Free radical and superoxide reactivity detection in semen quality assessment: past, present, and future

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Abstract Oxidative stress is a well-established cause of male infertility, with reactive oxygen species (ROS) impairing sperm production, motility, membrane, and DNA integrity. Currently, most clinics do not test infertile patients for the imbalance between ROS generation and the ability of the antioxidants to scavenge them, although there is a clear need for andrology laboratories to be able to identify and/or quantify seminal oxidative stress. As such there is a clinical urgency for an inexpensive and easy-to-perform assay able to identify oxidative stress in semen. The aim of this review is to provide information on the currently available methods to assess and quantify ROS and particularly superoxide in male reproductive cells, tissues, and fluids which may have a significant clinical utility in identifying men with impaired fertility associated with oxidative stress. Through a deeper understanding of oxidative stress and its assessment options, clinical andrology labs may better assist patients to achieve increased rates of fertility and pregnancy.

Keywords Antioxidants · Male infertility · Oxidative stress · Reactive oxygen species · Semen · Spermatozoa · Superoxide

Reactive oxygen species: damaging molecules acting at a cellular level

Aerobic life inevitably depends upon oxygen needed for a controlled oxidation of molecules containing carbon, followed by a subsequent controlled release of energy. However, any aerobic cell, including the spermatozoon, is constantly facing the so-called “Oxygen Paradox” [1]: oxygen is crucial to sustain aerobic life but at the same time, it is inherently dangerous to the cells’ existence. Normal aerobic metabolism comes hand in hand with the generation of by-products called reactive oxygen species (ROS) [2, 3], which are essential for normal cell functions [4]. However, if ROS levels become too high, either due to their intrinsic or extrinsic overproduction or low levels of antioxidant defense mechanisms, it can lead to oxidative stress (OS) [5], which is a serious threat to the sperm cell survival [6]. OS is currently recognized as a well-established cause of male reproductive dysfunction [7].

ROS include all molecules containing at least one oxygen atom and represent a broad category of intermediates including radical oxygen derivatives (molecules having one or more unpaired electrons in the outer valence molecular orbital, such as the hydroxyl and peroxy ion, superoxide, etc.) and non-radical oxygen derivatives (ozone, singlet oxygen, lipid peroxide, hydrogen peroxide, etc.) [8]. These short-lived, unstable, and highly reactive molecules can “steal” electrons from surrounding structures to achieve a ground state, hence causing other molecules to become free radicals. This chain reaction amplifies the degree of alterations in the neighboring cellular structures [9].

Low levels of ROS play a crucial role in the activation of the intracellular pathways responsible for spermatozoa maturation, capacitation, hyperactivation, acrosome reaction, and fusion with the female gamete [10–13]. On the other hand, high levels of ROS can damage the sperm membrane resulting

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in poor motility, premature capacitation and acrosome reaction, abnormal behavior of transport and communication ion channels, alterations in phosphatidylserine translocation, morphological abnormalities, and impaired sperm-oocyte fusion [10, 14, 15]. Oxidative chain reactions may furthermore lead to lipid peroxidation, protein degradation, alterations to the intracellular signaling pathways, and apoptosis. Moreover, OS has been linked with sperm DNA damage, which may in turn result in poor embryogenesis, miscarriage, and infertility [16–19].

The origin of ROS generation and the etiologies of OS in males with reproductive dysfunction have only recently been elucidated, offering multiple strategies to improve the management of OS-associated male infertility. Also, the role of ROS in male subfertility or infertility is an area that deserves continued research [20]. Above all, and from a clinical point of view, there is a clear need for andrology laboratories to be able to identify and/or quantify seminal OS [7, 21, 22].

Superoxide: the “mother” of ROS

The recognition that cellular systems produce substantial quantities of superoxide ($O_2^{\bullet-}$) through normal metabolic pathways [23] and that enzymes, particularly superoxide dismutase (SOD), help protect aerobic cells from the presumed toxicity of this free radical [24, 25] have triggered much scientific interest. These enzymatic tools both generate (via xanthine oxidase) and eliminate superoxide (via SOD), which has facilitated additional research in numerous areas of physiology and pathology. Indeed, for several decades, ROS-associated biology was considered “superoxide-centric”, owing largely to the fact that superoxide is quantitatively the predominant ROS produced by biological systems [26].

Superoxide is the principal ROS generated by respiring cells. It is created as a result of a monovalent reduction of oxygen and the addition of a single electron [27].

In the spermatozoon, there are two main sources of $O_2^{\bullet-}$: the NADH-dependent oxidoreductase [28] located in the inner mitochondrial membrane and the NAD(P)H-oxidase found in the plasma membrane [29]. Superoxide is a regular by-product of oxidative phosphorylation [30] and is created between complex I and III of the electron transport chain [31] as a result of an electron addition to the intracellular oxygen. Surprisingly, $O_2^{\bullet-}$ is an effective reducing agent in addition to being a mild oxidizing agent. In most dismutation reactions, one superoxide radical acts as an oxidant while the other behaves as a reductant [26].

Although $O_2^{\bullet-}$ is relatively unreactive [27], in the presence of H^+ it undergoes either a spontaneous or enzyme-catalyzed dismutation into hydrogen peroxide (H_2O_2)—a membrane permeable molecule [32], which has been established as the major initiator of peroxidative damage in the plasma membrane of the sperm cell [6, 33]. It can be scavenged by the

enzymatic antioxidants glutathione peroxidase or catalase, catalyzing the dismutation of H_2O_2 into water and oxygen.

At the same time, $O_2^{\bullet-}$ as well as H_2O_2 can undergo a series of cellular transformations to generate the extremely reactive hydroxyl radical ($OH^{\bullet}$) through the Fenton and Haber-Weiss reactions, which consist of a reduction of ferric (Fe^{3+}) to ferrous ion (Fe^{2+}) in the presence of $O_2^{\bullet-}$, followed by the H_2O_2 conversion to $OH^{\bullet}$. Furthermore, the superoxide anion has the ability to interact with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), subsequent reactions of which may lead to either apoptotic or necrotic cell death [34, 35].

Methods for detecting reactive oxygen species

The scientific interest in the role of OS in sperm dysfunction originally revolved around the relationships between ROS overproduction and male reproductive pathologies [4, 7, 8, 36, 37]. Further work has nevertheless shown that ROS are in fact naturally produced in semen and serve as important cellular messengers for both intra and intercellular communications [11, 12]. It is now clear that a very complex intracellular regulatory system involving ROS exists during sperm production and maturation. Male reproductive cells respond to ROS fluctuations in different ways depending on the intensity, duration, and context of the signaling [35].

Because high levels of ROS have been strongly associated with male reproductive dysfunction, measuring ROS levels in semen should be regarded as an important part of the initial evaluation as well as follow-up of subfertile and infertile men [36–38].

Early methods of ROS detection and quantification in semen were primarily based on absorbance measurements. Nevertheless, with millimolar reference values, absorbance based measurements played only an informative role in clinical settings. With the discovery that ROS act as intracellular messengers and regulators, new analytical methods emerged, bearing micromolar detection requirements in mind. These techniques are primarily fluorescence-based, but recently, luminometric approaches have been introduced.

Currently, there are over 30 different assays to assess and study seminal OS, which are generally based on the interaction of superoxide or other ROS with some other compound to create a measurable result [7, 39]. Chemiluminescent and fluorescent methods are most commonly used in clinical settings.

Chemiluminescence

Chemiluminescent methods are highly sensitive and specific, owing the possibility to investigate different ROS (including $O_2^{\bullet-}$, H_2O_2 , $OH^{\bullet}$, and singlet oxygen) simultaneously. Two major probes used to assess ROS generation by spermatozoa

are luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (10,10'-dimethyl-9,9'-biacridinium dinitrate) [40].

Because of very weak native luminescence phenomena, both luminol or lucigenin dependent chemiluminescent protocols have been used frequently for the detection of $O_2^{\bullet-}$ in biological systems. In both cases, light production depends directly on the formation of an unstable endoperoxide or dioxetane, which decomposes to an electronically excited product [41, 42]. Subsequently, this product releases a photon while falling to the ground state. The results are expressed in relative light units (RLU), counted photons per minute (cpm), or millivolts per second (mV/s) [42] (Fig. 1, Fig. 2).

Lucigenin carries a positive ionic charge, which makes it membrane-impermeable and responsive to ROS, particularly $O_2^{\bullet-}$. As such, the univalently reduced form of lucigenin will react with superoxide primarily in the extracellular space. Lucigenin is an excellent probe to evaluate $O_2^{\bullet-}$ production as a nonspecific redox marker for the enhanced electron transfer activity associated with defective sperm function. Both the sensitivity and specificity of this probe may be enhanced by its redox cycling activity [42].

Inversely, the uncharged luminol is relatively membrane permeable and its univalently oxidized form reacts with a variety of ROS including $O_2^{\bullet-}$, H_2O_2 , and $OH^{\bullet}$ in intra and extracellular spaces. In the case of luminol, H_2O_2 is more reactive than $O_2^{\bullet-}$. The advantages of luminol include its quick reactivity with both intracellular and intercellular free radicals; however, it does not differentiate between the types of free radicals and therefore measures global ROS [42, 43].

Both chemiluminescent assays are convenient for diagnostic purposes and have relatively well-established ranges for healthy donors as well as infertile men [36–39, 44–46]. Numerous studies have revealed significant negative associations between increased ROS levels detected by luminometry and traditional semen quality parameters including sperm count, motility, viability, and morphology [36, 37] as well as positive correlations with specific markers linked to male

reproductive dysfunction, such as DNA fragmentation, leukocyte concentration, and the incidence of apoptotic or necrotic spermatozoa [47, 48].

On the other hand, significant set-up costs have to be taken into consideration, and the data generated by chemiluminescent assays must be interpreted carefully because a variety of factors can affect the signals obtained, such as incubation time, medium composition, pH, seminal plasma contamination, and the presence of leukocytes [49, 50]. Finally, Kobayashi et al. [50] hypothesized that chemiluminescence may be accurate and reliable but only in samples with sperm concentration >1 million/mL, as its sensitivity declines significantly even in specimen with sperm concentrations <5 million/mL [43].

Fluorescence

A possible solution to the disadvantages associated with the chemiluminescent approach can be found in a variety of redox-sensitive fluorescence probes that can be loaded into spermatozoa and subsequently monitored by flow cytometry or fluorescent microscopy. Using flow cytometry, the assessment can focus on a specific sperm population using the appropriate setting of gates [49]. The cell suspension is adjusted to a density of 10^5 – 10^7 cells/mL and 10,000 events are usually measured.

Different fluorescent probes may be used, depending on the nature and objective of the subsequent assessment. Cellular $O_2^{\bullet-}$ production can be visualized by dihydroethidium or hydroethidine (DHE). It is a non-fluorescent probe that is oxidized by superoxide and to a much lesser extent other reactive oxygen or nitrogen species. DHE oxidation results in hydroxylation at the 2-position forming 2-hydroxyethidium with red fluorescence emission at 488 nm, which will stain the mitochondrial and nuclear DNA [51–53]. DHE can be used along with a vitality marker (SYTOX green) in order to identify those cells that are alive and generating ROS [54].

Fig. 1 Autolumat 953 Plus Luminometer used for the measurement of ROS by chemiluminescence assay. **a** external view. **b** internal view. Multiple tubes can be loaded simultaneously for measuring ROS

Fig. 2 Preparation of the tubes for ROS measurement. A total of 11 tubes are labeled from S1-S11: Blank, negative control, test sample, and positive control. Luminol is added to all tubes except the blank. Hydrogen peroxide is added to the positive control exclusively

Another fluorescent probe is 2,7-dichlorofluorescein diacetate (H₂DCFDA). H₂DCFDA is a stable, non-fluorescent cell-permeable probe that de-esterifies in the presence of intracellular H₂O₂ to form a fluorescent 2,7-dichlorofluorescein (DCF) [55]. Other ROS such as peroxynitrite, hypochlorous acid, and OH[•] can also oxidize this probe and may significantly contribute to the positive signals observed in defective spermatozoa [56, 57].

Hydrocyanine dyes are fluorogenic sensors specifically designed for O₂^{•-} and OH[•]. These dyes are synthesized by reducing the cyanine (Cy) dyes with sodium borohydride. While weakly fluorescent in normal state, upon oxidation their fluorescence intensity significantly increases. In addition to being fluorescent, oxidation also converts the Cy molecule from being membrane permeable to an ionic impermeable moiety [58]. The most used probes are Hydro-Cy3 and Hydro-Cy5 [59].

Considering that mitochondria are the main source of cellular ROS, superoxide specific to the mitochondria can be visualized using fluorescence microscopy with the commercially available MitoSOX™ Red reagent (Life Technologies). The probe is a cationic derivative of DHE, which only reacts with superoxide, coupled with triphenylphosphonium cation that directs the probe to actively respiring mitochondria of living cells. As with DHE, the probe intercalates with mitochondrial DNA resulting in red fluorescence. Fluorescence measurements can be performed using an excitation wavelength of 510 nm with an emission

detection at 590 nm, although it has been reported that a lesser excitation peak at 400 nm that is absent in the excitation spectrum of the ethidium oxidation product generated by ROS other than superoxide may provide better discrimination of O₂^{•-} [53, 60].

Fluorescent techniques have a higher specificity, accuracy, sensitivity, and reproducibility than relevant chemiluminescence for intracellular ROS. With regard to specific analyses of spermatozoa, flow cytometry is advantageous in that it can be focused exclusively on the male germ cells. Also, a large number of cells can easily be analyzed, leading to high specificity and sensitivity [49, 57].

Fluorescent dyes have been very useful in shedding more light on the role of mitochondria in the intricate oxidative milieu of male gametes. Using DHE, Treulen et al. [61] revealed that mitochondrial permeability transition induced by calcium overload leads to increased ROS production and DNA fragmentation in human spermatozoa. Moreover, Koppers et al. [62] used the MitoSOX red probe to uncover ROS generation from complex I or III as a result of disruptions of the mitochondrial electron transport flow in human spermatozoa via mechanisms independent of the mitochondrial membrane potential.

One major disadvantage of fluorescent probes is that sophisticated and expensive hardware is needed. Also, the results do not quantify the target ROS but simply indicate the percentage of cells exhibiting a high level of activity without indicating the concentration or a cellular content of the metabolites being evaluated [22, 49].

NBT test

The nitro blue tetrazolium (NBT) assay has emerged as a simple yet effective laboratory method to quantify cellular oxidative metabolism and neutrophil function [63]. The NBT test is based on the use of NBT, a yellow water-soluble, nitro substituted aromatic tetrazolium molecule (2,2'-bis (4-nitrophenyl)-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride), that interacts with cellular superoxide to form formazan, which can be monitored microscopically or spectrophotometrically [63, 64]. The sperm cytoplasm contains glucose-6-phosphate dehydrogenase using glucose to produce nicotinamide adenine dinucleotide phosphate (NADPH) via the hexose monophosphate shunt. NADPH subsequently serves as an electron donor for the generation of superoxide anions via the NADPH oxidase present in spermatozoa, which in turn converts NBT into formazan. Furthermore, the oxidase system in the cytoplasm facilitates the transfer of electrons from NADPH to NBT formazan [22, 63]. As such, the NBT reaction indirectly reveals the ROS-generating activity of the cellular cytoplasm and hence may help detect the cellular origin of ROS in complex and heterogeneous suspensions such as semen [22, 65].

The principle of the NBT test is relatively simple and straightforward: target cells are incubated in the NBT solution and subsequently take up the tetrazolium salt into their cytoplasm where it is converted by the activity of superoxide anions to water insoluble purple-blue formazan crystals [59]. While the formazan crystals are trapped intracellularly, these can be visualized within the target cells using an optical microscope (Fig. 3). Alternatively, formazan crystals can be released using a solubilization reagent and subsequently quantified by measuring the absorbance of the resulting purple-blue suspension [66, 67].

Although being a relatively recently introduced technique, different studies have already emphasized on significant associations between NBT-positive staining, abnormal spermatozoa and the occurrence of leukocytes, sperm DNA fragmentation and apoptosis [22, 68] followed by negative correlations with sperm motility, concentration and morphology in fertile as well as infertile subjects [65, 68].

Although the NBT test is relatively easy and inexpensive to perform, its use within clinical andrology laboratories is hampered by a lack of published normal ranges. Even though previous reports have established cut-off values for the amount of formazan to determine the fertility status of individual subjects with a high sensitivity and specificity, these studies are relatively small and need to be validated by other large multi-center trials. Furthermore, the method was reported to be relatively ineffective in samples with leukocytic contamination or low sperm concentration [65, 68, 69].

Comparative studies

As ROS generation by spermatozoa significantly contributes to the etiology of male reproductive dysfunction, assessment of OS markers has become a valuable investigative tool in the diagnosis or management of male infertility. As outlined earlier, chemiluminescence and fluorescence are two major research strategies to unravel the contribution of ROS to a decreased male fertility, with the NBT test turning out as a promising alternative in the detection and/or quantification of the superoxide produced by defective spermatozoa. Given this relatively vast availability of methods for the diagnostic analysis of seminal OS, reports are increasingly emerging with the aim to compare the performance characteristics of such techniques in practical settings.

Fig. 3 Sperm reactivity to nitro blue tetrazolium (NBT) after oxisperm reaction. **a** Whole microscope field observed under bright field microscopy ($\times 40$) showing spermatozoa positive to NBT reaction and some of them free of formazan deposits. **b–d** Selected and enlarged

spermatozoa from Fig. 3a to show the absence of formazan precipitates (**b**), precipitates localized in the sperm head (**c**), and precipitates mainly affecting the mid-piece where mitochondria are localized (**d**)

Mahfouz et al. [70] assessed the efficiency of ROS quantification using luminol- and lucigenin-based chemiluminescence as well as flow cytometry employing H₂DCFDA and DHE in different sperm fractions collected from 18 healthy donors before and after exposure to H₂O₂. The study revealed that regardless of the chosen technique, immature sperm fractions exhibited significantly higher ROS levels when compared to the neat and mature fraction. Both mature and immature sperm fractions had a significantly higher percentage of cells positive for H₂O₂ in comparison with neat semen samples. The authors speculate that flow cytometry and chemiluminescence may be comparable with respect to specimens generating high ROS levels. Nevertheless, flow cytometry was more accurate particularly in case of specimens producing low ROS levels as well as in oligospermic semen samples. While the use of adjusted ROS levels may be a more useful tool in ROS assessment by chemiluminescence, specimens tested negative by the luminometric assay may still be producing intracellular H₂O₂, which may be detected by flow cytometry. Furthermore, by using both fluorescent dyes, two main types of ROS generated intracellularly (O₂[•] and H₂O₂) may be evaluated at the same time.

Employing a variety of reagents triggering ROS generation in semen (including 2-hydroxyestradiol, menadione, 4-hydroxynonenal, and arachidonic acid), the objective of Aitken et al. [71] was to systematically evaluate the ability of a variety of assays, including flow cytometry (MitoSOX Red, DHE, and H₂DCFDA), luminometry (luminol and lucigenin), as well as NBT to detect elevated ROS levels in specimens collected from normozoospermic donors. The study revealed that ROS released into the extracellular space may be readily detected by luminol and, to a lesser extent, H₂DCFDA. On the other hand, these assays were particularly vulnerable to interference by leukocytes. Intracellular ROS generation by the sperm mitochondria could be optimally detected using MitoSOX Red and DHE. Assessment of spontaneous ROS generation by defective spermatozoa revealed that MitoSOX Red was the most effective indicator of OS.

In order to differentiate the ROS-generating activity in the predominant cells from semen (spermatozoa and leukocytes) based on their morphological characteristics acquired by the deposition of formazan, Esfandiari et al. [65] applied the NBT test using a histochemical approach in whole ejaculates, leukocytes, and abnormal spermatozoa from 9 healthy donors as well as 21 infertile patients. Furthermore, the study examined the association between NBT staining and the ROS-TAC (reactive oxygen species-total antioxidant capacity) score, an index to evaluate seminal OS measured by chemiluminescence. The percentage of NBT-positive cells was significantly higher in the sperm suspensions contaminated with leukocytes than in those from the non-leukocytospermic group and donors. A strong positive correlation was observed between the ROS levels in whole ejaculates and NBT-positive staining in

leukocytes as well as in leukocyte fractions following density gradient separation. The ROS-TAC score was furthermore correlated with the NBT staining in leukocytes and spermatozoa with cytoplasmic retention. The authors conclude that the NBT test could be used to assess the occurrence and contribution of seminal leukocytes to the oxidative balance in semen as a convenient alternative to other cytochemical methods. Moreover, the study showed that the NBT assay was equally effective in evaluating the ROS-generating ability of abnormal or immature spermatozoa.

Indirect methods

Other methods to assess the oxidative balance in semen include indirect measurements such as the total antioxidant assay (TAA). This technique measures the ability of a compound or biological fluid to scavenge ROS with any specific or nonspecific mechanism available [72]. Different TAA methods include phycoerythrin fluorescence-based assays [73], ferric reducing ability of plasma (FRAP) [74], total radical trapping antioxidant parameter (TRAP) [75], and oxygen radical absorbance capacity (ORAC) [76]. Furthermore, commercially available colorimetric assays have been successfully used with semen samples or fractions. Such techniques are based on the cumulative ability of antioxidants in the sample to inhibit the oxidation of 2,2'-azino-di-3-ethylbenzthiazoline sulphonate (ABTS) to ABTS⁺ by metmyoglobin. As such, the antioxidants in the sample suppress the absorbance at 750 nm to a degree that is proportional to their final concentration [77]. Alternatively, an enhanced chemiluminescence protocol involving a cell-free ROS-generating system containing horseradish peroxidase, H₂O₂ and luminol may be used to measure the total nonenzymatic activity in biological fluids [78]. The system generates ROS at a known and steady rate, where the luminescence intensity remains almost constant for several minutes. This steady light emission is temporarily interrupted when an antioxidant is added to the system. The emission is subsequently restored once the ROS scavenging ability is depleted [72]. TAA of a sample is compared with that of a standard, generally Trolox, a water-soluble tocopherol analog, and the results are reported as micromoles of Trolox equivalent. TAA may provide more relevant biological information compared to that obtained by the measurement of individual components, as it considers the sum of endogenous and food-derived antioxidants present in plasma or body fluids, including glutathione, proteins, lipids, vitamins, and uric acid [38, 72].

Another option is to assess the activity of antioxidant enzymes (SOD, catalase, glutathione peroxidase, and reductase) as well as the redox potential defined by the ratio of oxidized and reduced glutathione (GSH:GSSH) [54]. Superoxide dismutase (SOD) activity may be assessed employing xanthine and xanthine oxidase (XO) to generate superoxide radicals,

which will react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. SOD activity is subsequently measured by the inhibition degree of the reaction at 505 nm [79]. Catalase (CAT) activity may be quantified by monitoring the decrease of H_2O_2 added to the sample at 240 nm. The calculation is subsequently based on the rate of H_2O_2 decomposition, proportional to the reduction of the absorbance during 1 min [80]. Glutathione peroxidase (GPx) activity may be evaluated applying the method of Paglia and Valentine [81]. GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase (Gr) and NADPH the oxidized glutathione is subsequently converted to the reduced form with a concomitant oxidation of NADPH to NADP^+ . The decrease of absorbance is measured at 340 nm. Reduced glutathione (GSH) can be determined by the Ellman method [82]. The sample is treated with DTNB (5,50-dithiobis-2-nitrobenzoic acid; Ellman's reagent) which interacts with the thiol groups of GSH, cleaving the disulfide bond to give 2-nitro-5-thiobenzoate (NTB^-) and creating the NTB^{2-} dianion in water at alkaline pH. This ion has a yellow color and may be quantified at 412 nm. Alternatively, individual antioxidants may be assessed using commercially available assay kits and following the methodology provided by the manufacturer [83].

A popular option is the measurement of oxidative end-products, including protein carbonyls [84], lipid hydroperoxides [85], malondialdehyde [86], and oxidative DNA adduct 8-hydroxy 2-deoxyguanosine [58].

The most commonly used marker of protein oxidation is the protein carbonyl content [28]. The most convenient procedure is the reaction between 2, 4-dinitro-phenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be assessed spectrophotometrically at 360–385 nm [84]. The molar absorption coefficient of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ is then used to quantify the concentration of protein carbonyl groups.

Thiobarbituric acid-reacting substances (TBARS) are the most common methods to quantify primarily malondialdehyde (MDA), which is derived from lipid peroxidation. Samples are pre-treated with sodium dodecyl sulfate, subjected to thiobarbituric acid (TBA) dissolved in 20% acetic acid and subsequently boiled at 90–100 °C. The concentration of thiobarbituric acid-reactive substances is determined spectrophotometrically at 530–540 nm and expressed by considering the coefficient of molar absorptivity of the product [86].

Immunohistochemistry or western blot analysis has been used to study and quantify oxidative DNA adduct 8-hydroxy 2-deoxyguanosine (8-oxo-dG) [87]. Immunohistochemistry can be performed on paraffin sections using specific anti-8-oxo-dG antibodies and diaminobenzidine staining. Finally, commercial kits are readily available to assess 8-oxo-dG directly. Prior to the analysis, DNA has to be extracted from the sample and

quantified using spectrophotometry at 260/280 nm. The isolated DNA is subsequently processed with the help of a fluorescent or colorimetric kit enabling a direct quantification of 8-oxo-dG using a strip-well microplate format and a fluorimeter or a photometer.

Oxidative stress assessment in clinical andrology

Although increased ROS levels have been repeatedly implicated as an important factor contributing to male reproductive dysfunction, the American Society for Reproductive Medicine (ASRM) [88] as well as the American Urological Association (AUA) [89] disclose that the assessment of seminal OS has a very limited role in the actual evaluation of male infertility due to insufficient clinical utility and little impact on any eventual treatment. According to both associations, direct ROS testing is limited by the short duration of activity of the molecules, which is why most studies have to rely on indirect methods that measure the by- or final products of oxidative damage. ASRM and AUA guidelines argue that reports correlating seminal ROS levels to pregnancy outcomes are sparse or contradictory, limited by lack of controls or standardized testing methods for ROS which makes an unbiased comparison between the studies complicated. As such, routine clinical testing or possible treatment options of seminal OS are not indicated at this time [88, 89].

Despite ASRM and AUA do not advocate for the assessment OS markers in clinical andrology, there are scenarios, where testing the extent of oxidative damage to the reproductive cells may provide helpful feedback and contribute to a faster and more effective diagnosis and/or management of male subfertility.

Varicocele

OS seems to play a key role in the pathophysiology of varicocele-related infertility by several mechanisms of action which are still under vigorous investigation. Agarwal et al. [90] hypothesize that OS is the central and common pathogenic mediator of testicular damage in varicocele, and that the exposure to heat, hypoxia, and toxic adrenal or renal metabolites significantly contribute to ROS overgeneration in the male reproductive system.

Pivotal clinical studies have revealed that the assessment of oxidative profile may significantly contribute to a more accurate diagnosis of varicocele emphasizing that even fertile men suffering from varicocele exhibit a disturbed oxidative homeostasis in reproductive structures, and that varicocele grade is correlated with the severity of OS in male reproductive fluids [91–93]. A number of variables can be measured to determine the extent of OS in men with varicocele, such as seminal levels of ROS, lipid peroxidation, and TAA. Interestingly, patients diagnosed with varicocele often have low levels of TAA in their seminal plasma and, therefore,

may benefit from antioxidant supplementation [94]. As for the actual antioxidant therapy, well-designed and controlled trials are needed in order to obtain solid evidence concerned with the effectiveness of antioxidants in the management of varicocele-related subfertility.

Idiopathic infertility

Men are diagnosed with idiopathic infertility when no cause of reproductive dysfunction can be found using common clinical, instrumental, or laboratory methods [7]. OS may be a contributing factor to infertility in such otherwise healthy males. While the exact cause of idiopathic infertility is still widely unknown, studies have reported that 25 to 40% of males with idiopathic infertility have higher ROS and lower antioxidant levels than fertile men [95, 96]. The sperm mitochondria have been found to be damaged in men with idiopathic infertility, leading to the release of ROS and apoptotic proteins into sperm structural compartments, resulting in DNA damage and cell death [95]. A different cause of idiopathic infertility may be morphologically abnormal spermatozoa which are often seen to be increased in infertile patients [37]. Such gametes exhibit a tendency to generate ROS, followed a reduced antioxidant capacity [95–98].

Similarly to varicocele, assessment of OS markers in healthy, yet infertile men may provide an additional set of information in order to distinguish such patients more accurately, and to decide on the best management and/or treatment option for idiopathic infertility. As men diagnosed with idiopathic infertility have often a decreased capacity to counteract ROS overproduction, it may be expected that such patients could benefit from eventual antioxidant treatment [97, 98].

Iatrogenic sperm damage

The assessment of OS has become a valuable tool in shedding more light on the iatrogenic sperm damage resulting from commonly used semen processing protocols, which has been linked to significant cell loss in assisted reproductive technologies (ART) [7]. During in vitro fertilization and intrauterine insemination semen specimens are often centrifuged to separate spermatozoa from the seminal plasma. This process may exacerbate OS as centrifugation significantly increases ROS production by spermatozoa, while removing sperm cells from the protective antioxidant mechanisms contained within the seminal plasma [99]. Furthermore, sperm cryopreservation, a commonly used technique in ART, is associated with ROS overproduction due to mechanical injury, cold shock, and exposure to atmospheric oxygen, which in turn increase the susceptibility to lipid peroxidation due to a higher ROS production [100]. Sperm preparation techniques, such as density gradient centrifugation, migration-sedimentation, glass wool filtration, or swim-up can be used to decrease ROS production in order to enhance and maintain

sperm quality after ejaculation. Currently, the use of antioxidants to prevent ROS generation during sperm preparation processes is under investigation [101, 102].

Conclusions

Oxidative stress is implicated in the etiology of male reproductive dysfunction, resulting in lipid peroxidation, abnormal sperm function, and increased mitochondrial and nuclear DNA damage. Therefore, there is an intense interest in the development and standardization of simple, convenient techniques to monitor the ROS generation by male reproductive cells, tissues, and fluids. As infertility rates continue to rise and male factor plays an ever-increasing role in contributing to infertility in couples of reproductive age, it is crucial for andrology laboratories to fully understand the importance of carefully assessing the seminal oxidative balance in order to provide a better care for infertile patients.

New tests are being developed to assess seminal OS, to evaluate the differential contribution of cellular components of semen to ROS generation, to identify oxidatively damaged spermatozoa, and to determine the state of activation of seminal leukocytes. More accurate, low-cost, and easy-to-perform assays have the potential to be added to the routine clinical andrology workup for the evaluation of seminal OS without the need for expensive technical equipment. Despite significant progress in the evolution of distinct techniques to evaluate seminal OS, simpler, less expensive assays with well-defined, clinically significant normal ranges based on physiological sperm functions have yet to be established for ROS to become a standard clinical marker in andrology laboratories. At the same time, the data obtained from newly developed test should be compared with other methods for the assessment of sperm structural integrity, functional activity, oxidative balance, and DNA damage.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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