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An update on the techniques used to measure oxidative stress in seminal plasma

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Abstract

Reactive oxygen species (ROS) are produced in significant amounts by spermatozoa and leucocytes. They are necessary to carry out various physiological functions such as sperm capacitation, hyperactivation, acrosome reaction, sperm-zona binding and cellular signalling pathways. Oxidative stress (OS) results when the ROS levels overwhelm the available antioxidant reserve. A number of direct and indirect tests have been developed to assess oxidative stress. In this manuscript, we discuss these common direct and indirect tests as well as their advantages and disadvantages. Tests measuring sperm dysfunction secondary to oxidative stress such as lipid peroxidation, DNA fragmentation and protein alterations are also described.

KEYWORDS

DNA integrity, lipid peroxidation, oxidation–reduction potential, reactive oxygen species, spermatozoa

1 | BACKGROUND

1.1 | Male infertility

Globally, about 15% of couples desiring pregnancy are infertile and male factor is responsible for about 30% to 50% of these cases (Agarwal, et al., 2019; Sigman, Lipshultz, & Howards, 2009). Environmental, physiological and genetic factors affect sperm function leading to infertility (Durairajanayagam, 2018). Semen analysis is the first step in the laboratory evaluation of male infertility. A semen analysis by itself is only a diagnostic test, it cannot determine the cause of infertility. In about 20% of the cases termed as idiopathic infertility, the cause is unknown (Agarwal, et al., 2019; Cooper et al., 2010; World Health Organization, 2010). This diagnostic limitation of semen analysis introduced the need to identify markers to understand the underlying molecular mechanism responsible for infertility. One such marker is oxidative stress (OS). Oxidative stress is a major player in the pathology of male infertility (Agarwal et al., 2006; Agarwal, Virk, Ong, & du Plessis, 2014).

OS is involved in the majority of known clinical, environmental and lifestyle causes of male infertility (Agarwal & Sengupta, 2020; Durairajanayagam, 2018). Furthermore, OS is implicated in the pathophysiology of infertility due to varicocele, genitourinary tract infection, prostatitis, obesity, tobacco smoking, endocrine imbalance and testicular dysfunction (Darbandi et al., 2018; Tremellen, 2008; Wagner, Cheng, & Ko, 2018; Wright, Milne, & Leeson, 2014). The plasma membrane of the spermatozoa is rich in polyunsaturated fatty acids (PUFAs) and therefore very susceptible to oxidation causing sperm dysfunction. This results in infertility, fertilisation failure, pregnancy loss, poor embryonic development and even childhood cancer (Fujii & Imai, 2014; Wahab, Yazmie, Isa, & Lokman, 2015). In this review, we will highlight the significance of OS in male infertility and describe the assays currently available for its evaluation.

1.2 | Mechanism of oxidative stress as a cause of infertility

Much like every other biological cell, oxygen is required by the mitochondria for energy producing processes via the oxidative phosphorylation pathway. This involves the oxidation and reduction of molecules consequently producing highly reactive molecules known as free radicals (Bisht & Dada, 2017; Bisht, Faiq, Tolahunase,

& Dada, 2017; Sharma & Agarwal, 2020). Reactive oxygen species (ROS) are highly reactive and very unstable molecules which possess one or more unpaired electrons (Nakajima et al., 2002; Nohl et al., 2000; Vezin et al., 2002). Superoxide anion ($O_2^{-\bullet}$), hydroxyl-radical (OH•), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and reactive nitrogen species such as peroxynitrite ($ONOO^-$) and nitric oxide (•NO) are all examples of ROS. ROS can be produced endogenously by seminal polymorphonuclear leucocytes (Aitken & Baker, 2013; Henkel, 2011; Mupfiga, Fisher, Kruger, & Henkel, 2013) and morphologically abnormal spermatozoa. Spermatozoa with excessive residual cytoplasm as a result of incomplete sperm maturation also have the ability to produce ROS (Aitken, 2017; Gomez et al., 1996). Furthermore, abnormalities in the mitochondria occur due to leakage of electrons from cytosolic L-amino acid oxidases and plasma membrane nicotinamide adenine dinucleotide phosphate (NADP) oxidases (Aitken, 2017; Ford, 2004; Koppers, De Iuliis, Finnie, McLaughlin, & Aitken, 2008). Exogenous sources include tobacco smoking, alcohol usage, radiation, pollution, testicular heat and other environmental toxicants (Tremellen, 2008; Wright et al., 2014; Figure 1).

Redox balance is essential for normal sperm function (Aitken, 2017; Aitken & Curry, 2011; Du Plessis, Agarwal, Halabi, & Tvrda, 2015; de Lamirande, Jiang, Zini, Kodama, & Gagnon, 1997; Wagner et al., 2018). OS is a result of high levels of ROS or a depleted antioxidant capacity in sperm cells (Bisht & Dada, 2017). Spermatozoa are exposed to OS during spermatogenesis, epididymal storage and transit through the reproductive tract and at the time of ejaculation (Aitken & Curry, 2011; Sakkas & Alvarez, 2010; Tremellen, 2008). Limited availability of intracellular antioxidants and elevated ROS levels expose the vulnerable molecules to oxidative attack (Aitken, 2017; Aitken & Curry, 2011).

2 | ROLE OF ROS IN SEMINAL PLASMA

2.1 | Physiological role

Controlled amounts of ROS are essential for the spermatozoa to acquire fertilising capacity. Physiological levels of ROS regulate the intracellular calcium concentration as well as the enzymatic tyrosine kinase activity required for capacitation and hyperactivation; a physiological condition where the sperm motility is dramatically enhanced. Only capacitated spermatozoa display hyperactivated motility and undergo the acrosome reaction necessary for fertilisation. Incubation of spermatozoa with exogenous oxidants such as $O_2^{-\bullet}$, H_2O_2 and •NO results in capacitation, hyperactivation and acrosome reaction and helps in oocyte fusion (Aitken, 2017; Aitken & Curry, 2011; Dutta, Henkel, Sengupta, & Agarwal, 2020; Makker, Agarwal, & Sharma, 2009; Tremellen, 2008). Physiological levels of ROS are also essential for chromatin and flagellar protein modifications during spermatogenesis (O'Flaherty & Matsushita-Fournier, 2017).

2.2 | Pathological role

Although ROS is involved in the regulation of physiological processes of the spermatozoa, an excess of ROS can impair cellular constituents affecting cell signalling and sperm function. The PUFAs render the spermatozoa more susceptible to lipid peroxidation, which results in the formation of potentially toxic and mutagenic aldehydes and alkanols causing DNA fragmentation (Figure 1) (Aitken, 2017; Aitken, Gibb, Baker, Drevet, & Gharagozloo, 2016; Henkel, 2011; Moazamian et al., 2015; Sharma & Agarwal, 2020).

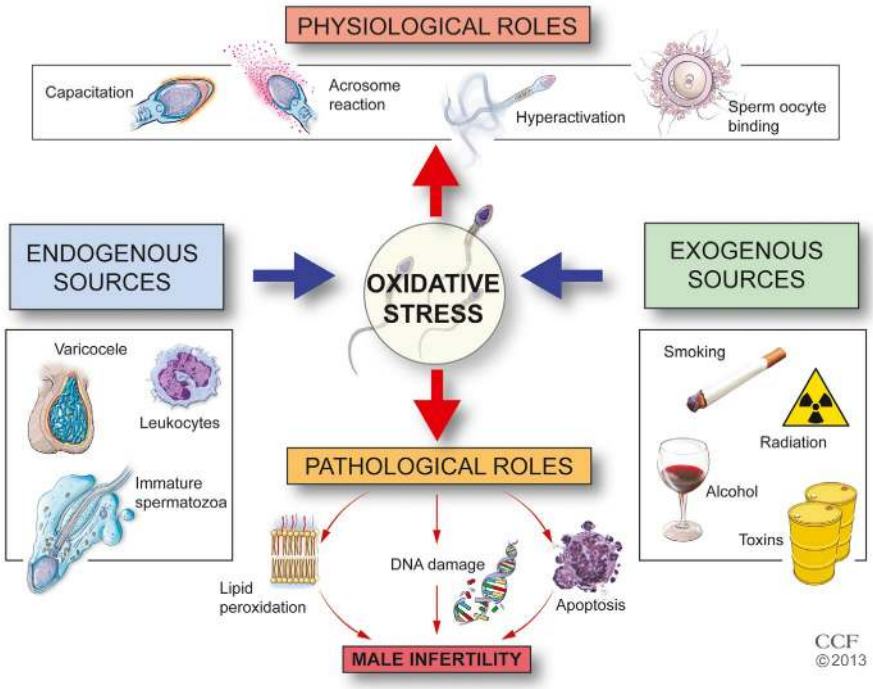


FIGURE 1 Physiological and pathological role of reactive oxygen species (ROS) and generation of excessive ROS by endogenous and exogenous sources of seminal reactive oxygen species

OS results in increased DNA damage, reduced motility, impaired acrosome reaction and decreased implantation rates in vitro fertilisation (IVF) (Aitken, 2017; Aitken & Curry, 2011; Bakos, Henshaw, Mitchell, & Lane, 2011; Palmer, Bakos, Fullston, & Lane, 2012; Tremellen, 2008; Wahab, Isa, & Ramli, 2016). It also interferes with epigenetic modifications and may result in abnormallities in sperm gene methylation (Darbandi et al., 2018; Menezo, Silvestris, Dale, & Elder, 2016; Tunc & Tremellen, 2009). Consequently, DNA damage may also result in impairment of embryo development, increased risk of gene mutations and miscarriage, congenital malformations and increased incidence of diseases in the offspring (Aitken & Curry, 2011; Aitken et al., 2016; Aitken & Koppers, 2011; Bisht & Dada, 2017; Muratori et al., 2015; Sakkas & Alvarez, 2010; Wright et al., 2014). The role of OS in male infertility is well established and has led to a number of direct and indirect laboratory tests to measure OS in the semen.

3 | LABORATORY EVALUATION OF OXIDATIVE STRESS

OS levels can be evaluated by various direct and indirect methods, which are listed in Table 1 and further described below.

3.1 | Direct methods

Direct tests measure the concentration of oxidant molecules and include chemiluminescence assay, nitro blue tetrazolium (NBT) assay, cytochrome C reduction test, electron spin resonance technique and oxidation–reduction potential (ORP).

3.1.1 | ROS measurement by chemiluminescence assay

Chemiluminescence assay is the most widely used direct test for quantification of ROS in semen (Agarwal, Ahmad, & Sharma, 2015). The two commonly used probes for measurement of ROS are luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (10,10'-dimethyl-9,9'-biacridiniumdinitrate). Luminol is a yellow-coloured, membrane permeable cyclic diacylhydrazide used to detect both global intracellular and extracellular ROS. However, it cannot discriminate the different types of oxidants. Lucigenin is a membrane-impermeable probe used to measure the extracellular $O_2^{-\bullet}$(Agarwal, Ahmad, et al., 2015). When measuring global ROS with luminol, a working solution (5 mM) of luminol is prepared in dimethyl sulfoxide (DMSO) and a blank, negative and a positive control is used along with the test sample. (Figure 2). The analysis is conducted using a luminometer (Figure 3). The chemiluminescent signals are expressed in relative light units (RLU). The results for test samples are obtained by subtracting the average RLU of negative control from the test sample (Agarwal, Ahmad, et al., 2015). The samples are normalised for sperm concentration by dividing the ROS value by the sperm concentration and results are expressed as RLU/10^6 sperm/mL of semen (Agarwal, Ahmad, et al., 2015). A cut-off of <102.2 RLU/s/10^6 sperm/mL can discriminate fertile from infertile men (Agarwal, Ahmad, et al., 2015).

3.1.2 | Nitroblue tetrazolium test

Nitroblue tetrazolium test (NBT) assesses the intracellular ROS produced by spermatozoa and leucocytes. Nitroblue tetrazolium (2,2'-bis (4-nitrophenyl)-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride is a yellow-coloured water-soluble nitro-substituted aromatic tetrazolium compound. In the presence of cellular $O_2^{-\bullet}$, it is converted to a water-insoluble formazan crystal (Baehner, Boxer, & Davis, 1976; Gosalvez, Tvrda, & Agarwal, 2017; Tunc, Thompson, & Tremellen, 2010). In the sperm cytoplasm, NADPH is synthesised by glucose-6-phosphate dehydrogenase via the hexosemonophosphate pathway. NADPH contributes to the synthesis of superoxide anions by NADPH oxidase. The same enzyme catalyses the reduction of NBT into formazan. It also indirectly provides a measure for ROS generation in cytoplasm. The reduced formazan is easily detected microscopically (Esfandiari, Sharma, Saleh, Thomas, & Agarwal, 2003) or spectrophotometrically (Gosalvez et al., 2017; Tunc et al., 2010). Alternately, it can also be detected using kit such as Oxisperm®kit (Halotech®DNA).

3.1.3 | Cytochrome C reduction test

The Cytochrome C reduction test is a colorimetric assay used to detect extracellular $O_2\bullet^-$ released by cells and causes the reduction

TABLE 1 Direct and indirect assays used to evaluate sperm DNA fragmentation

Test	Test principle
Direct	
Comet assay	Evaluates the integrity of DNA, double- and single-strand breaks
TUNEL assay	Evaluates DNA fragmentation, double- and single-strand breaks
Indirect	
SCSA	Evaluates the susceptibility of sperm DNA to acid denaturation
SCD or Halo test	Evaluates the susceptibility of sperm DNA to acid denaturation
AOT	Evaluates double- and single-strand breaks

Abbreviation: AOT, acridine orange test; SCSA, sperm chromatin structure assay; SCD, sperm chromatin dispersion; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

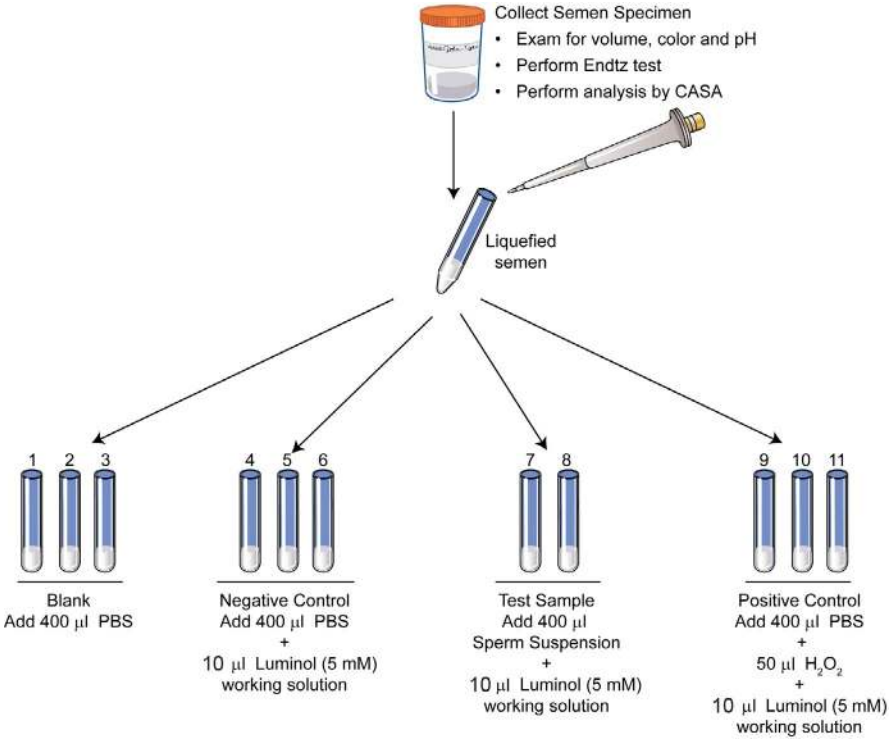


FIGURE 2 Set-up of the tubes for reactive oxygen species measurement. A total of 11 tubes are labelled from S1–S11: Blank, negative control, test sample and positive control. Luminol is added to all tubes except the blank. Hydrogen peroxide is added only to the positive control

FIGURE 3 Autolumat 953 Plus Luminometer used in the measurement of reactive oxygen species (ROS) by chemiluminescence assay. Multiple tubes can be loaded simultaneously for measuring ROS. The luminometer can be connected with a computer and monitor

of ferricytochrome C to ferrocytochrome C by NADPH dependent cytochrome C reductase. The reaction is analysed spectrophotometrically at 550nm absorbance (Dikalov & Harrison, 2014). Various commercially available kits can be used with a cuvette or in a microplate. The spectrophotometer is set at 550 nm in the kinetic programme. A blank ($NADH^+$ Buffer) and a positive control ($NADPH^+$ Cytochrome C Reductase enzyme) are included in the assay. The test is performed by adding superoxide dismutase (SOD) to selectively measure $O_2\bullet^-$ mediated reduction (Dikalov & Harrison, 2014). Dismutation of $O_2\bullet^-$ into (H_2O_2) is catalysed by SOD, and the resulting SOD-inhibited signal is used to normalise the results. The results are expressed as NADPH-Cytochrome c Reductase (NCR) unit. One unit of NCR activity is the enzyme that generates 1 micromole of reduced cytochrome C per minute.

3.1.4 | Electron spin resonance/electron paramagnetic resonance

Electron spin resonance (ESR) spin-trapping uses the nitrone compound to detect oxygen free radicals such as $O_2\bullet^-$ and HO• utilising magnetic resonance spectroscopy (Kohno, 2010). Electrons are characterised by the angular momentum of the electron demonstrated as a spin quantum number. When an external magnetic field is applied, the electrons orient themselves either parallel or antiparallel to the magnetic field. This results in two distinct energy levels for the unpaired electrons. By using a fixed frequency of microwave irradiation, some of the electrons in the lower energy level are excited to the higher energy level leading to absorption of energy (Kopáni, Celec, Danišovič, Michalka, & Biró, 2006).

ROS molecules can be detected by the using the 'spin-trap' strategy as well as by hydroxylamine spin probes because they have a very short half-life (Dikalov, Polienko, & Kirilyuk, 2018; Kohno, 2010). The spin-trap approach is based on diamagnetic nitrone or nitroso compounds which 'trap' a radical molecule, generating a paramagnetic spin adduct radical, which are detectable by ESR. These adducts have a specific 'signature' electron paramagnetic resonance spectrum based on the type of trapped radical. 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) is the most common nitrone spin-trapping molecule. Hydroxylamine spin probes are different from spin-trap compounds; they do not bind the radicals but they form stable nitroxide when they are oxidised. The oxidation process requires the transfer of one electron. The most common electron is $O2^{-\bullet}$ (Dikalov et al., 2018; Kohno, 2010). ESR measurements provide information about the quantity, type, nature, surrounding environment and behaviour of unpaired electrons (Dikalov et al., 2018; Kohno, 2010).

3.1.5 | Oxidation–reduction potential

Oxidation–reduction potential (ORP) is a new metric of redox equilibrium in a specified biological system. It measures the transfer of electrons from antioxidants (reductants) to oxidants. The MiOXSYS system comprises of an Analyzer consisting of an ultrahigh impedance electrometer (Rael, Bar-Or, Kelly, Carrick, & Bar-Or, 2015) and a sensor with the reference and the working electrodes. To perform the test, a MiOXSYS sensor is placed on the sample port and 30 μl of the sample is placed on the pre-inserted sensor (Figure 4). The sample fills the reference electrode on the sensor, completing the electrochemical circuit and the test then commences (Agarwal, Sharma, Roychoudhury, Du Plessis, & Sabanegh, 2016). The test is based on the electrical conductance relative to an internal reference standard (Shapiro, 1972) according to Nernst equation.

$$E\,(\mathrm{ORP}) = E_0 - RT/nF,$$

Where E is the Redox potential or ORP. E_0 is the standard potential of a redox system measured with respect to hydrogen electron, which is arbitrarily assigned an E_0 of 0 volts. R = gas constant. T = absolute temperature measured in degrees Kelvin. n = number of moles of electrons transferred in the balanced equation for the reaction occurring in the cell. F = Faradays constant.

This galvanostat-based system measures the static ORP (sORP) by measuring the voltage between the reference and the working electrode. MiOXSYS analyzer detects the voltage between the reference and the working electrodes at an interval of 0.5 s, and the final ORP is calculated after ~120 s; an average of the last 10 s (or the last 20 readings) of the run. ORP results are expressed in millivolt (mV) and are a snapshot of the balance of the redox system. ORP is manually normalised by dividing with the sperm concentration to give the adjusted ORP and expressed as mV/10^6 sperm/mL (Agarwal al., 2016). A cut-off of 1.34 mV/10^6 cells/mL was shown to discriminate samples based on semen quality (Agarwal et al., 2019). The advantages and of disadvantages of direct tests are listed in Table 2.

3.2 | Indirect tests

Indirect tests measure the concentration of antioxidants or the ROS-induced damage on cellular components such as lipids, DNA or proteins (Table 3). The advantages and disadvantages of indirect tests are shown in Table 3.

3.2.1 | Endtz test

Leucocytes, particularly polymorphonuclear neutrophils and macrophages, can produce significantly higher levels of ROS than abnormal spermatozoa and negatively affect sperm quality (Sharma, Pasqualotto, Nelson, & Agarwal, 2001). White blood cells (WBC) can be differentiated from the immature germ cells by the peroxidase or the Endtz test (Shekarriz, Sharma, Thomas, & Agarwal, 1995). The Endtz test is based on the evaluation of peroxidase, an enzyme present in the leucocyte granules which utilises H_2O_2 to oxidise the colourless substrate benzidine to an insoluble blue/brown derivative (Agarwal, Gupta, & Sharma, 2016b). The test can differentiate peroxidase positive granulocytes such as neutrophils, polymorphonuclear leucocytes and macrophages from other germ cells (Endtz, 1974). The concentration of seminal leucocytes more than 1x10^6 WBC/mL of the sample is indicative of leucocytospermia (Cooper et al., 2010; World Health Organization, 2010).

3.2.2 | Measurement of lipid peroxidation products

Unlike the somatic cells, the lipid bilayer in the plasma membrane of the human spermatozoa has high levels of PUFAs, which render them particularly susceptible to damage caused by excess ROS (Alvarez & Storey, 1995; Mack, Everingham, & Zaneveld, 1986; Poulos, Darin-Bennett, & White, 1973). The breakdown of PUFAs to form lipid peroxides is known as lipid peroxidation (Halliwell & Chirico, 1993). Lipid peroxidation generates end products such as

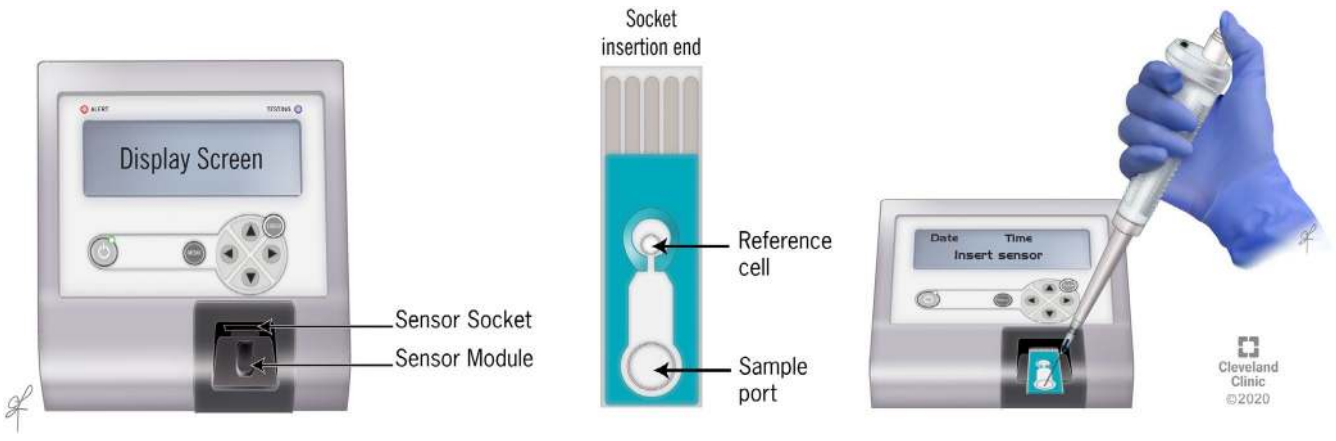


FIGURE 4 Measurement of ORP by the MiOXSYS system. (a) MiOXSYS analyzer showing the socket and the sensor module, (b) sensor showing the reference cell and the sample port where the sample is loaded and (c) loading of semen samples on the sample port of the sensor strip

TABLE 2 Advantages and disadvantages of direct tests used to measure oxidative stress levels in the seminal plasma

Test	Advantages	Disadvantages
Chemiluminescence assay	1. Robust 2. High sensitivity and specificity	1. Requires large sample volume 2. Time-consuming 3. Expensive equipment 4. Cannot be used to measure ROS in frozen, viscous or azoospermic samples 5. Affected by changes in pH, centrifugation and the presence of NADPH, cysteine, ascorbic acid or uric acid
Nitroblue tetrazolium test	1. Readily available, inexpensive and has high sensitivity 2. Provides information about the differential contribution of leucocytes and abnormal spermatozoa in the production of ROS i.e. the cellular origin of ROS in the sample 3. It detects intracellular ROS, therefore it can be used to discriminate the cellular source of ROS in a heterogeneous cell population 4. It can detect much lower concentrations of (neutrophils) leucocytes (0.5×10^6/ml) than he established World Health Organization (2010) cut-off for leucocytospermia (1.0×10^6/ml)	1. Presence of other cellular reductases may also reduce NBT 2. Changes in the cellular content of various oxido-reductases may also alter the rates of NBT reduction 3. Specificity of ROS detected is questionable because the assay is based on the reduction of NBT
Cytochrome C reduction test	1. It can quantify O_2•− released during the respiratory burst of neutrophils or by isolated enzymes 2. Can measure high level of ROS production	1. Electrons donated from enzymes and other molecules can directly reduce ferricytochrome c, and the resultant change in absorbance is not specific for O_2•− 2. Small quantities of O_2•− cannot be detected. The enzyme cannot access to the intracellular space, so, only the extracellular ROS fraction can be detected
Electron spin resonance: Spin-trap	1. Spin trap can differentiate between different kinds of oxidative molecules 2. In Hydroxylamine Spin, oxidation of hydroxyl spin probes generates very stable nitroxide, whose half-life can last hours	1. The spin-trap can be chemically modified by several enzymes 2. Hydroxylamine Spin generates the same type of nitroxide, therefore cannot discriminate between different oxidative molecules. 3. Probes are basic and at physiological pH are partially inactive in hydroxylamine spin 4. Spontaneous oxidation of hydroxylamine increases in presence of transition metal ions, therefore addition of Fe/Cu chelators to solutions and buffer is required 5. Adduct formation may be hampered by the scavenging action of antioxidants in both techniques
Oxidation–reduction potential	1. Simple, rapid (less than 4 min) method. 2. Can be used in fresh and frozen samples without any prior treatment 3. High sensitivity and specificity 4. Results are stable for up to 2 hr. 5. It estimates all the oxidants and antioxidants present in a given sample (and not just one end of the spectrum as do other tests that detect oxidative stress directly or indirectly) 6. ORP measurements are more stable than standard semen analysis 7. Can discriminate semen samples based on sperm parameters or fertility status	1. Viscous samples are difficult to load and can affect the reading 2. Cannot be used to analyse azoospermic samples

Abbreviations: NBT, nitroblue toluidine; ROS, reactive oxygen species; WHO, World Health Organization.

malondialdehyde (MDA), hydroxynonenal, 2-propenal (acrolein) and isoprostanes, all of which are indicators of OS (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006). These unstable lipid peroxides decompose to form cytotoxic second messengers of oxidative stress such as MDA, 4-hydroxynonenal (4-HNE) and acrolein (ACR). These complex compounds modify relatively more stable DNA, lipids and proteins thereby altering sperm functions (Spickett, 2013; Zarkovic, 2003).

TABLE 3 The advantages and disadvantages of indirect tests used to measure oxidative stress levels in the seminal plasma

Test	Advantages	Disadvantages
Myeloperoxidase	1. Rapid, easy to perform and inexpensive 2. Recommended by WHO to assess leucocytospermia in semen 3. $> 1 \times 10^6$ peroxidase positive WBC/mL of semen (leucocytospermia)	1. Peroxidase-positive leucocytes (PMNs and macrophages) account for 50%–60% and 20%–30% respectively of all seminal leucocytes. 2. Cannot detect the ROS generation by spermatozoa
Total antioxidant capacity	1. Measures all antioxidants in seminal plasma 2. Automated 3. Established cut off ≥1950 μM Trolox indicative of good antioxidant reserves	1. Requires expensive assay kit and microplate reader
Lipid peroxidation		
HNE-HIS adduct ELISA	1. Rapid	1. Cross reactivity
Malondialdehyde assay	1. Simple, Measures lipid peroxidation 2. Detects MDA-TBA adduct by colorimetry or fluoroscopy	1. Expensive instrumentation 2. Rigorous controls required 3. Nonspecific for MDA
DNA fragmentation	1. Multiple methods available – TUNEL, SCSA, Comet, SCD, and 8-OHdG 2. Comet assay is simple, versatile, sensitive and rapid assay and has demonstrated some correlation with other assays such as SCSA and TUNEL 3. SCD test is simple, fast, and reproducible with comparable results to those of the SCSA 4. SCSA and TUNEL with flow cytometry is robust and sensitive method 5. TUNEL is a direct method that measures single- and double-strand DNA breaks	1. Multiple cut-offs 2. Inter- and intra-observer variability 3. Lack of standardised reference value 4. The 8-OHdG technique can itself cause DNA oxidation interfering with basal level 5. Cost of is a major concern in TUNEL and SCSA assays
Post-translational modifications	1. Commercially available assay kits	1. Costly and time consuming 2. Does not provide an overall assessment of the OS
Protein alterations	1. Highly specific and sensitive 2. Selected proteins can be validated by western blot analysis, ELISA or immunochemistry. Sperm protein alterations specific to male infertility conditions can be identified	1. Requires expensive instrumentation, software programs and specific skills 2. Costly and time consuming

Abbreviation: 8-OHdG, 8-hydroxy-2-deoxyguanosine; ELISA, enzyme linked immunosorbent assay; MDA, malondialdehyde; OS, oxidative stress; OS, oxidative stress; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; SCD, sperm chromatin dispersion; SCSA, sperm chromatin structure assay; TBA, thiobarbituric acid; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling; WBC, white blood cell; WHO, World Health Organization.

3.2.3 | Malondialdehyde measurement by thiobarbituric acid—reactive substances (TBARS) assay

Malondialdehyde (MDA) is a reactive and mutagenic end product of lipid peroxidation in semen and can be measured using the TBA assay. In this assay, MDA (an aldehyde) derived from peroxides and unsaturated fatty acids binds with 2 molecules of TBA to form the MDA-TBA adduct which is measured colorimetrically or fluorometrically (Halliwell & Chirico, 1993). MDA levels in the seminal plasma are 5–10-fold higher compared with those in the spermatozoa (Tavilani, Doosti, & Saeidi, 2005). Low concentrations of sperm MDA can be detected by the sensitive high-performance liquid chromatography (Li, Shang, & Chen, 2004; Shang et al., 2004), or by spectrofluorometry (Aitken, Harkiss, & Buckingham, 1993).

Malondialdehyde levels show a significant positive correlation with seminal ROS levels in men with infertility, compared with fertile controls or normozoospermic individuals, highlighting its clinical utility (Hsieh, Chang, & Lin, 2006; Tavilani et al., 2005). ROS-induced abnormalities in motility, sperm DNA integrity and sperm-oocyte fusion are associated with an increase in MDA concentration (Aitken, Clarkson, & Fishel, 1989; Aitken et al., 1993).

Highly reactive aldehyde 4-hydroxynonenal (4-HNE), an end product of lipid peroxidation, is a second messenger of free radicals and a signalling molecule. HNE-protein adducts are quantified using ELISA with a monoclonal antibody (Borovic, Rabuzin, Waeg, & Zarkovic, 2006). 4-HNE may impair sperm capacitation by targeting the protein kinase A affecting the tyrosine phosphorylation pathway and thus reducing sperm motility (Baker et al., 2015).

Peroxidation of arachidonic acid results in the formation of isoprostanes which are a series of prostaglandin F2-like compounds. They can be determined by quantification of the amount of F2-isoprostanes (F2-IsoP) present in the semen (Signorini, Comporti, & Giorgi, 2003; Signorini et al., 2008).

8-iso-PGF2α is determined by immunocytochemical staining with polyclonal antibody and visualised under fluorescence microscope. Significantly higher levels of free 8-iso-PGF2α levels in the seminal plasma are reported in infertile men when compared with normozoospermic men (Khosrowbeygi & Zarghami, 2007). In addition, it negatively correlates with MDA and seminal SOD activity in normozoospermic men (Tavilani et al., 2008).

3.2.4 | Measurement of total antioxidants in seminal plasma

Total antioxidant capacity (TAC) is the measure of the reducing capacity of seminal antioxidants against an oxidative reagent. It evaluates enzymatic antioxidants in the seminal plasma such as superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic antioxidants such as α-tocopherol (vitamin E), ascorbate (vitamin C), β-carotene (vitamin A), folic acid (vitamin B9), ferritin and carnitines, N-acetyl L-cysteine, coenzyme Q10, ceruloplasmin, selenium, L-arginine, urate and zinc (Henkel, Sandhu, Agarwal, & A., 2019; Mahfouz, Sharma, Lackner, Aziz, & Agarwal, 2009a; Mahfouz, Sharma, Said, Erenpreiss, & Agarwal, 2009b). Over the years, a number of assays have been proposed which include colorimetric, spectrophotometric and chemiluminescence-based assays.

In the colorimetric assay, the clear seminal plasma is used with the TAC kit. The principle of the assay is based upon the ability of all antioxidants in the seminal plasma to inhibit the oxidation of the 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS+. (Agarwal Gupta, & Sharma, 2016a, 2016b, 2016c, 2016d). The antioxidants in the seminal plasma suppress the absorbance at 750 nm; this suppression is proportional to the concentration of antioxidant. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogueis used as the standard. The amount of antioxidant in a given sample is measured by a colorimeter (Agarwal, Gupta, & Sharma, 2016a, 2016b, 2016c, 2016d). Antioxidant level is reported as micromoles of Trolox equivalent. The normal antioxidant concentration in a sample is >1,950 micromoles of Trolox equivalent (Figure 5). Lower TAC values reflect increased OS or poor ROS scavenging ability. Although this test is rapid, it does not measure any specific enzymatic antioxidants (Roychoudhury, Sharma, Sikka, & Agarwal, 2016).

3.2.5 | ROS-TAC score

ROS-TAC score is derived from the individual values obtained for ROS levels and seminal TAC values. Principal component analysis is used to analyse the standardised values of ROS and TAC. The linear combinations or weighted sums accounts for the most variability among correlated variables. The ROS and TAC values of the control samples are used to generate a reference point (Sharma, Pasqualotto, Nelson, Thomas, & Agarwal, 1999).

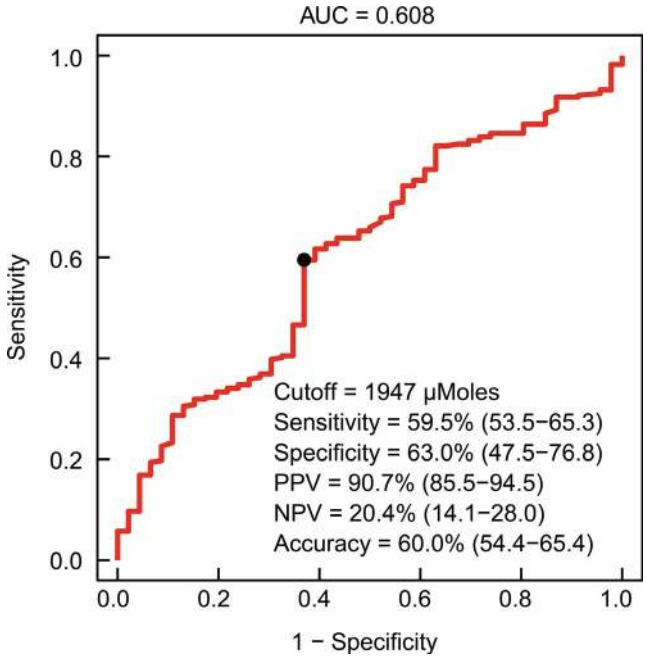


FIGURE 5 Total antioxidant capacity receiver operating characteristics (ROC) curve showing the area under curve (AUC), cut-off, sensitivity, specificity, positive predictive value, negative predictive value and accuracy of the assay

Patients with high OS have a lower ROS-TAC score than do fertile healthy men (Sharma et al., 1999). ROS-TAC score can discriminate between fertile and infertile men better than ROS or TAC alone (Sharma et al., 1999). Significantly lower ROS–TAC scores than controls are seen in infertile men with male factor or idiopathic infertility. Furthermore, significantly higher ROS–TAC scores are seen in men with male factor diagnoses who are able to initiate a successful pregnancy compared to those who do not. Male partners of couples who achieve pregnancy have ROS–TAC scores similar to those of the controls. ROS–TAC score may help identify patients with a clinical diagnosis of male infertility who are likely to achieve a pregnancy over a period of time (Pasqualotto, Sharma, Pasqualotto, & Agarwal, 2008).

3.2.6 | Measurement of sperm DNA fragmentation

OS and apoptosis contribute to sperm DNA fragmentation (SDF; Henkel et al., 2005; Mahfouz et al., 2010; Mahfouz et al., 2009a; Mahfouz et al., 2009b; Sakkas et al., 1999). In addition, advanced age, cigarette smoking, chemotherapy, radiation, cancer, varicocele, leucocytospermia and elevated levels of ROS all contribute to SDF (Agarwal, Varghese, & Sharma, 2009; Zini, Boman, Belzile, & Ciampi, 2008). Several authors have demonstrated high amount

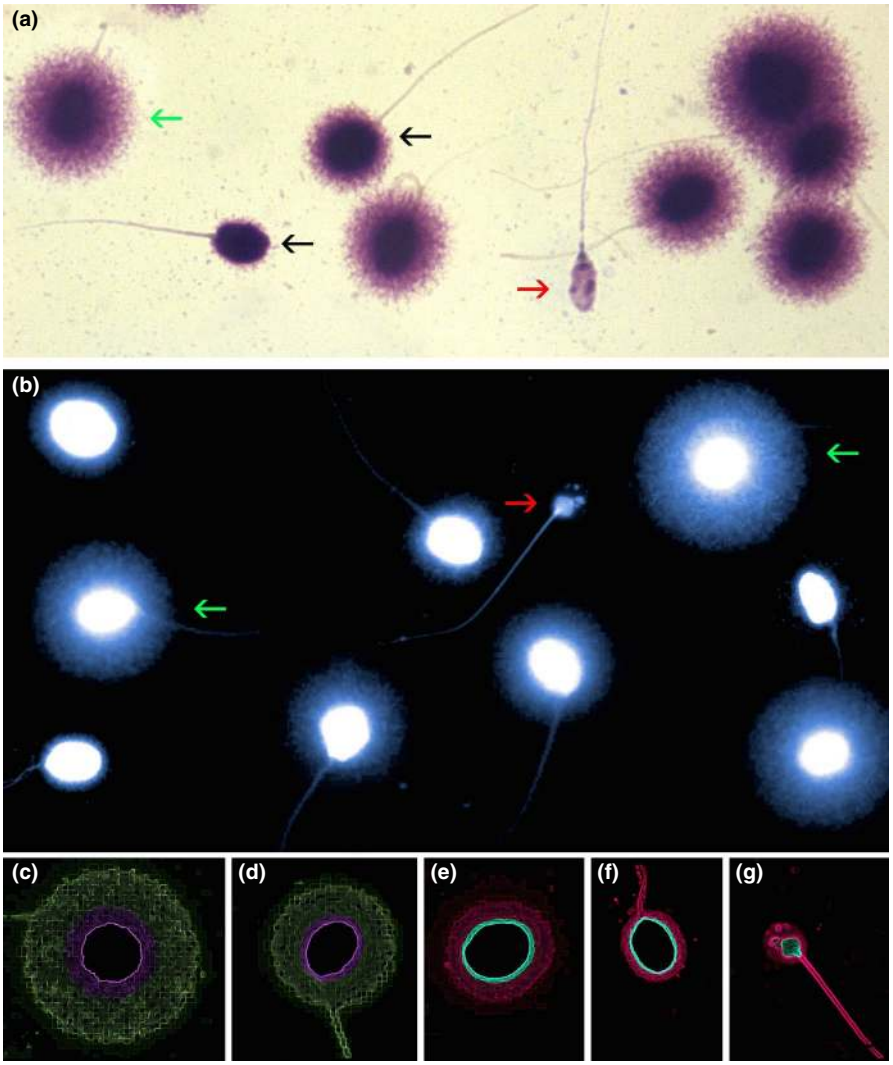


FIGURE 6 Assessment of sperm DNA fragmentation using the sperm chromatin dispersion (SCD) test. Nucleoids from human spermatozoa obtained with the improved SCD procedure (Halosperm, Halotech DNA, SL, Madrid, Spain) under (a) bright-field microscopy and Wright's stain (b) under fluorescence microscopy and DAPI staining. Green arrows target spermatozoa containing a normal DNA molecule. Red arrows target a highly fragmented spermatozoon (degraded spermatozoa). (c–g) Electronic filtered images showing a series of nucleoids with different levels of sperm DNA damage. Nucleoids with highlighted core delineation in green correspond to (c) large (d) and medium halos of dispersed chromatin representing a normal DNA molecule. Nucleoids in red are spermatozoa containing fragmented DNA and are represented by (e) small or (f) no halos of dispersed chromatin and (g) degraded spermatozoa. Bright-field and fluorescence microphotographs were obtained using a motorised fluorescence microscope controlled with software for automatic scanning and image digitisation (Leica Microsystems). The microscope was equipped with a Leica EL6000 metal halide fluorescence light source and Plan-Fluotar 60 × objectives with three independent filter blocks (DAPI-5060B; FITC- 3540B and TRITC-A; Semrock). A charge-coupled device (Leica DFC350 FX, Leica Microsystems) was used for image capture. (Courtesy of Prof. Jaime Gonsálvez, Madrid, Spain)

of impaired DNA integrity in infertile men (Garolla et al., 2015; Majzoub, Agarwal, Cho, & Esteves, 2017; Ribas-Maynou et al., 2013).

The more commonly used tests are summarised below.

Sperm chromatin dispersion assay

In this assay, spermatozoa are treated with an acid solution prior to lysis buffer, spermatozoa with fragmented DNA produce a minimal halo (Fernández et al., 2003). When spermatozoa with non-fragmented DNA are immersed in an agarose matrix and directly exposed to lysing solutions, the resulting deproteinised nuclei (nucleoids) show a central (core) and peripheral halo of dispersed DNA caused by release of DNA loops. These halos can be observed either by bright-field microscopy using Wright's stain or fluorescent microscopy with DAPI (4′,6-diamidino-2-phenylindole; 2 μg/ml; Feijó & Esteves, 2014; Fernández et al., 2005; Figure 6). SDF is negatively correlated with fertilisation rates and embryo quality in IVF/ICSI. However, SDF was not related with clinical pregnancy rates or births (de la Calle et al., 2008; Muriel et al., 2006).

Comet assay or Single-cell gel electrophoresis

The comet assay, also called the single-cell gel electrophoresis, is based on the electrophoretic migration of cleaved fragments of

DNA. It is based on the principle that the negatively charged DNA loops or fragments are drawn through an agarose gel in response to an electrical field. Neutral comet assay detects only double-stranded DNA breaks, whereas alkaline comet assay detects both single- and double-stranded DNA breaks (Singh et al., 1989; Singh, McCoy, Tice, & Schneider, 1988).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling (TUNEL) assay

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling or the TUNEL assay uses a template-independent DNA polymerase enzyme called terminal deoxynucleotidyl transferase (TdT), which adds deoxyribonucleotides to 3′ hydroxyl (OH) end of DNA breaks (Figure 7). TdT enzyme adds fluorescent deoxyuridine triphosphate (dUTP) to the free 3'-OH break-ends of DNA and are quantified by flow cytometry (Agarwal et al., 2016c, 2016d; Gupta, Sharma, & Agarwal, 2017; Sharma, Ahmad, Esteves, & Agarwal, 2016; Sharma et al., 2010). TUNEL assay measures both single- and double-strand DNA fragmentation.

DNA fragmentation can be measured using an APO-DIRECT Kit (BD Pharmingen™). The percentage of negative (TUNEL-negative) and positive (TUNEL-positive) cells is calculated on the flow cytometer software (Figure 8a–e). The reference value of 16.7% has been established for DNA fragmentation (Figure 9a). Significantly higher levels of SDF are seen in infertile men compared with control (healthy men) (Agarwal et al., 2016a; Agarwal et al., 2016b; Agarwal et al., 2016c; Agarwal et al., 2016d; Gupta et al., 2017; Sharma et al., 2016) (Figure 9b).

Sperm Chromatin Structure Assay (SCSA)

Impaired sperm chromatin is susceptible to partial DNA denaturation induced in situ either with heat or acid treatment. This change in the conformation following acid or heat treatment is determined by measuring the metachromatic shift of acridine orange fluorescence from green (native DNA) to red (denatured DNA). The extent of SDF measured as % of sperm with fragmented DNA is called DNA fragmentation index or DFI (Evenson, 2016).

$$\text{Percent DFI} = \frac{\text{Red fluorescence}}{\text{Total(red + green)fluorescence}} \times 100.$$

Intact spermatozoa (native double stranded) fluorescent green whereas those with fragmented DNA (single-stranded DNA) fluoresce red. SCSA is sensitive and robust assay that accurately measures the percentage of DNA-damaged spermatozoa. A DFI of 30% can differentiate between fertile and infertile samples (Evenson, Larson, & Jost, 2002; Evenson & Wixon, 2006).

8-hydroxy-2-deoxyguanosine (8-OHdG)

8-hydroxy-2-deoxyguanosine (8-OHdG) assay measures levels of 8-hydroxy-2-deoxyguanosine, which is a byproduct of oxidant-induced DNA damage in the spermatozoa. It can be detected at a single-cell level using 8-OHdG specific antibodies producing fluorescence that can be visualised by flow cytometry or fluorescent microscopy (Vorilhon et al., 2018).

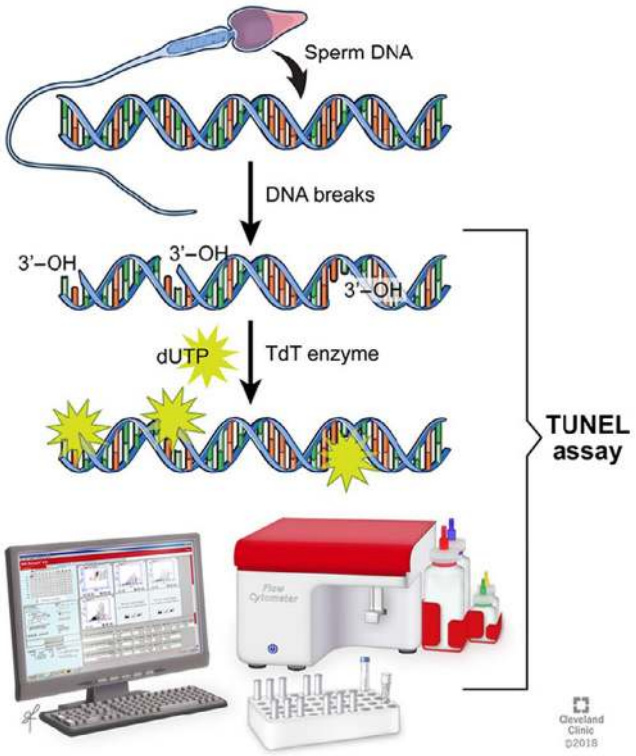


FIGURE 7 Schematic of the DNA staining principal by the TUNEL assay

8-OHdG provides the most direct evidence that SDF due to oxidative stress is involved in male infertility. 8-OHdG is inversely associated with sperm concentration (Kodama, Yamaguchi, Fukuda, Kasai, & Tanaka, 1997). SDF measured by 8-OHdG and TUNEL assay are highly correlated with each other (Aitken, De Iuliis, Finnie, Hedges, & McLachlan, 2010). In addition, 8-OHdG levels are highly correlated with the extent of disruption of chromatin remodelling (De Iuliis et al., 2009).

3.2.7 | Detection of post-translational modifications of proteins

Spermatozoa do not have an active protein synthesis; however, a dynamic change in protein profile occurs as a result of acquisition of new proteins through vesicular transport and several post-translational modifications (PTMs) (Samanta, Swain, Ayaz, Venugopal, & Agarwal, 2016). PTMs are chemical alterations in the protein structure that are typically catalysed by substrate-specific enzymes. These modifications regulate the stability, distribution and function of proteins. Post-translational modifications increase the diversity of the proteome, and specific modifications are introduced that can be translated into functional changes in the affected proteins (Brohi & Huo, 2017).

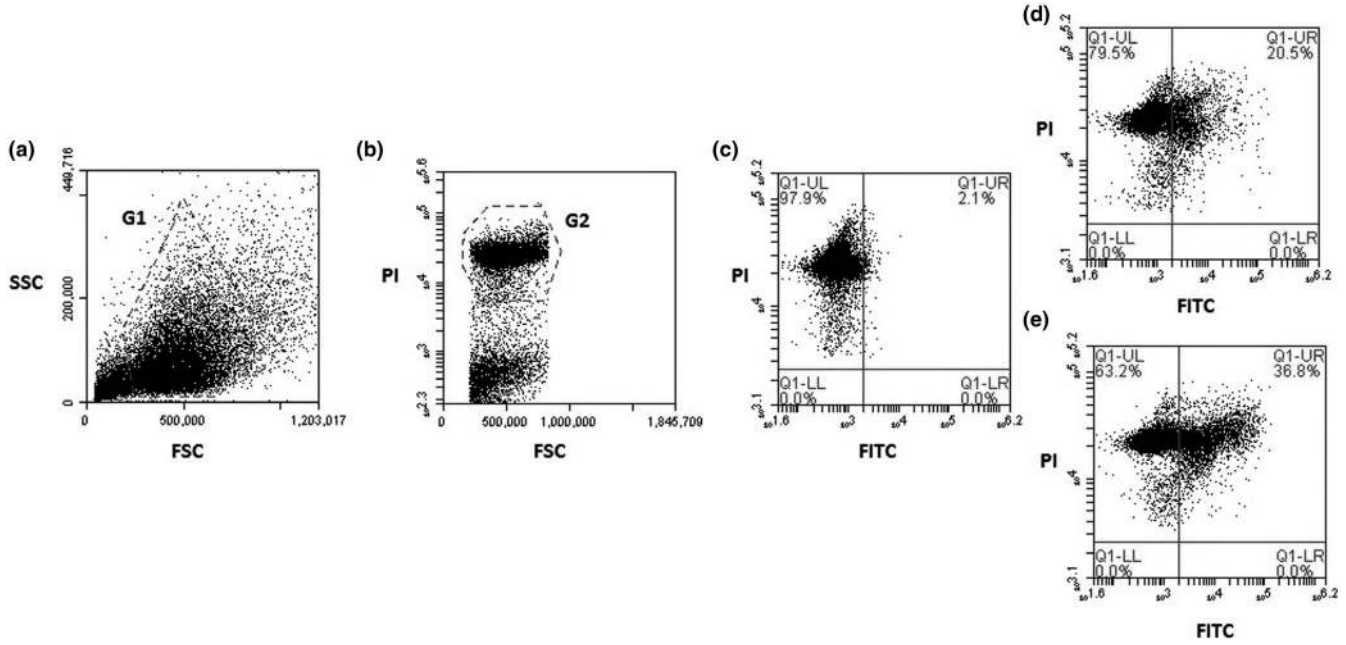
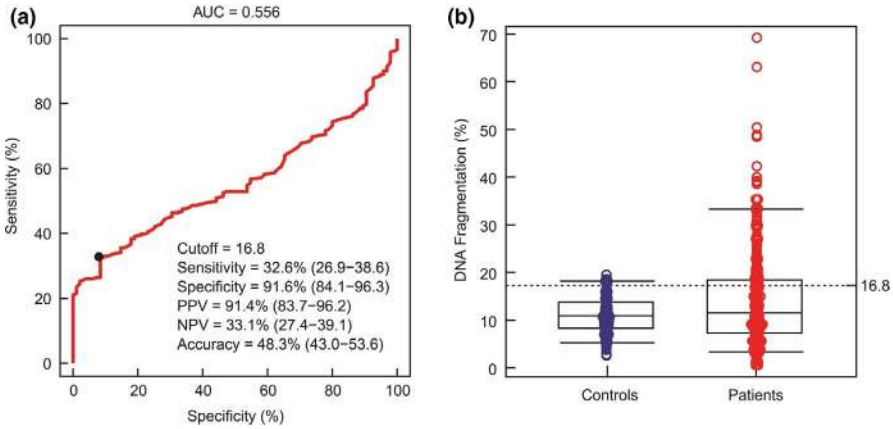


FIGURE 8 Example of Accuri C6 Workspace and gating strategy used in both laboratories for TUNEL data analysis. (a) FSC/SSC plot showing the gate used for spermatozoa selection (G1). (b) PI/FSC plot with gating for PI positivity (G2). (c) PI/FITC plot of negative control sample (TdT enzyme omitted). (d) PI/FITC plot of standard sample. (e) PI/FITC plot of positive control sample. FSC, forward scatter; SSC, side scatter; PI, propidium iodide fluorescence; FITC, fluorescein isothiocyanate fluorescence; Q1-UL, upper left quadrant; Q1-UR, upper right quadrant; Q1-LL, lower left quadrant; Q1-LR, lower right quadrant

FIGURE 9 Receiver operator characteristic (ROC) curve showing (a) TUNEL cut-off and the area under the curve. Values within the parentheses represent the 95% confidence interval and (b) distribution of TUNEL values between controls and infertile men



Failure to control complex molecular processes can be detrimental or lethal for the cell. There are about 300 reported covalent modifications and of these modifications, the most common are phosphorylation, nitrosylation, glycosylation, methylation, lipid modification, ubiquitination and acetylation (Zhou et al., 2015). Protein modifications by ROS results in formation of end products such as s-glutathione, nitrotyrosine and reactive carbonyls (Dalle-Donne et al., 2006; Radi, 2004; Samanta et al., 2016). These modifications result in altered protein functions that can be measured by ELISA using protein-specific antibodies (Agarwal, Sharma, et al., 2015; Esteves et al., 2015; Radi, 2004). PTMs are overexpressed in the seminal plasma proteome of men with high levels of ROS when compared to fertile control group (Agarwal, Ayaz, et al., 2015).

3.2.8 | Proteomic analysis of spermatozoa and seminal plasma

Protein alterations present both in the spermatozoa and seminal plasma vary at different levels of OS (Agarwal, Ayaz, et al., 2015; Sharma et al., 2013). Exposure of seminal proteome to different amounts of OS showed that proteins involved in biomolecule metabolism, protein folding and degradation were differentially modulated in infertile patients when compared to fertile controls (Agarwal, Ayaz, et al., 2015; Sharma et al., 2013). Exposure of the sperm proteome to different levels of OS in infertile men showed differentially expressed proteins (DEPs) exhibiting distinct reproductive functions (Ayaz et al., 2015). These modified DEPs can be identified by proteomic and bioinformatic analysis and validated as a

potential biomarkers of ROS by Western blot analysis using protein-specific antibodies or ELISA followed by immunochemistry. Most commonly employed techniques to detect sperm-specific protein alterations utilising proteomic analysis include 2D polyacrylamide gel electrophoresis (2D-PAGE), differential in gel electrophoresis (DIGE) and liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS). Global proteomic analysis involves analysis of pooled or individual test samples of spermatozoa or seminal plasma (Agarwal et al., 2016; Ayaz et al., 2015; Samanta et al., 2016). The quantification and identification of a target protein can be achieved by Western blot analysis, (Agarwal et al., 2016) or by immunocytochemistry (Engvall, Jonsson, Jonsson, & Perlmann, 1971; Mathews, Plaisance, & Kim, 2009; Samanta et al., 2018).

3.3 | Measurement of intracellular ROS

Intracellular ROS is measured by flow cytometry using two specific dyes 2',7' dichlorofluorescin diacetate (DCFDA) and dihydroethidium or hydroethidine (DHE). DCFDA is deacetylated within the sperm cell and oxidised by ROS to form 2',7'-dichlorofluorescein (DCF), a fluorescent green compound. It measures peroxyl, alkoxyl, $NO_2^{\bullet}$, carbonate ($CO_3^{\bullet}-$) and $OH^{\bullet}$ radicals (Mupfiga et al., 2013). Peroxynitrite, hypochlorous acid and OH• in defective spermatozoa can also oxidise DCF and significantly contribute to the positive signals (Mahfouz et al., 2009a; Mahfouz et al., 2009b; Myhre, Andersen, Aarnes, & Fonnum, 2003). Cellular production of $O_2^{\bullet-}$ can be visualised by oxidation of $O_2^{\bullet-}$ by DHE and results in hydroxylation and formation of 2-hydroxyethidium with red florescence emission at 488 nm (Henkel et al., 2003; Rothe & Valet, 1990; Zielonka, Vasquez-Vivar, & Kalyanaraman, 2008). Furthermore, viable cells generating ROS can be measured by DHE along with a vitality marker (SYTOX green; De Iuliis, Wingate, Koppers, McLaughlin, & Aitken, 2006; Mahfouz et al., 2009a; Mahfouz et al., 2009b). Fluorescent techniques have a higher specificity, accuracy, sensitivity and reproducibility (Gosalvez et al., 2017).

4 | TYPES OF SAMPLES USED FOR ROS MEASUREMENT

ROS levels can be measured in unprocessed seminal ejaculate or semen sample processed by simple wash, swim up, migration-sedimentation or density-gradient centrifugation (Benjamin, Sharma, Moazzam, & Agarwal, 2012).

4.1 | Neat (or unprocessed) seminal ejaculate

Levels of ROS in the neat sample or unprocessed samples are reflective of the ROS produced by spermatozoa and all the other cells present in the seminal ejaculate. These include secretions from prostate, seminal vesicles and other accessory glands and cellular components such as round cells, leucocytes and epithelial cells.

4.2 | Sperm wash

In this method, culture medium is added to the ejaculate and the sample is centrifuged twice and the seminal plasma is removed. However, this does not eliminate other cells such as leucocytes, round cells and debris. The centrifugation force (<500 *g*) and numbers of centrifugation steps must be kept to a minimum in order to reduce the ROS-induced damage by nonviable, morphologically abnormal spermatozoa and leucocytes (Björndahl et al., 2010; Ren, Sun, Ku, Chen, & Wu, 2004).

4.3 | Swim-up

In the conventional swim-up technique, 0.5 ml of post-liquefaction sample is gently placed at the bottom of a tube and layered with about 2 ml of the sperm wash medium. Swim up can also be performed using a prewashed sperm pellet obtained by low speed centrifugation and placing the tube gently at an angle of 45° and incubating for 60 min. Clear supernatant is carefully aspirated using sterile pipette and centrifuged to obtain a highly motile, morphologically normal intact spermatozoa enriched in the absence of other cells, proteins and debris within the supernatant (Agarwal et al., 2016d). Swim-up method is inexpensive; however, the sperm recovery is relatively low and only 5 to 10 per cent of sperm cells are retrieved.

4.4 | Migration-sedimentation

Migration-sedimentation is usually used for samples with low motility. It uses the swim-up technique and the natural settling of spermatozoa due to gravity. It is performed in special tubes called Tea-Jondet tubes (Mortimer, 1994). The amount of ROS produced is not very significant as this technique is gentle (Henkel & Schill, 2003).

4.5 | Discontinuous density gradient

Density gradient separation is a process where the cells are separated based on their density, motility and the centrifugation speed. The gradient is a colloidal suspension of silica particles, which are stabilised with covalently bonded hydrophilic Silane in HEPES. Two distinct gradients are used and consist of a lower phase or 80% gradient and an upper phase or a 40% gradient. Two mL of lower phase is placed in a 15 ml graduated centrifuge tube and 2 ml of upper phase is placed gently on the lower phase. Up to 2 ml of a completely liquefied semen sample is gently layered on top and centrifuged at

300 *g* for 20 min. The highly purified motile sperm cells are enriched in the soft pellet at the bottom. (Agarwal et al., 2016a, 2016b, 2016c, 2016d). Two mL of sperm washing medium (modified HTF with 5.0 mg/ml human albumin) is used to wash the gradient. The sample is centrifuged at 300 *g* for 7 min. The final pellet is resuspended in 0.5 ml of the sperm washing medium. This method allows for the enrichment of mature and motile spermatozoa. Sperm recovery rates of 30%–80% can be achieved depending on the quality of the initial semen sample as well as the technical skill of the operator.

5 | FACTORS AFFECTING THE ROS MEASUREMENT

Semen samples with a higher concentration of morphologically abnormal spermatozoa with excessive presence of cytoplasm will produce higher levels of ROS (De Lamirande & Gagnon, 1995; Plante, de Lamirande, & Gagnon, 1994). The measurement of intracellular ROS will also be affected by the scavenging action of the antioxidants present in the seminal plasma, producing a result lower than the actual ROS concentration at a given time point. Hence, it would be ideal to separate the cells from the reactants-containing seminal fluid. The time of assessment is important wherein viable samples have higher oxidative damage when exposed to ROS for longer periods (Bourne, Archer, Edgar, & Gordan Baker, 2009). The semen samples undergo sperm preparation post-liquefaction by various methods to obtain a rich population of morphologically normal and highly motile spermatozoa. Paradoxically, sperm preparation techniques that include centrifugation also increase the ROS production.

6 | LIMITATIONS OF CURRENT OXIDATIVE STRESS MARKERS

Enzymes and other molecules can modify the reagents used in assays such as cytochrome C reduction test, electron spin resonance and NBT reducing the accuracy of the results. Lipid peroxidation cross-reactivity is a drawback with HNE-HIS adduct ELISA, whereas the reagent used in MDA-TBARS is not specific for MDA. Most of the above described assays require large sample volumes, specific reagents and expensive instrumentation and are laborious. The majority of these assays measure only a single parameter and do not provide a comprehensive assessment of OS. Sperm preparation performed prior to the assay can further enhance the ROS production as a result of repeated centrifugation. Measurement of ORP overcomes majority of these limitations being rapid and cost-effective and can be measured in fresh and frozen samples without any prior sample preparation.

7 | CONCLUSION

Although OS has a central role in male infertility, a physiological level is required for normal sperm function. Both direct and indirect methods for measuring OS are available, but establishing the reference ranges of these markers is challenging. ORP provides a global value of redox potential and can help categorise men with normal, high or low OS. There is ongoing need for more efficient and safer technique that avoids the excessive production of ROS during the preparation of spermatozoa.

Take Home Message

1. There is no 'gold standard' for the evaluation of oxidative stress.
2. The direct and indirect tests for the detection of oxidative stress present different strengths and weakness, which limit their use in clinics.
3. There is an urgent need for laboratory tests that are novel, simple and provide a comprehensive picture of oxidative status in the infertile male.
4. ORP represents a promising marker to be used in the andrology laboratory, as it provides an evaluation of the global redox status, unlike other techniques which assess only the oxidants or the antioxidants.

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