

# Chapter 2

## Methods to Measure Reactive Oxygen Species (ROS) and Total Antioxidant Capacity (TAC) in the Reproductive System

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### 2.1 Introduction

ROS production in the male reproductive tract has become a real concern because of their potential toxic effects on quality and function of sperm [1–3]. ROS is produced by abnormal, immature, morphologically abnormal spermatozoa and contaminated white blood cells especially the polymorphonuclear granulocytes in the seminal ejaculates [4–7]. Examples of ROS include the superoxide anion ( $\bullet\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), the extremely reactive hydroxyl radical ( $\bullet\text{OH}$ ) and the peroxy radical ( $\text{HO}_2^-$ ) [8]. Reactive nitrogen species are often considered to be a subclass of ROS. It includes nitric oxide (NO), nitrous oxide ( $\text{N}_2\text{O}$ ), peroxynitrite ( $\text{NO}_3^-$ ), nitroxyl anion (HNO), and peroxynitrous acid ( $\text{HNO}_3$ ) [5–7, 9]. High levels of ROS have a detrimental effect on sperm concentration [10–16], motility [10, 12, 14, 16], abnormal sperm morphology [12, 17–21] as well as increase DNA damage [22–24], apoptosis [25] and result in sperm dysfunction [26–30]. Increased presence of ROS with progressive depletion of antioxidant reserves results in oxidative stress, which is strongly correlated, with the etiology of male infertility [28, 31–33].

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There are two methods by which ROS can be measured; direct and the indirect methods. The direct methods measure the ROS directly whereas indirect methods measure their oxidized products. Direct assays include chemiluminescence, nitroblue tetrazolium test (NBT), cytochrome *c* reduction, flow cytometry, electron spin resonance, and xylenol orange-based assay. Indirect methods include measurement by myeloperoxidase test, measurement of redox potential, lipid peroxidation levels, levels of chemokines, antioxidants, and antioxidant enzymes measuring levels of DNA damage and proteomic alterations (Table 2.1).

### ***2.1.1 Types of Semen Sample Used to Detect ROS***

Various types of semen samples can be used to detect ROS levels, some of which include the unprocessed seminal ejaculate, the processed semen sample by swim up and by density gradient centrifugation [20, 42]. Seminal ejaculate comprises of not only spermatozoa but also all other secretions from prostate and seminal vesicles and other accessory glands and cellular components such as round cells, leukocytes and epithelial cells. Levels of ROS are reflective of the de novo status of the ROS in the sample. In a simple wash and resuspend sample, the seminal plasma is removed but the leukocytes, round cells and debris remain in the sample. The sperm prepared by swim-up separates the actively motile sperm from the non-motile sperm and the debris. Similarly, in the density gradient separation, the spermatozoa are separated on the basis of their densities, which results in the separation of actively motile and morphologically normal sperm. The density gradient technique is used to measure ROS levels in both mature and immature spermatozoa [20, 42].

### ***2.1.2 Measurement of ROS***

#### **2.1.2.1 Nitroblue Tetrazolium Test**

Nitroblue Tetrazolium or the NBT test is based on the generation of ROS by sperm and leukocytes by using the compound Nitroblue Tetrazolium. NBT is a yellow water-soluble nitro-substituted aromatic tetrazolium compound that reacts with cellular superoxide ions to form formazan derivative that can be monitored spectrophotometrically [72]. This test is based on the principle that when heterogeneous samples such as seminal ejaculate are stained with NBT; it results in the formation of colored formazan due to reduction of NBT. This has been shown to correlate with impaired sperm function [72]. NBT is an electron acceptor that becomes reduced in the presence of free oxygen radicals to form a blue-black compound, formazan [73]. The spermatozoa containing this formazan can be also be stained histochemically and scored under microscope.

The method involves preparation of NBT solution by adding phosphate- buffered saline (PBS) with NBT powder. This is then used to stain the whole ejaculate, i.e., leukocytes and abnormal spermatozoa. The tubes are centrifuged and the pellet

**Table 2.1** Direct and indirect assays measuring ROS

Method	Type	Principle	Advantage	Reference
Cytochrome c reduction test	Direct	The reduction of ferricytochrome c to ferrocytochrome c is used to detect superoxide formation	Gold standard for measuring extracellular superoxide anions	[34]
Electron spin resonance (ESR)	Direct	The magnetic properties of unpaired electrons in free radicals enable them to absorb electromagnetic radiation on application of external magnetic field and this then generate absorption spectra utilizing the energy of electron spin state, which is measured by ESR spectrophotometers	This is used to measure oxidative stress on proteins and lipids	[5, 25, 34, 35]
Electron paramagnetic resonance (EPR)	Direct	Provides direct detection of the “instantaneous” presence of free radical species in a sample Plays a major role in the assessment of most of the oxidants characterized by very short half-life (nanoseconds to microseconds) usually by using stabilizing molecules called spin-traps/probes	Simple, high sensitivity and specificity Detects free radicals and paramagnetic molecules. The magnetic field-based EPR detection enables nondestructive (in vitro) and noninvasive (in vivo) measurements of biological samples	[36, 37]
Xylenol orange based assay	Direct	This uses automated analyzer. The ROS in semen oxidizes ferrous to ferric ion and this forms a colored complex with xylenol orange in an acidic medium, the color intensity of which can be measured spectrophotometrically. Results are expressed in $\mu\text{mol H}_2\text{O}_2$ equiv./L	EPR spectroscopy, coupled with the use of paramagnetic probes, is a potential technique for accurate and precise determination of ROS concentrations in a variety of biological samples	[38]
Aromatic traps	Direct	Used to measure ROS produced in vivo. Salicylates and phenylalanine are used which reacts with free radicals to form more stable products	It is rapid, easy, stable, inexpensive, reliable and sensitive	[35]
ROS measurement by chemiluminescence	Direct	Measures real time production of ROS. Uses two probes—luminol and Lucigenin. Luminol measures Global ROS levels both extracellular and intracellular (superoxide anion, hydrogen peroxide, hydroxyl radical) Lucigenin is specific for superoxide anion and hydroxyl radical	Used to measure ROS in cardiovascular and cerebrovascular systems	[39–45]

(continued)

**Table 2.1** (continued)

Method	Type	Principle	Advantage	Reference
Flow cytometry	Direct	ROS measurement of hydrogen peroxide and superoxide anion by flow cytometry. Dihydroethidium measures intracellular superoxide anion and dichlorofluorescein diacetate for intracellular hydrogen peroxide	Requires very low amounts of spermatozoa, high specificity gofer intracellular ROS in spermatozoa	[46–48]
Endtz test	Indirect	ROS is mainly generated by leukocytes. The myeloperoxidase is used to stain polymorphonuclear granulocytes. But does not provide any information regarding ROS generation by spermatozoa	Indirect indicator of excessive ROS generation by leukocytes in semen	[4, 38, 49]
Redox potential GSH/GSSG	Indirect	Balance of reduced glutathione and its oxidized form (GSSG) gives an indication of ROS levels in vivo. GSH/GSSG levels are measured biochemically or using high performance liquid chromatography	Can be used to measure oxidative stress in-vitro and in-vivo	[7, 20, 50]
Total antioxidant capacity	Indirect	Measures total antioxidants in seminal plasma	Rapid colorimeter method	[51, 52]
Thiobarbituric acid assay (TBARS)	Indirect	Measures lipid peroxidation. Detects malondialdehyde (MDA-TBA) adduct by colorimetry or fluoroscopy	Simple but non specific	[53, 54]
Isoprostone	Indirect	Liquid chromatography-tandem mass spectrometry	Specific, stable compound	[55]
HNE-HIS Adduct ELISA	Indirect	ELISA	Rapid, helps in quantification	[56–58]
DNA damage	Indirect	Measures single and double stranded DNA fragmentation by sperm chromatin structure assay, TUNEL assay, sperm chromatin dispersion assay or comet assay	Measure single or double strand DNA breaks, robust, sensitive method (SCSA and TUNEL)	[6, 59–68]
Oxidation reduction potential	Indirect	Measures the redox balance in a given biological system. It measures all known and unknown oxidants and antioxidants in a given sample	High sensitivity, specificity and accuracy. Can be measured both in seminal ejaculates and in seminal plasma (both fresh and frozen)	[69–71]

formed at the bottom is used to make smears. The smears are air dried and using the Wright stain, the slides are stained again and scored under microscope [73]. Leukocytes are scored as: no detectable formazan (–), scattered or few formazan granules (+), intermediate density (++), and cells filled with formazan (+++). Spermatozoa are scored as follows: formazan occupying 50 % or less of the cytoplasm (+) and more than 50 % of cytoplasm (++) [73]. NBT reaction reflects the ROS generating activity in the cytoplasm of cells, and therefore it can help determine the cellular origin of ROS in semen [74].

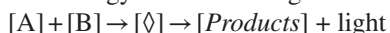
The cells stained with NBT are NBT positive cells and there exists a relation between the NBT+ cells and the levels of ROS in the same suspension [73]. This test helps in identifying the source of ROS whether it's sperm or the infiltrating leukocytes in the semen. It is important to distinguish between the sources of ROS, as the clinical implications of infiltrating leukocytes are different from the pathological conditions in which sperm are themselves the source of ROS [75]. The advantages of this method include that it is readily available, inexpensive and has high sensitivity. It provides information about the differential contribution of leukocytes and abnormal spermatozoa in the production of ROS i.e., the cellular origin of ROS in the sample [73]. The major limitation of this test is that presence of other cellular reductases may also reduce NBT. Furthermore, changes in the cellular content of various oxido-reductases may also alter the rates of NBT reduction [74].

### 2.1.2.2 Chemiluminescence Assay

Chemiluminescence is one of the most commonly employed methods used to detect ROS in semen sample [44, 75, 76]. The reaction causes emission of light, which is measured with a luminometer. The two major types of luminometer include the photon counting luminometer and the direct current luminometer. The photon counting measures the individual photon whereas the direct counting measures the current passing through a luminometer. These are measured as photons per minute or relative light units, respectively [77].

Luminometers are also classified as single tube luminometer, which can measure only single sample, or multiple tube luminometer, which can measure multiple samples at any time, and this is the one used in research laboratories. The third one is the plate luminometer, which utilizes a 96 well plate to read multiple samples at a time [77].

The basic principle of chemiluminescence is the measurement of emitted light due to a chemical reaction occurring between chemical reagents and the ROS generated. The following equation shows two reactants A and B in presence of an excited intermediate [ $\diamond$ ] resulting in emission of light. The decay of this excited state [ $\diamond$ ] to a lower energy level causes light emission.



There are two major types of probes used in chemiluminescence which include Luminol and Lucigenin (Table 2.2).

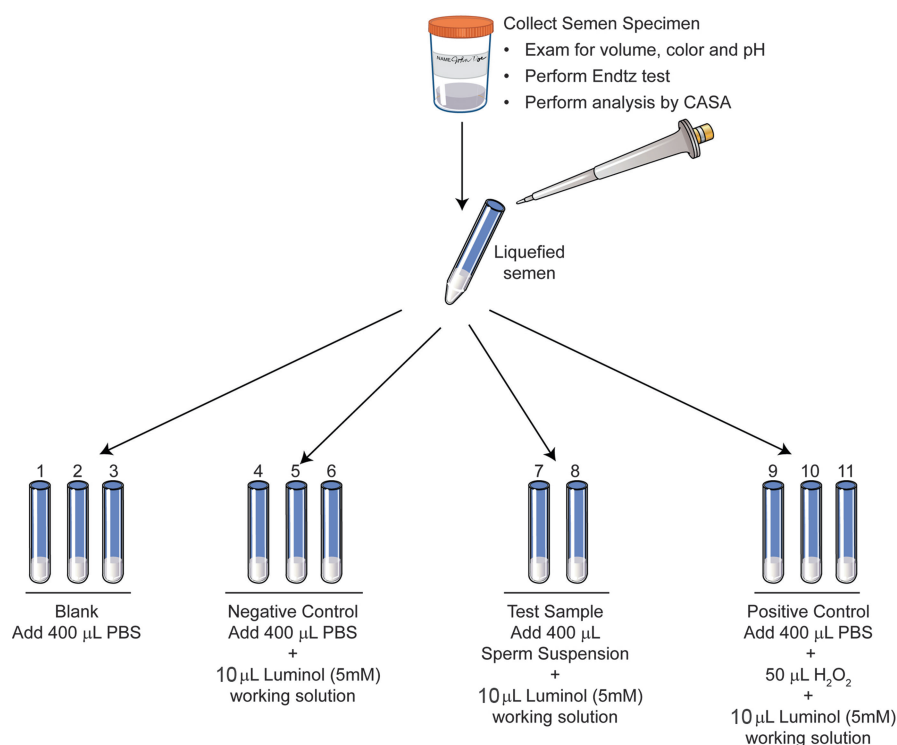
**Table 2.2** Major types of probes for measuring ROS by chemiluminescence

Luminol	Lucigenin
1. It works through one electron oxidation	1. This works through one electron reduction
2. Measures both intracellular as well as extracellular ROS	2. It measures only the extracellular ROS
3. Hydrogen peroxide radical and oxygen radical are involved	3. It involves measuring the superoxide anion

**Fig. 2.1** Autolumat 953 Plus Luminometer used in the measurement of ROS by chemiluminescence assay. Multiple tubes can be loaded simultaneously for measuring ROS. The luminometer can be connected with a computer and monitor

The reagents used are the stock luminol probe (100 mM), the working luminol (5 mM) and the dimethylsulfoxide (DMSO) solution [78]. The procedure is performed in an indirect light. A luminometer is attached to a computer (Fig. 2.1). A total of 11 tubes are used that include 3 blank tubes which contain only the PBS, 3 negative controls which contain PBS + luminol (working solution), 2 tubes which contain the patient sample and + luminol, 3 positive control which contain PBS + hydrogen peroxide (50  $\mu$ L) + luminol (Fig. 2.2). The tubes are loaded into the luminometer (Berthold, Autolumat Plus LB 953) and a real time plot of the ROS levels produced in each sample is visualized on the computer monitor (Fig. 2.3) and the results can be visualized and printed in an excel sheet [78].

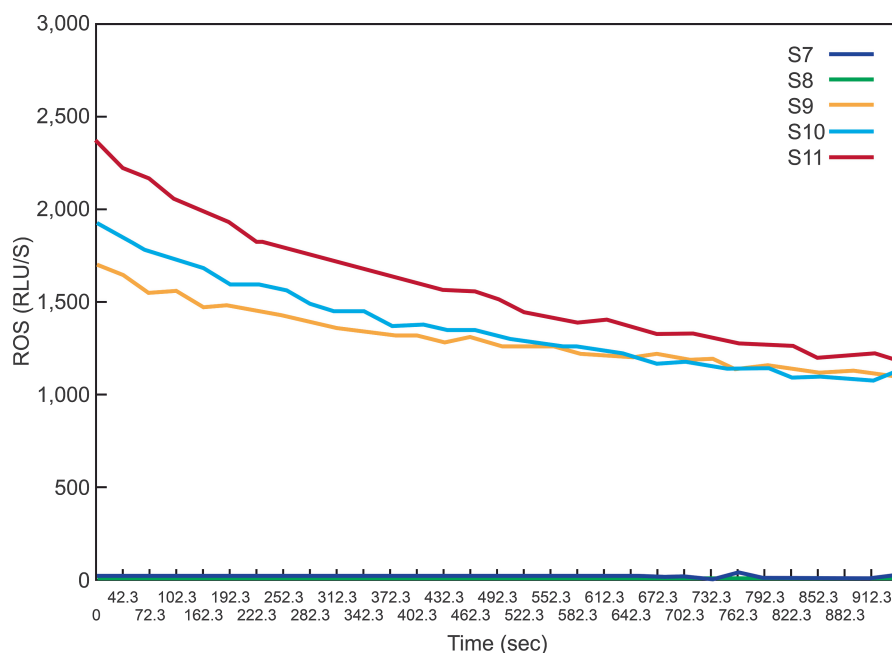
The factors affecting chemiluminescence reactions include sample volume, time of analysis, viscosity of the sample, concentration of reactants, reagent injection, temperature control, human error, background luminescence. The major advantages include that it is high specificity and sensitivity and can measure both intracellular as well as extracellular ROS [41]. The major disadvantages are: (1) it cannot measure multiple markers simultaneously and (2) the level of ROS declines with time after ejaculation due to short half-life of ROS. Factors affecting ROS measurement are: (1) luminometer calibration; (2) sensitivity and the dynamic range as well as



**Fig. 2.2** Preparing the tubes for ROS measurement. A total of 11 tubes are labeled from S1 to 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

the units used; (3) concentration and the type of probe used; (4) concentration and the volume of semen used, and (5) temperature of the instrument at the time of measurement. Semen age, viscosity of the sample, repeated centrifugation, use of media containing albumin that can generate spurious signals; spikes and sensitivity of luminol to pH changes are other variables that influence ROS production. ROS is an independent factor of male factor infertility [26]. We have reported different cutoff values for ROS in processed semen samples [4, 6, 17] and seminal ejaculates [17, 21, 24, 43].

We have recently revised the reference range of ROS in seminal ejaculates [43]. ROS levels  $>102$  RLU/s/ $10^6$  sperm are considered abnormal. At this cutoff ROS sensitivity is 76% with a positive predictive value of 82.1%. When the controls were strictly comprised of individuals who had established pregnancy, the cutoff was slightly lower at  $<93$  RLU/s/ $\times 10^6$  sperm. The sensitivity increased to 93.8% indicating that the test can differentiate subjects that are fertile from those that are not. Levels of ROS  $>102$  RLU/s/ $\times 10^6$  sperm must be considered pathological.



**Fig. 2.3** A typical graph showing the ROS levels in the 11 tubes (S1–S11). As seen here, only the positive controls have significantly higher levels of ROS. Those producing low levels (Tubes S1–S8) of ROS are seen very close to the X axis

The major diagnostic application of chemiluminescence is that it provides independent assessment of the quality of the ejaculate and this is extremely important in patients with unexplained infertility as these patients demonstrate high levels of ROS despite conventional semen parameters within normal ranges [20, 77, 79–81]. The reproducibility of ROS by chemiluminescence assay makes the test sensitive and reliable in measuring ROS levels. ROS levels in the mature spermatozoa may have both diagnostic and prognostic importance as elevated ROS levels in mature spermatozoa may reflect oxidative stress in semen samples that will be used for ART purpose and may also be used to predict the fertilizing potential of the spermatozoa. This can be accomplished by characterizing the semen samples that are used in ART based on the established reference values of ROS.

The intracellular levels of ROS can be measured by flow cytometry using dihydrofluorescein diacetate (DCFH) to detect intracellular hydrogen peroxide radicals. This dye is oxidized to the highly fluorescent derivative dichlorofluorescein (DCF), which is detected by the use of flow cytometer [46–48, 82]. A counterstain dye for nucleic acid (propidium iodide) is used to exclude the apoptotic spermatozoa [83]. Dihydroethidium (DE) can be used to detect intracellular levels of superoxide anions [46, 48, 83]. The results are interpreted as percentage of fluorescent spermatozoa [83].

### **2.1.3 Measurement of DNA Fragmentation**

Reduced fertility, embryo development, increased rates of miscarriages has been reported in cases of higher sperm DNA damage [84–86]. Several etiological factors such as cigarette smoking, irradiation, chemotherapy, leukocytospermia, varicocele, cancer, elevated levels of ROS, abnormalities during chromatin packaging and advancing age have demonstrated compromised sperm DNA quality [31, 83, 87–91].

Oxidative stress is responsible for single strand breaks in DNA [20, 91]. Furthermore, apoptosis can also occur as a result of increased oxidative stress and result in DNA fragmentation. Several studies show that infertile men have high number of sperm with single or double stranded fragmentation [59, 92–94].

Several tests have been introduced to measure the sperm DNA damage [64, 88, 95–101]. The methodological approaches by which sperm DNA damage is investigated in these tests are varied. Some tests measure abnormalities in sperm chromatin whereas others measure direct DNA strand fragmentation. Among such test the most commonly used are the sperm chromatin structure assay (SCSA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) [87, 97, 99, 102, 103]. These are briefly described below:

#### **2.1.3.1 Sperm Chromatin Structure Assay (SCSA)**

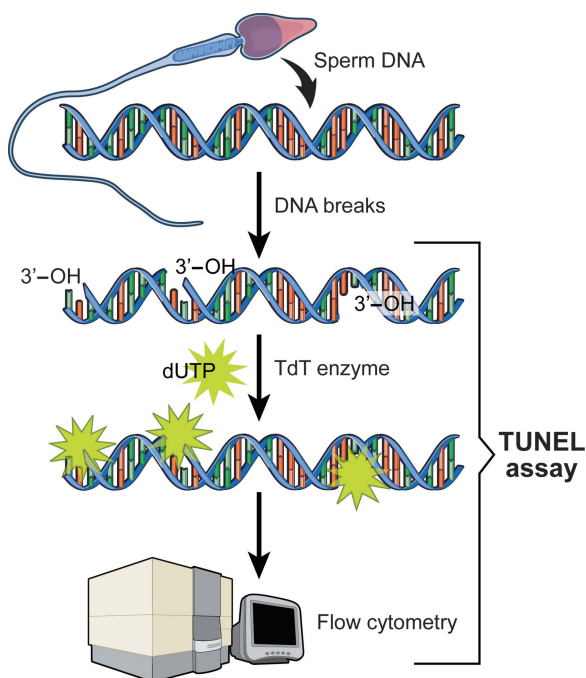
Sperm Chromatin Structure Assay (SCSA) detects damaged sperm DNA using flow cytometry of acridine orange stained sperm. It is based on the susceptibility of DNA breaks to acid denaturation. Low pH treatment opens the DNA strands at the sites of breaks. Staining by acridine orange is highly precise and repeatable and comparable between fresh and frozen samples. The DNA damage is induced by exposing to denaturing conditions. This utilizes the metachromatic properties of acridine orange to distinguish single stranded/red fluorescence and double stranded/native DNA/green fluorescence [59, 60, 83]. The DNA fragmentation index (DFI) is the ratio of percentage of sperms showing red fluorescence/total fluorescence (red+green) [48, 59].

The SCSA also measures sperm with high DNA stainability (%HDS) which is related to the nuclear histones retained in immature sperm and shown to be predictive of pregnancy failure [60]. The current clinical threshold 25 % DFI that categorized patient into a statistical probability of the following: (a) longer time to natural pregnancy, (b) low odds of IUI pregnancy, (c) more miscarriages, or (d) no pregnancy [60]. The test is precise, repeatable with acceptable DNA fragmentation that has a threshold of placing a man at risk of infertility.

#### **2.1.3.2 Terminal deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay**

TUNEL assay utilizes a template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT) that non-preferentially adds deoxyribonucleotides to 3' hydroxyl (OH) single- and double-stranded DNA. Deoxyuridine

**Fig. 2.4** Schematic of the DNA staining by the TUNEL assay



**Fig. 2.5** Set up of the bench top flow cytometer



triphosphate (dUTP) is the substrate that is added by the TdT enzyme to the free 3'-OH break-ends of DNA (Fig. 2.4) [96, 99].

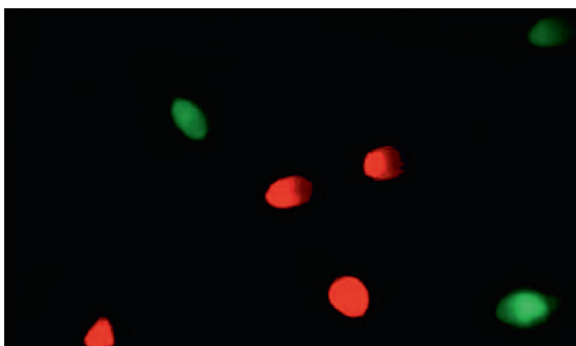
The more DNA strand breaks sites that are present, the more labels are incorporated within a cell. This identifies the in situ DNA breaks. Here, the 3' hydroxyl free ends are labeled using a fluorescent label which on passing through a flow cytometer generates fluorescence, which is directly proportional to the number of strand breaks [96, 99]. Various protocols are used to detect DNA damage via TUNEL assay like fluorescein isothiocyanate labeled dUTP system and Apoptosis detection kit. DNA fragmentation can be measured by both a simple bench top flow cytometer (Fig. 2.5). This test is highly sensitive and specific [96, 99]. It measures a definite end point and is considered to provide better prediction regarding the potential of embryo implantation [86, 102].

Many factors involved in the processing, fixation and permeabilization of the specimen adversely affect the clinical implication of TUNEL assay [104]. The difficulty in the permeabilization of sperm chromatin due to its highly dense compaction and tightly packed protamines plays a major role in reduced sensitivity preventing TdT from directly interacting with DNA strand breaks [105]. Unlike SCSA, the cutoff values of sperm DNA fragmentation for TUNEL have not been clearly established [61]. Studies have reported DNA fragmentation measured by TUNEL assay to range from 12 to 36.5 % at which no pregnancies were reported [106, 107]. A cutoff value of >19.2 % and 16.8 % has been recently shown to have >90 % specificity [61]. The specificities can be further increased by including only men with established pregnancies as controls. The high specificity and positive predictive value is important particularly in cases of idiopathic and unexplained infertility.

### 2.1.3.3 Epifluorescence Using Acridine Orange Dye

Acridine orange is a nucleic acid specific, fluorescent, cationic dye. It interacts with DNA by intercalation and by electrostatic interaction with RNA or single stranded DNA [96]. Fluorescence microscopy is used along with acridine orange dye. There is exposure to acid, which denatures DNA with single- or double stranded breaks. The dye acridine orange binds to DNA. The double stranded DNA fluoresces green and single stranded DNA gives red color. In addition the sperm suspension after staining with the Apo-direct kit for the TUNEL assay can also be visualized for DNA fragmentation by the fluorescence microscope (Fig. 2.6). Sperm stained green indicate sperm with DNA fragmentation [96]. This method uses fluorescence, which is relatively rapid, simple and inexpensive. The major disadvantage of this technique is the heterogeneous staining and color fading of the slides. Also, the presence of indistinct colors ranging from red to green interferes with the results [108].

**Fig. 2.6** Fluorescent staining showing intact (*red*) spermatozoa and spermatozoa with DNA fragmentation (*green*)



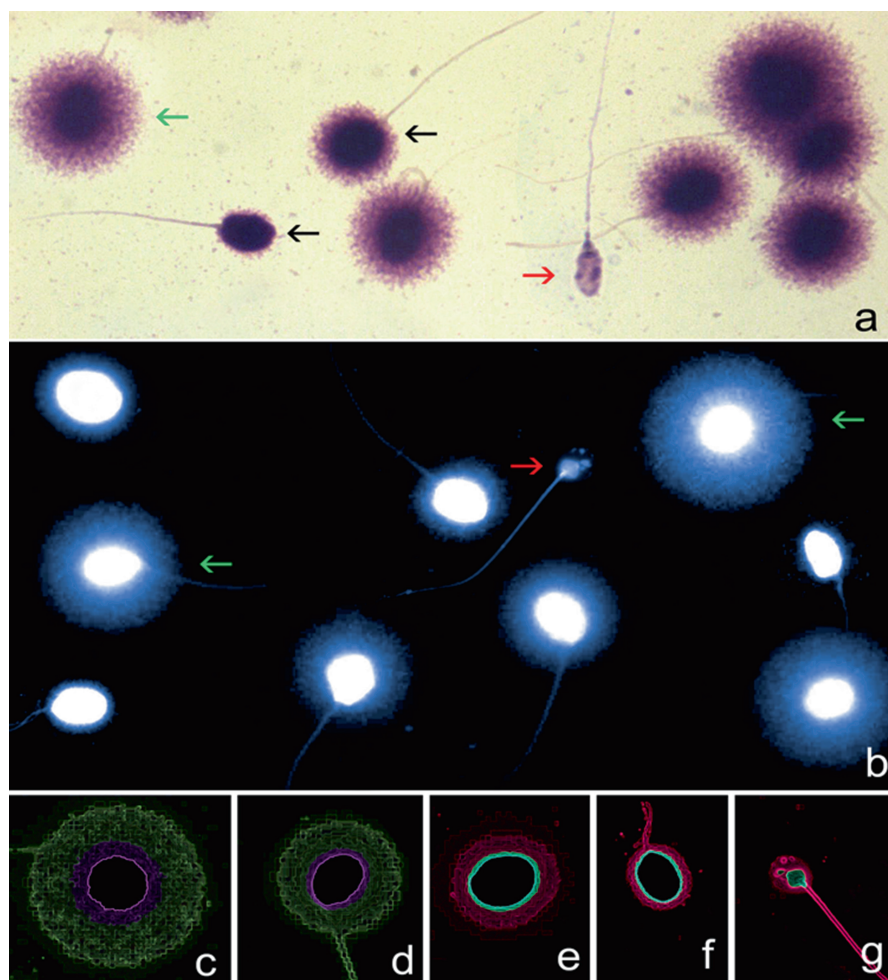
#### 2.1.3.4 Comet Assay

This is a single gel electrophoresis method, which basically measures the breaks in DNA [66]. Electrophoresis is used to mobilize DNA fragments that are produced from nucleoids after being depleted of proteins. It is based on the general concept that DNA fragments resulting from pre-existing DNA breaks have different mobility in the electrophoretic field depending on the relative size of the fragment. This generates morphological differences between nuclei containing fragmented DNA when examined under fluorescent microscopy. The resulting image represents a “comet” that consists of a head and a tail chromatin in the direction of the anode. Larger the size of the comet, higher is the level of DNA fragmentation [109, 110]. Thus, sperm with more DNA breaks shows intense comet tail [111]. At the end of electrophoresis, all the broken strands of DNA migrate towards the anode and this forms a comet tail, which can be used to assess the DNA damage using the fluorescence microscope or cytometer [64]. The comet’s tail length and fluorescent intensity is directly proportional to the degree of DNA fragmentation [65].

The assay can be performed in both neutral as well as alkaline environments. In neutral buffer, double stranded DNA damage is measured while in alkaline environment DNA can be denatured, both single– (SS) and double stranded (DS) DNA damage can be measured due to unwinding of the DNA strands [112]. Under the influence of electric field, there is separation of broken DNA strands (SS and DS) [65, 113–116]. After separation, the broken DNA fragments migrate towards the tail forming comet tail and the intact DNA remains confined to the head forming the comet’s head [117]. The major limitation of the comet assay for its routine use in fertility labs is (1) slide processing is time consuming and (2) it requires electrophoresis equipment and fluorescence microscopy.

#### 2.1.3.5 Sperm Chromatin Dispersion (SCD) Assay

Sperm chromatin dispersion (SCD) assay uses the Halosperm kit to differentiate between non-fragmented spermatozoa from the fragmented spermatozoa [64]. This test is used in laboratories with no access to flow cytometry. It can be visualized using bright field or fluorescence microscopy. It is based on a controlled species-specific DNA denaturation to produce single-stranded DNA stretches from any DNA breaks, coupled with controlled DNA depletion [67, 101, 118, 119]. The process involves (1) integration of sperm sample into an inert agarose microgel on pretreated slide, (2) controlled acid denaturation of DNA, (3) controlled protein depletion. Normal sperm produce halos of dispersed chromatin around a dense core. In fragmented DNA, no halos of dispersed chromatin are produced [120, 121]. The halos can further be classified according to their morphology and the results can be expressed for each patient against the established cutoff criteria (Fig. 2.7). The results show a strong correlation when compared with indirect assessments of DNA damage such as the SCSA or comet assay [64, 122]. The advantages and disadvantages of the sperm DNA fragmentation assays are shown in Table 2.3.



**Fig. 2.7** 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 sperm). (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 motorized fluorescence microscope controlled with software for automatic scanning and image digitization (Leica Microsystems, Barcelona, Spain). 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, Rechestern NY, USA). A charge coupled device (Leica DFC350 FX, Leica Microsystems, Barcelona, Spain) was used for image capture (Courtesy of Prof. Jaime Gosálvez, Madrid, Spain)

**Table 2.3** Assays measuring DNA fragmentation

Assay	Advantage	Disadvantage	Reference
SCSA	Established clinical thresholds, robust and sensitive assay; uses metachromatic acridine orange staining and flow cytometry; requires only 10,000 cells; can be done in fresh or frozen samples	Not available in commercial kits, calculations are complex, acid induced denaturation. Not performed in routine andrology labs	[59, 60]
TUNEL	High sensitivity and specificity. Can be done on fresh or frozen samples. Associated with fertility and available in commercial kits. Measures definite end point	Thresholds not standardized, not specific to oxidative damage, need for special equipment (flow cytometer or fluorescence microscope). Results affected by fixation and permeabilization of sperm. Although can be measured by fluorescence microscopy, results are subjective and prone to inaccuracy due to heterogenous nature of staining and instability of the stain	[61, 86, 96, 99, 104–107]
Comet	Requires small number of cells; high sensitivity, measures breaks in DNA; correlates with seminal parameters. Assay can be performed in neutral and in alkaline environment	Time and labor intensive, not specific to oxidative damage, requires special imaging software, lacks correlation with fertility. Requires electrophoresis and fluorescence microscopy. Slide processing is time consuming	[63, 64, 66, 111]
SCD	Differentiates fragmented from non-fragmented spermatozoa. Does not require flow Cytometry. Can be visualized both by bright field and fluorescence microscopy Strong correlation with SCSA or Comet assay	Interobserver subjectivity to categorize the halos is a limitation of SCD	[64, 67, 68, 101, 118, 119, 122]

SCSA=Sperm chromatin structure assay, TUNEL=Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), SCD=Sperm chromatin dispersion

### 2.1.4 Other Methods for Measuring ROS

In addition to the more popular methods of measuring DNA damage described above there are other less common techniques reported in literature. These are shown in Tables 2.4 and 2.5.

**Table 2.4** Different methods to measure total antioxidants

Technique	Principle	Advantage	Disadvantage	Reference
TEAC	Inhibition method	Standard by other assays		[123]
ORAC	Inhibition method	High specificity, responds to numerous antioxidants, gross differentiation of aqueous and lipid soluble antioxidants	Time consuming	[124]
FRAP	Reduction of $\text{Fe}^3 \rightarrow \text{Fe}^2$	Simple, inexpensive	Does not measure SH-group containing radicals	[125]
Enhanced Chemiluminescence	Chemiluminescence	Accurate	Cumbersome, expensive instrumentation, time consuming, signal reagent might reduce in intensity	[126]
Colorimetric	Colorimetric analysis	Less time consuming, relatively inexpensive, convenient, can be used as an in office test	Significantly expensive reagents	[51, 52]
ROS-TAC Score <sup>a</sup>	Chemiluminescence	Better predictor compared to ROS and TAC alone	Requires statistical modeling	[6, 127]
ORP <sup>a</sup> using the RedoxSYS Diagnostic System	Galvanostat based mechanism	Prognostic marker (cORP), easy, less time consuming, requires less expertise, can be used for frozen specimens	Affected by viscosity of the sample	[69–71, 128]

*TAEC*=Trolox equivalent antioxidant capacity, *ORAC* = Oxidation radical absorbance capacity, *FRAP* = Ferric reducing ability assay, *ORP* = Oxidation reduction potential

<sup>a</sup> These techniques do not directly measure ROS or TAC but rather predict the OS status in the sample

### 2.1.4.1 Measurement of Total Antioxidant Capacity

The total antioxidant capacity (TAC) is a parameter that can be measured by evaluating the reducing ability of various antioxidants present in semen against an oxidative reagent such as hydrogen peroxide, and measuring the effect on the substrate [51, 52]. The reaction can be measured with a spectrophotometer, colorimeter, depending on

**Table 2.5** Various techniques to measure lipid peroxidation

Technique	Principle	Advantage	Disadvantage	Reference
Thiobarbituric acid assay (TBARS)	MDA-TBA adduct detection by colorimetry or fluoroscopy	Simple but non specific	Rigorous controls are required	[53, 54]
Isoprostane	EIA/Liquid chromatography-tandem mass spectrometry	Specificity, stable compound	Labor intensive and expensive cost of equipment	[55]
HNE-His Adduct ELISA	ELISA	Rapid, helps in quantification	Chances of cross reactivity	[56–58, 129]

*MDA* = malondialdehyde, *TBA* = Thiobarbituric acid, *HNE-His* = hydroxynonenal histidine

the substrate. Most techniques employed to estimate TAC measure the low molecular weight, chain breaking antioxidants and do not include the contribution of antioxidant enzymes (glutathione group of enzymes, catalase, and superoxide dismutase) and metal binding proteins.

### Colorimetric Analysis

The antioxidant assay is based on the principle that aqueous and lipid antioxidants in the seminal plasma specimens inhibit the oxidation of the 2, 20-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS<sup>+</sup>. Under the reaction conditions used, the antioxidants in the seminal plasma cause suppression of the absorbance at 750 nm to a degree that is proportional to their concentration. A stable blue–green color is formed [49, 50]. The capacity of the antioxidants present in the sample to prevent ABTS oxidation is compared with that of standard Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue. Results are reported as micromoles of Trolox equivalent. Clear seminal plasma is used for the assay.

Ten microliters of metmyoglobin and 150 µL of chromogen are added to all standard/sample wells. The reaction is initiated by adding 40 µL of hydrogen peroxide as quickly as possible. The plate is incubated for 5 min at room temperature on a horizontal shaker and absorbance monitored at 750 nm using a microplate reader. The total antioxidant concentration of each sample can be calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation:

$$\text{Antioxidant } (\mu\text{M}) = \frac{\text{Unknown average absorbance} - Y \text{ intercept}}{\text{Slope}} \times \text{dilution} \times 1000$$

Men with oxidative stress have reduced levels of total antioxidants in their seminal plasma.

Some of the other methods measuring antioxidants are illustrated in Table 2.4.

## Oxygen Radical Absorbance

Oxygen radical absorbance capacity (ORAC) is a technique that works on utilizing the unique property of phycoerthrins. It works on the same principle as Trolox equivalent antioxidant capacity (TEAC) [123, 124].

## Ferric Reducing Ability Assay

Ferric Reducing Ability Assay (FRAP) is a simple and automated test working on the principle of reduction of ferric to ferrous ions. Antioxidants present in the seminal plasma reduce ferric-tripyridyltriazine ( $\text{Fe}^{3+}$ -TPTZ) complex to  $\text{Fe}^{2+}$ -TPTZ and form a blue colored complex. Comparing the changes in absorbance occurring at 593 nm gives an estimate of the antioxidant capacity [125].

### 2.1.4.2 ROS-TAC Score

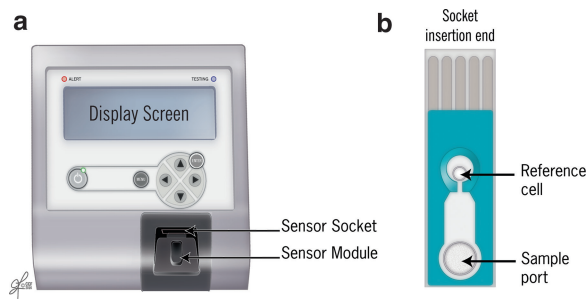
It is a novel parameter derived from the ROS concentration and seminal TAC values called ROS-TAC score. The ROS and TAC values from controls are used to create a scale of these two variables using controls as reference point. Both values are normalized for distribution after converting ROS to the log of ROS+1. Both log ROS+1 and TAC are standardized to Z scores so that both will have the same variability. Standardized scores are calculated by subtracting the mean value of the controls from the individuals observed values and dividing by the standard deviation of the control population.

The standardized values are analyzed with principal component analysis, which provides linear combinations or weighted sums that account for most variability among correlated variables [6]. The ROS-TAC scores are novel measures of oxidative stress and superior to the individual ROS and TAC alone as it is capable of discriminating fertile from infertile men [6, 49, 127]. Infertile men with male factor or idiopathic diagnoses were reported to show low ROS-TAC scores. Infertile men with higher ROS-TAC scores were able to initiate pregnancies compared to those who had low scores and had failed pregnancy [6].

### 2.1.4.3 Measurement of Redox Potential

Redox potential or oxidation-reduction potential (ORP) is a novel measure of oxidative stress or a redox balance in a given biological system. It has been shown to correlate with illness or injury that is related to a state of oxidative stress [69, 70, 130, 131]. Unlike measuring a single marker of oxidative stress, the Redox system allows the assessment of all known and unknown oxidants and antioxidants in a given sample. It is based on the electrical conductance relative to an internal reference standard according to Nernst equation

**Fig. 2.8** Measurement of oxidation reduction potential (ORP) in semen sample using (a) MiOXSYS analyzer and (b) Sensor. A 30  $\mu$ L sample is loaded on the sensor sample port and the sensor is inserted in the sensor socket for ORP measurement



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

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 V,  $R$  is the gas constant,  $T$  is the absolute temperature measured in degrees Kelvin and  $F$  is Faraday's constant [69, 128]. It has been measured in whole blood, plasma, urine, cerebrospinal fluid, saliva, tears and amniotic fluid [70, 130, 131]. ORP values higher than the established reference values are indicative of oxidative stress [132]. More recently ORP values have been reported in fresh and frozen semen and seminal plasma samples [71]. It involves the RedoxSYS or MiOXSYS Analyzer and the sensor strip which has an application port where the sample is loaded (Fig. 2.8).

It measures two parameters. The first parameter, static ORP or sORP is snapshot of the current oxidative stress in the sample that may be induced due to infection, inflammation or disease. The second parameter is capacity ORP or cORP, and this parameter gives us an estimate of the antioxidants reserves present. The sperm concentration in the sample is calculated and the sORP and cORP results are normalized and expressed as sORP/ $10^6$  sperm or cORP/ $10^6$  sperm. Initial results have shown high sensitivity, specificity and accuracy of ORP reflecting its potential application as a diagnostic tool in oxidative stress related male infertility [133].

### 2.1.5 Measurement of Lipid Peroxidation

Polyunsaturated fatty acids (PUFAs) containing cellular macromolecules are particularly susceptible to ROS. Lipid peroxidation is characterized by breakdown of these PUFAs into lipid peroxides due to oxidative stress [55]. Lipid peroxides are unstable indicators which on decomposition form more complex and reactive compounds such as 4-hydroxynonenal (4-HNE), acrolein (ACR) and malondialdehyde (MDA).

Compared to free radicals, these aldehydic products are relatively stable and are able to move freely and react with molecules like DNA, proteins and lipids. These

products not only modify proteins and alter their function but are also considered as cytotoxic second messengers of oxidative stress. This property of these aldehydic fragments makes them highly utilized in biomedical research [56, 57]. Measurement of the end products of lipid peroxidation is a widely accepted marker of oxidative stress. Some of the common methods to measure lipid peroxidation are described below:

### **2.1.5.1 HNE-His Adduct ELISA/HNE Adduct Competitive ELISA**

HNE-His Adduct ELISA functions on the principle of enzyme immunoassay and has been developed for quantification and rapid detection of HNE-His protein adducts. 4-HNE can react with lysine, histidine or cysteine residues in protein to form adducts. A 96-well titer ELISA plate is taken and protein samples (10 µg/mL) or BSA standards 0–200 µg/mL are adsorbed onto it. The HNE-protein adducts present in the standard or sample are probed with a primary antibody, (anti-HNE-His antibody). This is followed by addition of a HRP conjugated secondary antibody. Standard curves prepared from predetermined HNE-BSA standards are used to compare and quantify the HNE-protein adduct content in an unknown sample. Using a primary wavelength of 450 nm, the absorbance of each well is read on a microplate reader. Results are expressed as pmol/mg [58].

### **2.1.5.2 Isprostane (IsoP) Method**

Another important biomarker of lipid peroxidation is 8-isoprostane (IsoP) measured as ng/mL [129]. It is a specific end product belonging to family of eicosanoids derived from nonenzymatic peroxidation of polyunsaturated fatty acids [34]. The advantages of using the IsoP marker are: it is not produced by enzymatic pathways like cyclooxygenase and lipoxygenase pathways of arachidonic acid, it is stable and it can be quantified in seminal plasma.

### **2.1.5.3 TBARS Assay**

Malonaldehyde (MDA) is a reactive compound formed during lipid peroxidation [134]. The thiobarbituric acid (TBA) assay is one of the commonly used tests to assess changes in MDA. The assay detects TBA-reactive substances (TBARS) via spectrophotometry, high performance liquid chromatography (HPLC), colorimetry or spectrofluorescence [53]. MDA and TBA are reacted together to form the MDA-TBA adduct. This is carried out in acidic conditions and under a high temperature (90–100 °C). This adduct formation is measured colorimetrically at 530–540 nm or fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm [54]. Lipid peroxidation in sperm is expressed as nmol MDA 10<sup>-7</sup> sperm [135–137]. The advantages and disadvantages of various lipid peroxidation methods are shown in Table 2.5.

### **2.1.6 Measurement of ROS-Induced Post-Translational Modifications**

Reactive oxygen species can modify proteins resulting in altered functions such as activation or inhibition of transcription factors, signal transducers and enzymes [100, 138, 139]. These result in alterations in structural and functional integrity of specific proteins. Most of the oxidants will react with several amino acids to yield multiple products. Only a few stable and specific products those are characteristic of a selected oxidant that may be used as a signature for any selected type of ROS. Three principal type of post-translational modification of proteins are induced by ROS, namely, S-glutathionylation (GSS-R), nitrotyrosine modifications (Nitro-Y) and carbonylation. Carbonylation, nitration and thionylation of proteins are regarded as the most common post-translational modifications leading to dysfunction of proteins [139, 140].

Nitrotyrosine is formed by the reaction of peroxynitrite or donors of  $\text{NO}\bullet$  with tyrosine residues. It can be produced by the sperm cell by the reaction of superoxide and  $\text{NO}\bullet$ . The nitrotyrosine protein modification can result in alteration of protein function or structure. Higher amounts of nitrotyrosine were found in patients with impaired motility (athenozoospermia) or spermatic duct cord blood in varicocele patients [141–143]. Reactive carbonyls are produced by direct protein oxidation (oxidation on Trp, Lys, Arg, Pro, and Thr) reaction with low- or high-molecular weight dicarbonyls (modifications of Lys, Arg, and Cys) generated during lipid peroxidation and glycoxidation, and oxidative degradation of Amadori products.

Similarly, for detection of quantification of carbonyl modification of spermatozoa proteins, BSA standards or protein samples (10  $\mu\text{g/mL}$ ) are adsorbed onto a 96-well plate for 2 h at 37 °C. The protein carbonyls present in the sample or standard are derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody. The protein carbonyl content in unknown sample is determined by comparing with a standard curve prepared from a predetermined reduced and oxidized BSA standard.

Introduction of carbonyl group to protein involves oxidative modification of specific amino acids namely proline, arginine, lysine and threonine, to aldehyde or ketone and their eventual cleavage. Protein carbonylation is irreversible and leads to structural and functional alterations in the protein and the carbonylated proteins are generally destined to proteosomal breakdown [144].

Products of oxidation such as protein carbonyls are useful for detection and in estimation of ROS levels in a semen sample [145]. Protein carbonyls are chemically stable, more reliable and a frequently used marker for protein oxidation [146]. ELISA is commonly used to quantify the levels of protein carbonyl content in the seminal plasma by colorimetric analysis [147].

Both nitrosylation and carbonylation can be measured by ELISA. ELISA is a commonly used technique to detect and quantify a specific protein in a complex mixture. The analysis is done by immobilizing proteins in microplate wells using specific antibodies. The specific antibodies are chemically linked to biological enzymes.

The activities of these enzymes produce a measurable signal when they are mixed with solutions containing appropriate substances [148]. The end product is a stable color that can be measured colorimetrically or fluorometrically if fluorophore-labeled antibodies are used for signal generation, especially in multiplex arrays.

Competitive ELISA kit can be used for nitrotyrosine quantitation. In this assay, the unknown protein nitrotyrosine sample or nitrated BSA standards is first added to a nitrated BSA preabsorbed EIA plate. After a brief incubation, an anti-nitrotyrosine antibody is added, followed by an HRP conjugated secondary antibody. The protein nitrotyrosine content in unknown sample is determined by comparing with a standard curve prepared from predetermined nitrated BSA standard.

### ***2.1.7 Measurement of ROS-Induced Protein Alterations–Proteomic Analysis***

Proteomics and bioinformatics tools can be utilized to understand alterations in proteins as a result of exposure of spermatozoa to reactive oxygen species or oxidative stress. It also helps in demonstrating that how post-translational modifications, such as phosphorylation, proteolytic cleavages, glycosylation and mutations, bring about changes in the physiological functions of the spermatozoa. Global change occurs in proteomic profile of human spermatozoa and seminal plasma under oxidative stress conditions [149–151].

Differential regulation of protein expression in infertile patients has been reported with variations in ROS level as evidenced by global proteomic profiling [28]. Both spermatozoa and seminal plasma proteome influence fertilization and implantation in infertile men with various levels of ROS or oxidative stress [28, 150–152]. Most commonly employed techniques to understand sperm specific proteins include 2D polyacrylamide gel electrophoresis (2D-PAGE), differential in gel electrophoresis (DIGE) and Liquid Chromatography-Mass spectrometry or LC-MS/MS. Global proteomic analysis involves analysis of pooled or individual test samples (either spermatozoa or seminal plasma from semen samples of infertile men exhibiting oxidative stress).

Exposure to different levels of ROS has shown that in the seminal proteome, proteins involved in biomolecule metabolism, protein folding and protein degradation are differentially modulated infertile patient group exposed to low, ROS, medium ROS and high ROS in comparison to fertile controls. In the sperm proteome, differentially expressed proteins with distinct reproductive functions have been demonstrated only in men with low, medium or high ROS levels [28]. Similarly in the seminal plasma proteome, pathways involved in post-translational modification of proteins, protein folding (heat shock proteins, molecular chaperones) and developmental disorder are overexpressed in the high ROS group compared with fertile control group [152].

2.1.7.1 Validation of Proteins of Interest

Proteins of interest that are modified by ROS and identified by proteomic and bioinformatic analysis can be further validated in order to be categorized as a potential biomarker of ROS in spermatozoa or seminal plasma. Potential biomarker candidates of interest can be validated by Western blot analysis utilizing specific antibodies to proteins of interest, or by ELISA and Immunochemistry.

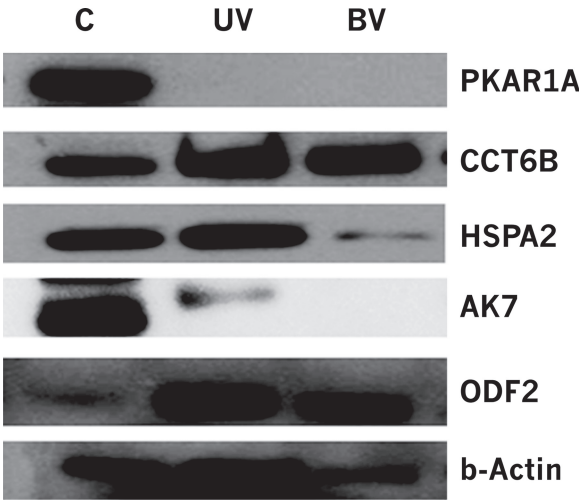
Western Blot Analysis

Western blot analysis is a commonly used technique that helps in quantification of proteins and also in identification of a target protein from a complex mixture of proteins extracted from a cell. It involves the separation of proteins using gel electrophoresis on the basis of their 3-D structure or denatured proteins by the length of the polypeptide. The gel is carefully transferred onto a membrane (PVDF), thus producing a band for each protein. After washing and blocking, it is incubated with primary antibodies specific to the protein of interest. Following incubation with a secondary antibody, the unbound antibodies are washed off. Bound antibodies are detected by chemiluminescence. Proteins are quantified by comparing it to a standard protein (Fig. 2.9) [153–155].

Immunochemistry

Immunocytochemistry is a technique employed on spermatozoa to localize antigen expression. It works on the principle of epitope-antibody interactions. Positive staining patterns are visualized using a molecular label, which can be fluorescent or

**Fig. 2.9** Validation of 5 proteins by western blot analysis in control (fertile men) and infertile men with unilateral varicocele (UV) or bilateral varicocele (BV)



chromogenic. Briefly, an aliquot of  $10 \times 10^6$  spermatozoa is centrifuged initially at 500 g for 5 min. It is then fixed for 15 min in 2 % paraformaldehyde. The fixed cells are then washed in 0.1 M phosphate buffered saline. The cells are then resuspended in 0.1 M glycine/PBS, and they are then transferred to poly-L-lysine coated coverslips to settle overnight. The spermatozoa are permeabilized using Triton X-100-PBS (0.2 %). This is carried out for 10 min at room temperature and is followed by a wash in PBS.

By mixing 3 % BSA solution (900 mg PBS, 30 mg BSA and 100  $\mu$ l goat serum) for 30 min, non-specific antibodies are inhibited. The cells are then rinsed in PBS and treated with the primary antibody of interest, diluted in PBS overnight at a temperature of 4 °C in a humidified chamber. The cells are washed in PBS and treated with the secondary antibody in PBS for 1 h at 37 °C. They are then re-washed in PBS. The cells are mounted on frosted slides and images are captured on a fluorescent microscope at 530 nm and positive staining patterns are identified [153]. The localization of the protein can be in the acrosome, neck, mid piece or the tail [156].

Proteins of interest can also be identified by ELISA utilizing the antibodies to proteins of interest.

## ELISA

Protein of interest can also be validated by ELISA. The proteins (antigen) is directly or indirectly immobilized and coated onto the surface of the wells of the microplate. All unsaturated surface binding sites are then covered by adding blocking antibodies. Antigen specific antibodies are then added and incubated. These antibodies bind to the antigens. A signal is generated by the primary or secondary tag present on the specific antibody when the antibody binds to the antigen. This signal is then detected and quantification of the protein is done by measuring the signal [148, 157].

## 2.2 Conclusion

Oxidative stress is recognized as an important factor in male infertility. Accurate assessment is therefore critical in the laboratory evaluation of male infertility attributed to oxidative stress. In this chapter we have described various techniques that can be used to measure reactive oxygen species or their end-products using simple techniques such as nitroblue testing to ROS measurement by chemiluminescence assay, measuring antioxidants or end products of oxidative stress such as lipid peroxidation, DNA damage or protein modifications. We have highlighted the value of the newer tools such as proteomics in identifying oxidatively modified proteins and validating these proteins. The ultimate goal is to identify potential markers of oxidative stress to assist in elucidating the underlying mechanism of oxidative stress related sperm dysfunction that ultimately results in male infertility and assists the clinicians in the management of these patients.

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