare     | ?              | +                           | ?                                 | ?                   | ?                 |
| Nigella sativa       | ?              | +                           | ?                                 | ?                   | +                 |
| Extract of Green tea | +              | v                           | ?                                 | ?                   | +                 |

+ positive impact; ? unexplored; v unchanged

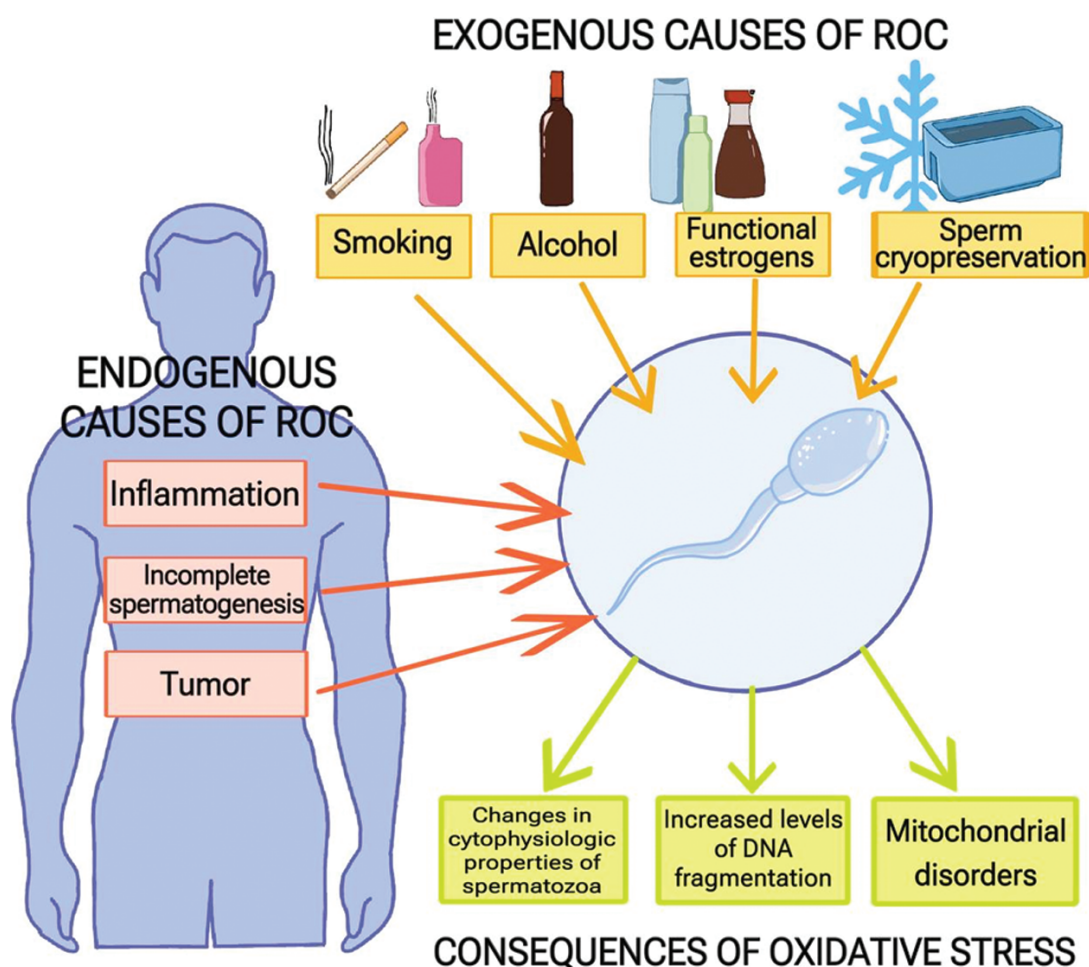


Figure 1. Causes of origin of oxidative stress.

sperm quality remains unclear and is of great interest. *Tribulus terrestris* aqueous extract, contains important free radical scavengers which subsequently may support the intercellular antioxidant system. Substances such as saponins and alkaloids are present in this plant and induce antioxidant activity (71).

### ***Extract of Origanum vulgare***

A perennial herbaceous plant of the genus *Origanum* in the family Lamiaceae. In this study, motility, level of ROS, and lipid peroxidation were evaluated after sperm thawing with the addition of oregano extract. It was concluded that the addition of an optimal concentration of 100 µg/ml oregano extract to the sperm freezing medium reduced early apoptosis, which may be due to the antioxidant activity of oregano extract and reduction of ROS. Oregano extract has antioxidant activity and also functions as a free radical scavenger and metal chelate (72).

### ***Extract of Nigella sativa***

An annual herbaceous plant of the buttercup family Ranunculaceae. In this work we studied the effect of *N. sativa* seed extract in concentrations from 1 to 6 %. Dihydroethidium was used to measure the intracellular production of ROS. Dihydroethidium is oxidised by superoxide anion, binds to DNA and gives red fluorescence. It was concluded that all concentrations of *N. sativa* seed extract resulted in a significant reduction in intracellular production of ROS and DNA damage compared to the control group of vitrified semen (73).

### ***Extract of Green tea***

Decaffeinated green tea extract, primarily containing epigallocatechin gallate, catechin epigallate, epicatechin, and epicatechin gallate, was tested as a cryoprotectant for human sperm. Adding a low concentration (1.0 ng/mL) of these substances significantly improved sperm motility and DNA integrity without affecting ROS levels. This suggests green tea extract may offer a clinically useful protective effect during sperm cryopreservation (74).

## **CONCLUSIONS**

This review compiles the latest research findings on the impact of OS on the structure and function of spermatozoa, further emphasizing the relevance of assessing OS levels in patients undergoing ART, as well as the implementation and routine use of diagnostic tests. Evaluating OS in spermatozoa may enhance the effectiveness of assisted reproductive technologies and provide valuable insights into embryonic development disorders and pathologies observed in offspring.

Additionally, proteomic analysis of ejaculate and other biological fluids should not be overlooked as an additional source of information on the cytomorphological capabilities of spermatozoa for fertilization. A small list of natural antioxidants tested at the ART stage of human sperm cryopreservation is also presented. The analyzed data demonstrated the efficacy of these supplements and their beneficial effects.

However, the number of studies remains insufficient, and there are no data on the potential long-term consequences of prolonged in vitro antioxidant use for embryo health and future offspring. At this stage, it is difficult to draw objective conclusions regarding the effects of antioxidants, and further research is required. Future studies may enable the integration of natural antioxidants into clinical practice.

This consolidated information may be useful for refining methodological aspects of OS assessment and approaches to mitigating its impact during the in vitro stage. Ultimately, this could contribute to improving the quality of obtained embryos.

## **ABBREVIATIONS**

ADP - adenosine diphosphate  
ART - assisted reproductive technology  
ATP - adenosine triphosphate  
DNA - Deoxyribonucleic acid  
mtDNA - mitochondrial DNA  
NADPH - beta nicotinamide adenine dinucleotide phosphate  
OS - oxidative stress  
ROS - reactive oxygen species  
WHO - World Health Organization  
4HNE - 4-hydroxynonenal

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