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10 Oxidative stress measurement in patients with male or female factor infertility

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Abstract

Both male and female infertility is associated with poor sperm or oocyte quality. Oxidative stress has been shown to be involved in the pathophysiology of infertility. It is caused by an imbalance between the formation of reactive oxygen species (ROS) and the ability of the antioxidants to scavenge them. It is important to understand the nature of these species as they are necessary for the many physiological functions but can be harmful when produced in excess.

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The measurement of ROS in seminal ejaculates as well as in follicular fluid is explained in detail. Other markers that are helpful in evaluating oxidative stress are also described. Andrology laboratories can standardize these protocols and further aid our understanding of the causes of both male and female infertility as well as help design strategies for reducing the harmful effects of ROS and thereby improve fertilization and pregnancy rates.

Introduction

Infertility is a problem with a large magnitude. Free oxygen radicals and other reactive oxygen species (ROS) affect both male and female gametes. ROS influence spermatozoa and oocytes as well as their local environments. Excessive production of ROS results in oxidative stress. Oxidative stress, in turn, affects spermatozoa quality, fertilization, early embryo development and implantation and ultimately, pregnancy rates. Oxidative stress affects both natural and assisted fertility. Therefore, understanding how ROS are produced and how they affect various functions is important. Also important is the ability to accurately measure them and establish reference values, which may help in identifying the possible causes leading to poor fertilization and subsequent stages of implantation and pregnancy. This will also be necessary to develop strategies that will help reduce oxidative stress especially during assisted reproductive techniques. This chapter aims to better our understanding on what free radicals are, their importance and how they can be measured accurately in a laboratory setting.

1. What are free radicals?

Free radicals are a group of highly reactive chemical molecules that have one or more unpaired electrons and can oxidatively modify biomolecules that they encounter. This causes them to react almost instantly with any substance in their vicinity [1]. Generally, free radicals attack the nearest stable molecule, "stealing" its electron **(Figure 1).** When the "attacked" molecule loses its electron, it becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade and ultimately lead to the disrupting of living cells.

2. Types of free radicals

ROS represent a broad category of molecules that indicate the collection of radicals and non-radical oxygen derivatives. In addition, there is another class of free radicals that are nitrogen derived called reactive nitrogen species (RNS) (2). These reactive species are readily converted into reactive non-radical species by enzymatic or nonenzymatic chemical reactions that in turn can give rise to new radicals **(Table 1)**.

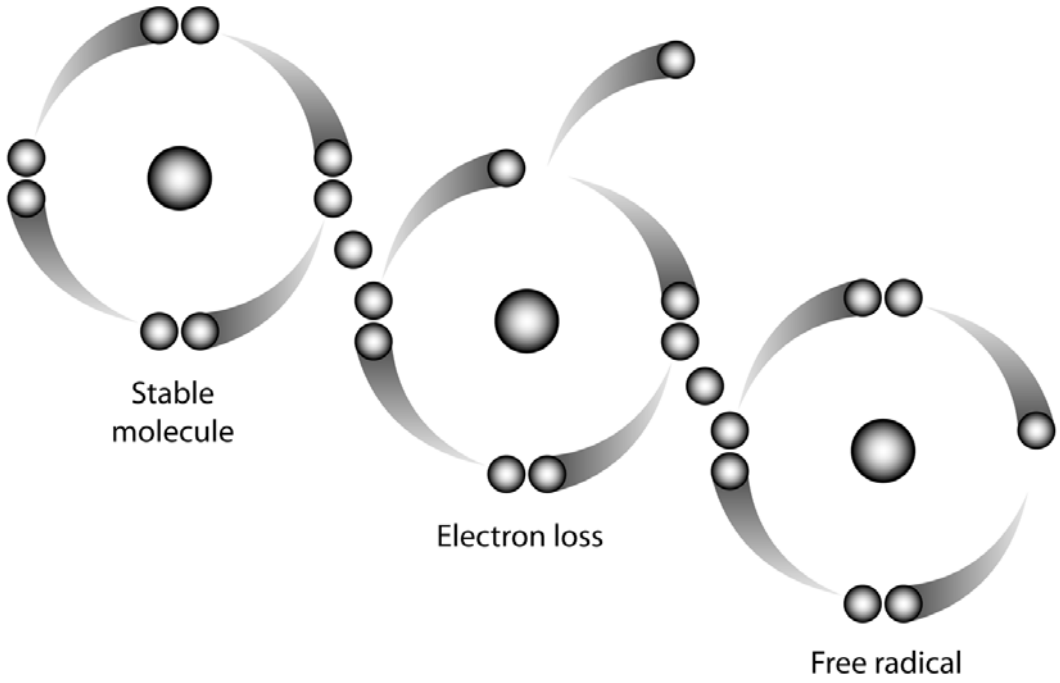

Figure 1. A schematic representation showing the formation of a free radical.

Table 1. Examples of free radicals.

Reactive oxygen species	Reactive nitrogen species
Superoxide anion ($O_2^{\bullet -}$)	Nitric oxide ($NO^{\bullet}$)
Hydrogen peroxide (H_2O_2)	Nitric dioxide ($NO_2^{\bullet}$)
Hydroxyl radical ($OH^{\bullet}$)	Peroxynitrite ($ONOO^-$)

3. Generation of free radicals in the seminal ejaculate

Human semen consists of different types of cells such as mature and immature spermatozoa, round cells from different stages of the spermatogenic process, leukocytes and epithelial cells. Of these, leukocytes (neutrophils and macrophages) and immature spermatozoa are the two main sources of ROS [3,4].

Reactive oxygen species are produced by spermatozoa when a defect occurs during spermatogenesis that results in the retention of cytoplasmic droplets [5]. The retention of excess residual cytoplasm is the link between poor sperm quality and elevated ROS. Spermatozoa carrying cytoplasmic droplets are thought to be immature and functionally defective [6]. There is a strong positive

correlation between immature spermatozoa and ROS production, which in turn is negatively correlated with sperm quality. Furthermore, as the concentration of immature spermatozoa in the human ejaculate increases, so does the concentration of mature spermatozoa with damaged DNA [7].

Peroxidase-positive leukocytes are believed to be the main source of ROS in semen. Reports suggest that positive peroxidase staining may be an accurate indicator of excessive ROS even at concentrations below the World Health Organization (WHO) cutoff value for leukocytospermia (concentration > $1x10^6$ peroxidase positive leukocytes/mL semen) [8,9]. The extent of damage caused by ROS resulting in sperm cell dysfunction depends on the nature, amount and duration of ROS exposure in addition to temperature, oxygen tension, concentration of ions, proteins and ROS scavengers [10].

4. Antioxidant defense mechanisms

Spermatozoa produce small amounts of ROS that are essential to many of the physiological processes such as capacitation, hyperactivation, and sperm-oocyte fusion [11,12]. Spermatozoa plasma membranes contain large quantities of polyunsaturated fatty acids (PUFA). Because their cytoplasm contains low concentrations of scavenging enzymes, they are particularly susceptible to the damage induced by excessive ROS [13,14]. The seminal plasma, however, contains two different types of antioxidants to minimize free radical-induced damage: enzymatic and non-enzymatic antioxidants **(Table 2)**.

Antioxidant defense mechanisms include three levels of protection: prevention, interception and repair. Prevention of ROS formation is the first line of defense against an oxidative insult. One example is the binding of metal ions, iron and copper ions in particular, which prevents them from initiating a chain reaction [12]. When transition metals become loosely bound to ROS, they can produce more reactive oxidants, particularly OH^- [13].

Table 2. Different classes of antioxidants that scavenge ROS in seminal plasma.

Enzymatic Antioxidants	Non-Enzymatic Antioxidants
Superoxide dismutase	Vitamin C, vitamin E
	and vitamin A (carotenoids)
Catalase	Proteins like Albumin, Transferrin,
	Haptoglobulin, Ceruloplasmin
Glutathione peroxidase	Glutathione (GSH)
Glutathione reductase	Pyruvate
	Ubiquinol

Free radicals have a tendency toward triggering a chain reaction. The interception of this reaction to prevent further damage is the process of deactivation, which leads to a nonradical end product formation [12]. Alpha-tocopherol, a chain-breaking antioxidant, inhibits lipid peroxidation by scavenging peroxyl (RO^-) and alkoxyl (ROO^-) radicals. The ability of α-tocopherol to maintain a steady-state rate of peroxyl radical reduction in the plasma membrane depends on the recycling of α-tocopherol by external reducing agents such as ascorbate or thiols. In this way, α-tocopherol is able to function again as a free radical chain-breaking antioxidant even though its concentration is low [14].

In most cases, free-radical induced damage can be repaired. Unfortunately, spermatozoa are unable to repair the damage induced by ROS because they lack the cytoplasmic enzyme systems required to accomplish this [15,16]. The pathological levels of ROS detected in the semen of infertile men are more likely caused by increased ROS production than by reduced antioxidant capacity of the seminal plasma [11].

5. What is oxidative stress?

Oxidative stress (OS) is the term applied when oxidants outnumber antioxidants [17]. It is a common condition caused by biological systems in aerobic conditions such that antioxidants cannot scavenge the free radicals. This causes an excessive generation of ROS, which damages cells, tissues and organs [18,19]. Evidence suggests that OS induced by ROS such as superoxide anion (O_2^-), hydroxyl radicals (OH^-) and a range of lipid peroxyl radicals produced in vascular cells is involved in the pathogenesis of a wide range of diseases of the reproductive system such as varicocele, endometriosis and infection [4,20].

6. ROS and infection

Sperm damage from ROS produced by leukocytes can occur when seminal plasma is removed during sperm preparation for assisted reproduction or when the seminal leukocyte concentration is abnormally high such as in leukocytospermia [21,22]. Both leukocytospermia and elevated ROS levels have been observed in patients with accessory gland infection [16]. In these clinical settings, impairment of sperm function is attributed to abnormal lipid peroxidation caused by elevated ROS [23]. The ability of polymorphonuclear neutrophils and macrophages to produce large amounts of ROS suggests that the ROS may be ultimately responsible for the leukocyte-induced sperm dysfunction [17,19,24].

During an infection, an imbalance of pro-oxidants and antioxidants favoring the former results in OS, which impairs the sperm functions

mentioned as well as motility and fertilization. Reactive oxygen species produced during infections of the testis and epididymis is especially harmful to spermatozoa due to the longer contact time and the lack of antioxidant protection [25]. In the final ejaculate, very high numbers of ROS-producing leukocytes are detrimental to sperm functions. An infectious injury involving ROS in the prostate gland, seminal vesicles or epididymis could impair sperm functions indirectly. Pro- and antioxidative properties of therapeutics are currently receiving more attention as part of anti-infectious therapies [18].

Leukocytes may have a role in stimulating ROS production by spermatozoa, but the exact mechanisms underlying such stimulation may involve direct sperm-leukocyte contact or may be mediated by soluble products released by the leukocytes [26].

7. Measurement of free radicals

Measurement of ROS is a helpful tool in the initial evaluation and follow-up of infertile male patients because high levels of OS seem to be strongly correlated with reduced fertility [17]. Numerous assays for ROS measurement have been introduced recently [20] **(Table 3; Fig. 2)**.

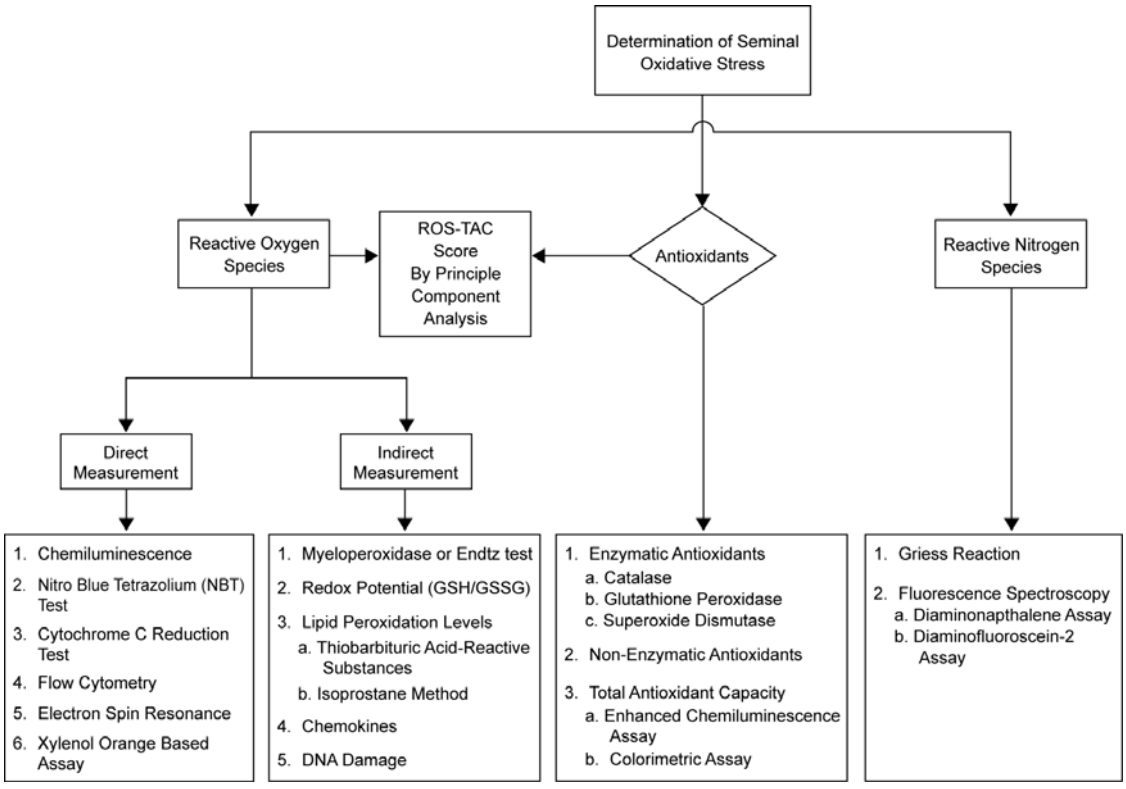

Figure 2. Flow diagram showing the various methods to measure seminal oxidative stress.

Table 3. Currently available tests for detection of reactive oxygen species (direct) or their oxidized products (indirect).

Assay	**Probe**	**Extracellular/ Intracellular**
Direct Measurement		
Tetrazolium nitroblue [24,27]	Ferricytochrome C	Extracellular
Chemiluminescence [25,28,29]	Luminol Lucigenin	Both Extracellular
Indirect Measurement		
Lipid peroxidation levels [27,30]	Thiobarbituric acid reactive substances	Measures oxidized component in the body fluids
Antioxidants, micronutrients, vitamins [31,32]	High-performance liquid chromatography	Serum and seminal plasma
Ascorbate [33]	High-performance liquid chromatography	Seminal plasma
Antioxidants enzymes [34-36]	Superoxide dismutase Catalase Glutathione peroxidase Glutathione reductase	Seminal plasma Seminal plasma Spermatozoa Spermatozoa
Chemokines [34,37]	ELISA	Seminal Plasma
Antioxidant-prooxidant status [11]	Total antioxidant capacity	Low molecular chain-breaking antioxidants

The chemiluminescence method is the most commonly used technique for measuring ROS produced by spermatozoa [38]. This assay quantifies both intracellular and extracellular ROS. Depending on the probe used, this method can differentiate between the production of superoxide and hydrogen peroxide by spermatozoa.

8. Luminometers

A variety of luminometers can be used to measure the light intensity resulting from the chemiluminescence reaction. Although all luminometers utilize photomultiplier tubes to detect photons, they differ in the processing of

signal input. Two different processing designs are presently found in luminometers. Photon counting luminometers count individual photons whereas direct current luminometers measure electric current that is maintained by, and is proportional to, the photon flux passing through the photomultiplier tube. The results are expressed as relative units (RLU), counted photons pre minute (cpm) or milivolts/sec.

Various models of luminometers are available and they differ in price, design and features **(Table 4)**. When comparing models, it is important to check their coefficient of variation and the lower limit of detection. Three types

Table 4. Some commercially available luminometer models: price and manufacturers [38].

Model	**Type**	**Sensitivity and dynamic range**	**Price (US $)**	**Manufacturer**
TD 20/20*	Single tube	0.1 fg luciferase, >5 orders	5,250	Turner biosystems Inc., Sunnyvale, CA, USA
FB-12*	Single tube	1000 molecules of luciferase, 6 orders	5,350	Zylux Corporation, Oak Ridge, TN, USA
Traithler	Single tube	1-10 pg ATP, 7 orders	6,000	Bioscan, Washington, D.C., USA
Zylux FB 15*	Single tube	1000 molecules of luciferase, >6 orders	7,450	Bio-World, Dublin, OH, USA
Optocomp-2*	Multiple tube	0.1 pg ATP, 6 orders	14,160	MGM Instruments, Inc., Hamden, CT, USA
Autolumat LB 953**	Multiple tube	5 amol of ATP, 6 orders	18,000	Berthold Technologies, Oak Ridge, TN, USA
MicroLumi XS*	Microplate	0.1 fg luciferase, >6 orders	9,000	Harta Instruments, Gaithersburg, MD, USA
Luminoskan*	Microplate	<0.5 fmol ATP, 6 orders	20,000	GMI, Inc., Albertville, MN, USA

ATP = adenosine 5' triphosphate.
* Instrument offers the option of direct data transfer to personal computer.
** Instrument has ability to measure in a range of $1\text{-}10^6$ counts without saturation. Beyond this range the instrument has a linear response to the signal input.

of luminometers are commercially available. Single/double tube luminometers are inexpensive and can measure only one or two samples at a given time. These are suitable for small research laboratories. Multiple tube luminometers are more expensive because they can measure multiple samples at one time. These are suitable for centers that are engaged in large scale research that measure ROS in samples by chemiluminescence very frequently. Plate luminometers can analyze multiple samples on a single plate. Each plate is disposable and is relatively inexpensive (approximately $5 each). However, the entire plate must be disposed of, even when measuring luminescence for a single sample. These luminometers are therefore more suitable for commercial entities and core research laboratories [38].

Multiple factors affect chemiluminescent reactions. These include the concentration of reaction mixture, sample volume, temperature control and background luminescence. The person who operates these instruments should be familiar with these factors, which will enable them to obtain consistently accurate results [39]. Our center currently uses the LB953 luminometer (Model: LKB 953, Wallac Inc., Gaithersburg, MD), which is a photon-counting instrument that covers a spectral range from 390 to 620nm.

9. Chemiluminescence measurement

A. Equipment and material

i). Disposable polystyrene tubes with caps (15mL)
ii). Eppendorf pipets (5 μL, 10 μL)
iii). Serological Pipets (1 mL, 2 mL, 10 mL)
iv). Desk top centrifuge
v). Disposable MicroCell Slides
vi). Dimethyl Sulfoxide (DMSO; Catalog # D8779, Sigma Chemical Co., St. Louis, MO)
vii). Luminol (5-amino-2,3 dihydro-1,4 phthalazinedione; Catalog # A8511, Sigma Chemical Co., St. Louis, MO)
viii). Polystyrene Round bottom tubes (6mL)
ix). Luminometer (Model: LKB 953, Wallac Inc., Gaithersburg, MD)
x). Dulbecco's Phosphate Buffered Saline Solution 1X (PBS-1X; Catalog #9235, Irvine Scientific, Santa Ana, CA)

B. Reagent preparation

a). Stock Luminol (100 mM): Weigh 177.09 mg of luminol and add it to 10 mL of DMSO solution in a polystyrene tube. The tube must be covered in aluminum foil due to the light sensitivity of the luminol. It can be stored at room temperature in the dark until the expiration date.

b). Working Luminol (5 mM): Mix 20 μL luminol stock solution with 380 μL DMSO in a foil-covered polystyrene tube. This must be done prior to every use. Store at room temperature in the dark until needed.

c). DMSO solution: Provided ready to use. Store at room temperature until the expiration date.

C. Specimen preparation and ROS measurement

Allow the semen sample to undergo liquefaction in a 37°C incubator for 20 minutes. Next, record the initial characteristics such as volume, pH and color and manually verify sperm count and motility. After liquefaction, process the semen specimens for ROS measurement as briefly described [40] **(Fig 3):**

1). Samples are centrifuged at 300 x g for 7 minutes, and the seminal plasma is removed.
2). The sperm pellet is suspended in 3 mL of Dulbecco's PBS solution (Irvine Scientific, Santa Ana, CA) and washed again at 300 x g for 7 minutes.
3). The sperm concentration is adjusted to 20 x 10^6/mL before ROS measurement. ROS formation is measured by a chemiluminescence assay using 5 μL of luminol (5 mM, 5-amino-2, 3-dihydro-1, 4-phthalazinedione, Sigma Chemical Company, St Louis, MO).
4). Chemiluminescence is measured in the integration mode using a LB 953 luminometer (Model: LKB 953, Wallac Inc., Gaithersburg, MD) at 37^{o}C for 15 minutes after the luminol is added. Reactive oxygen species production is expressed as counted photons per minute (cpm)/20 x10^6 sperm.

D. Types of samples for ROS measurement

ROS measurement can be performed in various types of samples such as:

1. Neat or unprocessed, whole seminal ejaculate [41].
2. Processed sample: Seminal plasma is removed by washing and the sample is resuspended in the culture media [38]. Measurement is done after liquefied semen specimens are centrifuged at x300*g* for 7 minutes and seminal plasma is removed. The sperm pellet is washed and resuspended to 1 mL volume in PBS. With this method the seminal plasma and other dissolved components are removed. However, all the cellular components such as debris, round cells, white blood cells and leukocytes are still present in the sample.
3. Sperm preparation by the swim-up procedure: After liquefaction, an aliquot of specimen is mixed with sperm wash media (Sage BioPharma, Bedminster, NJ) using a sterile Pasteur pipette. It is centrifuged at x330*g* for 10 minutes. The supernatant is carefully aspirated and the pellet resuspended

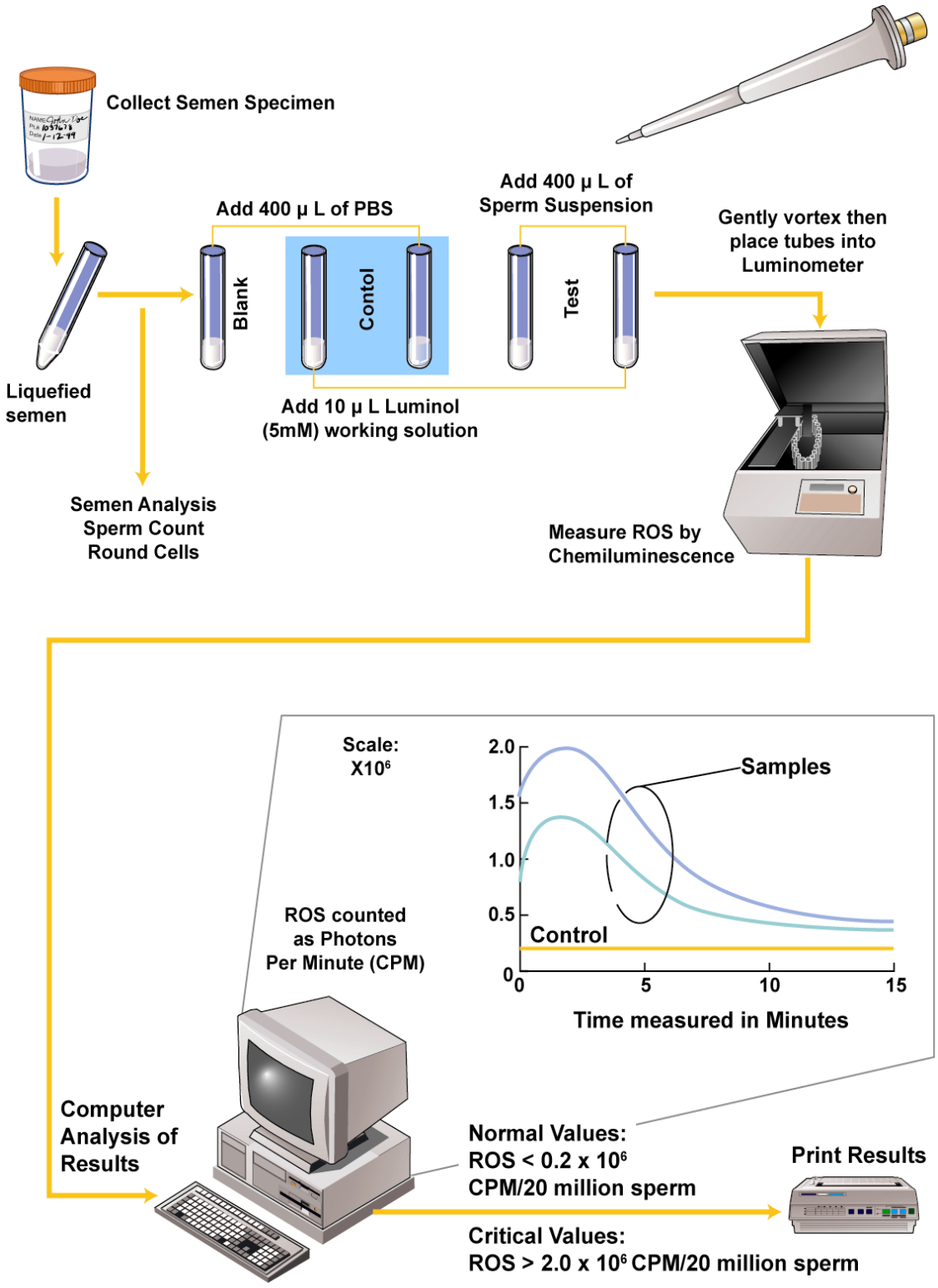

Figure 3. Laboratory measurement of ROS in the seminal ejaculates by chemiluminescence. The inset depicts a representative ROS signal for control (OS negative) and 2 ROS positive samples.

in 3 mL of fresh sperm wash media. The resuspended sample is carefully transferred in equal parts to two 15 mL sterile round-bottom test tubes and centrifuged at x330*g* for 5 minutes. Motile sperm are allowed to swim up during the incubation of test tubes at a 45° angle in 5% CO_2 at 37°C for 1 hour. Supernatant is aspirated into a clean test tube and centrifuged at x330*g* for 7 minutes. The final supernatant is aspirated and the sperm pellet re-suspended in 0.5 mL of sperm wash media. The final volume is measured, and the semen analysis is performed on an aliquot of the sample [42].

4. Sperm preparation by density gradients: With this method, a double density gradient (40% 'Upper phase' and 80% 'Lower phase') is used [42]. Both the density gradient and the sperm wash media is brought to 37°C or room temperature. Using a sterile pipette, 2.0 mL of the "lower phase' is transferred into a 15 mL conical centrifuge tube. Using a new sterile pipette, 2.0 mL of the "upper layer" is carefully placed on the top of the lower layer. The liquefied semen sample (1-2 mL) is placed on top of the upper layer, and the tube is centrifuged for 20 minutes at x330*g*. The upper and lower layers are carefully aspirated without disturbing the pellet. Using a transfer pipette, 2-3 mL of sperm wash media (Sage BioPharma, Bedminster, NJ) is added, and the resuspended pellet is centrifuged for 7 minutes at x330*g*. The supernatant is removed, and the pellet is suspended in 1.0 mL of sperm wash media. Sperm count, motility and ROS levels are measured in the recovered fractions.

E. ROS measurement by luminometer

After turning on the luminometer, label seven 6 mL tubes and add reagents as shown in **Table 5**. This procedure must occur in subdued light.

Table 5. Steps showing the assay protocol for measuring ROS.

No.	Labeled tube	PBS vol*.	Specimen Vol.	Luminol (5 mM)
1.	Blank	400 µL	--	
2.	Control 1	400 µL	--	10 µL
3.	Control 1	400 µL	--	10 µL
4.	Patient 1	--	400 µL	10 µL
5.	Patient 1	--	400 µL	10 µL
6	Patient 2	--	400 µL	10 µL
7	Patient 2	--	400 µL	10 µL

Note: To avoid contamination, change pipette tips after each addition.

*Blank and control tubes should ideally contain filtered seminal plasma. However, we found no differences in ROS levels between filtered seminal plasma and PBS. For convenience, PBS can be used instead of filtered seminal plasma.

F. Enter the information in the luminometer

A). Protocol: Enter the patient information, number of samples, single measuring time and data points measured in the integrated mode. Generally, the measurement time is 15 minutes.

B). Measurement: Here, the file name must be entered. Verify the information is accurate.

Run the assay, and when measurement is complete, add the details of each tube in the comments section. Go to "evaluation" and check the results, which can also be printed.

G. Calculation of ROS results

a). Calculate the average of the control tubes.
b). Calculate the average of each set of patient sample tubes. Subtract the control value from the test value.
c). The results must be expressed as $x10^6$ counted photons per minute (cpm).
d). Next, the results must be expressed as cpm /20 million sperm. To calculate this, plug in the actual sperm concentration and multiply by the appropriate factor.

For example:
Sperm count = 8.5 x 10^6 sperm/mL and ROS levels are 0.2 x 10^6cpm
To express this per 20 x 10^6 sperm/mL:

$$\frac{0.2}{8.5} \times 20 = 0.47 \times 10^6 \text{ cpm}/ 20 \times 10^6 \text{ sperm}$$

The cutoff values for abnormal ROS levels depend on the type of sample:

Values > 0.2 x 10^6 cpm/ 20 x 10^6 sperm for neat sample and > 1 x 10^6 cpm/ 20 x 10^6 sperm for washed samples are considered to be ROS positive.

10. Reference values for abnormal ROS

Seminal OS is a result of an imbalance between ROS production and antioxidant protection. So, OS can be measured either by assessing ROS or the total antioxidant capacity (TAC). It is important to have a reliable and reproducible method of ROS measurement for clinical purposes. Also, strict quality control must be in place for this assay to be valid in a clinical laboratory setting.

Neither measurement of ROS levels or TAC alone can exactly quantify OS. However, it is possible to combine these two parameters into one index score [17]. Briefly, the ROS and TAC values from the controls are used to create a scale of these two variables that uses the control values as reference points. The log of (ROS +1) is used in calculations so that both values are

normalized to the same distribution. First, both TAC and log (ROS +1) are standardized to z-scores so that both will have the same variability. These standardized scores are calculated by subtracting the mean values for the controls from the mean value for the patients and dividing by standard deviation of the control population.

For log (ROS + 1): Standardized ROS = [log (ROS + 1) - 1.3885]/0.7271

For TAC: Standardized TAC = (TAC – 1650.93)/532.22

These two standardized variables are then analyzed with the principal components analysis, which provided linear combinations (or weighted sums) that account for the most variability among correlated variables. The first principal component provides the following linear equation:

Principal component = (- 0.707 x standardized ROS) + (0.707 x standardized TAC)

To ensure that the distribution of the ROS-TAC score will have a mean of 50 and standard deviation of 10 in controls, the ROS/TAC score is transformed as:

ROS-TAC score = 50 + (Principal component x 10.629)

This index minimizes the variability of the parameters alone. The ROS-TAC score was based on a group of normal healthy fertile men who had very low levels of ROS. The ROS-TAC score was found to be better than ROS or TAC alone in discriminating between fertile and infertile men. Infertile men with male factor infertility or an idiopathic diagnosis had significantly lower ROS-TAC scores than the healthy controls, and men with a male factor diagnosis who eventually were able to initiate a pregnancy had significantly higher ROS-TAC scores than those who were not able to do so. The average ROS-TAC score for fertile healthy men was 50.0 ± 10, which was significantly higher ($p \leq 0.0002$) than that of the infertile patients (35.8 ± 15). The probability of successful pregnancy is estimated at <10% for values of ROS-TAC <30, but increased as the ROS-TAC score increased.

Recently, measurement of ROS in neat semen has proved to be an accurate and reliable test for assessing the OS status [41]. Also, assessing ROS directly in neat semen has diagnostic and prognostic capabilities identical to that of the ROS-TAC score. This methodology accurately represents the true in vivo OS status of an individual and overcomes the drawbacks of earlier methods as the processing of semen may generate ROS by itself. However, sperm preparation is necessary to enhance and maintain sperm quality and function following ejaculation before it can be used for assisted reproduction [41]. Levels of ROS were significantly lower in neat semen than in washed spermatozoa. However, ROS levels in neat semen showed a strong positive correlation with ROS

levels in washed semen [43,44]. The measurement of ROS levels for fertile donors with normal semen parameters was 1.5 x 10^4 cpm/20 million sperm/mL. Using this cutoff, infertile men can be classified as either OS-positive (>1.5 x 10^4 cpm/20 million sperm/mL) or OS-negative (≤1.5 x 10^4 cpm/20 million sperm/ml), irrespective of their clinical diagnosis or results of standard semen analysis [44].

11. Probes for extracellular and intracellular ROS measurement

Two probes may be used with the chemiluminescence assay: luminol and lucigenin. A luminol-mediated chemiluminescence signal in spermatozoa occurs when luminol oxidizes at the acrosomal level. Luminol reacts with a variety of ROS and allows both intracellular and extracellular ROS to be measured. Lucigenin, however, yields a chemiluminescence that is more specific for superoxide anions released extracellularly [29,45].

The luminol assay is more advantageous for a number of reasons. It can measure H_2O_2, $O_2^{\bullet-}$, and $OH^{\bullet-}$ levels, although it cannot distinguish these oxidants from one another [20]. It can also measure the global level of ROS under physiological conditions, and it is easy to use. In addition, the assay can measure both extracellular and intracellular ROS, which means it has a high sensitivity [20]. Multiple studies have correlated high chemiluminescent signals using luminol as a probe with adverse effects on sperm function. The assay can be sensitized by adding horseradish peroxidase to the sperm suspension, thereby increasing the spontaneous luminescence levels commonly observed in healthy semen samples [45].

12. Other methods for ROS measurement

Flow cytometry can be used to indirectly measure ROS by measuring the fluorescent intensity of the compounds oxidized by ROS. Oxidation of 2, 7 dichlorofluorescin diacetate (DCFH-DA) by ROS, which is generated within the cell, makes them highly fluorescent. The fluorescence can be quantified, which reflects the rate and quantity of the ROS produced [46].

Hydroethidine (HE) is another fluorescent probe that can be used for ROS measurement. It is a substance that is oxidized by the superoxide anion to become ethidium bromide with red florescence emission [47]. Although a flow cytometer can be used to accurately measure intracellular ROS, it is an expensive piece of equipment and lacks standardization [38]. An epifluorescence microscope can be used to assess the fluorescent probe, too, which is a more simple and inexpensive technique [48]. In addition, the thermochemiluminescence (TCL) assay has also been used to measure ROS.

This assay is based on the heat-induced oxidation of biological fluids, which leads to the formation of electronically excited species in the form of unstable carbonyls, which further decompose into stable carbonyls and light energy (low chemiluminescence) in the wavelength range of 300–650 nm [49].

13. ROS in frozen semen samples

Decreasing the temperature during the process of semen cryopreservation can stimulate semen cells to produce significant amounts of ROS. Higher levels of ROS are reported during cooling if the semen sample is contaminated by $>0.5 \times 10^6$ leukocytes. Removing leukocytes from semen samples or treating the samples with antioxidants prior to cryopreservation may improve sperm viability and function [50]. In some samples, ROS has been to shown to increase following cryopreservation. Significantly lower post-thaw recovery of sperm motility and vitality was seen compared to ROS-free samples. This was likely due to oxidative stress that led to the lipid peroxidation of the sperm membranes [51]. Levels of intracellular ROS, mitochondrial membrane potential, lipid peroxidation and DNA damage (all indirect measurements of ROS) also increase following cryopreservation. Furthermore, **e**vidence suggests that high levels of ROS can mediate damage to many cellular elements in the testis including the DNA of mature spermatozoa [52].

Studies looking at the effects of freezing on DNA damage have produced conflicting results. Whereas a significant increase in sperm DNA damage has been reported following cryopreservation-thawing in some studies [53-55], others have reported no evident deleterious effects on sperm DNA integrity; although motility, viability and mitochondrial function are frequently impaired [54,56,57]. In contrast, freezing of spermatozoa from infertile men has a significant detrimental effect on sperm DNA integrity, sperm motility, viability and mitochondrial function [54,56,58]. Cryopreservation by itself could decrease the sperm motility and increase DNA fragmentation [59]. Reactive oxygen species may play a role in decreasing motility and viability of cryopreserved sperm after thawing, which could be counteracted by adding Vitamin E to the freezing media [60].

14. Effects of ROS on post-fertilization events

Reactive oxygen species have deleterious effects on post-fertilization embryos through different mechanisms. They can penetrate the cell membrane of the embryos and produce mitochondrial damage, cell block or even embryo apoptosis. Reactive oxygen species can also increase the 2-cell block in mouse embryo as a result of an increase in lipid peroxidation [61]. Culture media with high levels of hydrogen peroxide show a higher incidence of fragmentation and apoptosis of the embryos [62].

15. ROS in female infertility

Peritoneal fluid containing immune-related cells are present in the vesicouterine cavity or the Douglas pouch. Peritoneal fluid may play a major role in controlling the peritoneal microenvironment. Reactive oxygen species are present in the peritoneal fluid of patients with endometriosis and idiopathic infertility. Wang et al. showed that levels of ROS are significantly higher in patients with idiopathic infertility than in healthy controls undergoing laparoscopic tubal ligation. Their study suggests that ROS in the peritoneal fluid may play a role in idiopathic infertility [63].

The follicular fluid environment may also play an important role in oocyte development. A positive correlation has been found between level of ROS in the intrafollicular fluid and the pregnancy outcome in patients undergoing in vitro fertilization (IVF). A low concentration of ROS in the follicular fluid may act as a marker for IVF success [64]. In addition, a positive relation was demonstrated between pregnancy rates and levels of lipid peroxides and the TAC in patients undergoing IVF or intracytoplasmic sperm injection (ICSI) [65]. Follicular fluid from women with endometriosis undergoing IVF may have higher levels of ROS to increase the chance of achieving pregnancy [65]. These differences may be attributed to differing participant recruitment practices [66].

16. Importance of free radicals in a clinical setting

A small percentage of apparently healthy men are unable to impregnate their spouses, even when there is no female factor infertility involved. These patients are usually classified as having idiopathic infertility. Men with idiopathic infertility generally present with significantly higher seminal ROS levels and lower antioxidant properties than healthy controls [19].

Recent studies have reported high levels of ROS in the semen of 25% to 40% of infertile men [67]. High levels of ROS are negatively correlated with sperm concentration and sperm motility [68]. High levels of seminal ROS have been observed in infertile men with varicocele as well. Patients with varicocele were also found to have low levels of TAC in their seminal plasma and may benefit from antioxidant supplementation [69]. Although reports of pregnancy rates after varicocelectomy are conflicting, rates have been shown to increase even if routine semen parameters do not improve [70]. This may be a result of decreased ROS levels [43].

Levels of ROS were found to be increased significantly in men with spinal cord injury and were associated with poor sperm motility and morphology [71]. In addition, OS has been shown to affect the integrity of the sperm genome by causing high frequencies of single and double-strand DNA breaks, which are often detected in the ejaculates of infertile patients [72]. Oxidative

stress can have damaging effects on female fertility and affect ovulation, fertilization, embryo development and implantation [73]. Thus, OS is considered a cause of female infertility, and this is particularly relevant in cases of endometriosis [74,75].

Studies concerning the role of ROS in female reproduction have reported that both natural and assisted fertility can be affected [73,76-78]. Oxidative stress-mediated precipitation of pathologies in the female reproductive tract is similar to those involved in male infertility. Oxidative stress influences the oocyte and embryo quality and thus, fertilization rates as well. Reactive oxygen species appear to play a significant role in the modulation of gamete interaction and are necessary for successful fertilization [79]. Reactive oxygen species in culture media may impact post-fertilization development, cleavage rate and blastocyst yield and quality, which are reliable indicators of assisted reproduction outcomes [73]. Also, generation of ROS in seminal plasma has lead to DNA fragmentation, which was negatively correlated with assisted reproduction outcomes [80].

17. Strategies to reduce ROS

Selection of the ideal sperm preparation technique is important to minimize the effects of ROS [41]. A study comparing the density gradient and swim-up technique for sperm preparation showed that the percentage of sperm with DNA fragmentation was significantly lower when the former technique was used knowledge that could be beneficial for couples undergoing assisted reproduction techniques [81]. The density gradient technique separates leukocytes and immature and damaged spermatozoa from the normal spermatozoa, which can be used in assisted reproduction [82]. Supplementing the media with appropriate antioxidants may also help counter the damaging effects of ROS.

In cases of IVF, insemination times of more than 16-20 hours have been associated with increased ROS production [83]. Shortening the insemination time (to 1-2 hours or even less) can reduce ROS levels the culture media [84] and potentially help improve fertilization, implantation and pregnancy rates.

18. Summary

In summary, ROS are necessary for various physiological functions but an imbalance in favor of ROS results in OS. Reactive oxygen species can be measured in a variety of fluids from both males and females and are involved in the pathophysiology of infertility. Various techniques as well as instruments are available for accurately measuring ROS. Supplementation with appropriate antioxidants may be beneficial in reducing the harmful effects of ROS.

References

1. Warren, J.S., K.J. Johnson and P.A. Ward: Oxygen radicals in cell injury and cell death. Pathol Immunopathol Res. 1987; 6:p. 301-15.
2. Sikka, S.C.: Relative impact of oxidative stress on male reproductive function. Curr Med Chem. 2001; 8:p. 851-62.
3. Aitken, R.J. and K.M. West: Analysis of the relationship between reactive oxygen species production and leucocyte infiltration in fractions of human semen separated on Percoll gradients. Int J Androl. 1990; 13:p. 433-51.
4. Hendin, B.N., P.N. Kolettis, R.K. Sharma, A.J. Thomas, Jr. and A. Agarwal: Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. J Urol. 1999; 161:p. 1831-4.
5. Gomez, E., D.W. Buckingham, J. Brindle, F. Lanzafame, D.S. Irvine and R.J. Aitken: Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J Androl. 1996; 17:p. 276-87.
6. Huszar, G., M. Sbracia, L. Vigue, D.J. Miller and B.D. Shur: Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane beta 1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. Biol Reprod. 1997; 56:p. 1020-4.
7. Gil-Guzman, E., M. Ollero, M.C. Lopez, R.K. Sharma, J.G. Alvarez, A.J. Thomas, Jr. and A. Agarwal: Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod. 2001; 16:p. 1922-30.
8. Sharma, R.K., F.F. Pasqualotto, D.R. Nelson, A.J. Thomas, Jr. and A. Agarwal: Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. J Androl. 2001; 22:p. 575-83.
9. Novotny, J., I.I. Oborna, J. Brezinova, M. Svobodova, J. Hrbac and H. Fingerova: The occurrence of reactive oxygen species in the semen of males from infertile couples. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2003; 147:p. 173-176.
10. Agarwal, A. and R.A. Saleh: Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am. 2002; 29:p. 817-27.
11. Lewis, S.E., P.M. Boyle, K.A. McKinney, I.S. Young and W. Thompson: Total antioxidant capacity of seminal plasma is different in fertile and infertile men. Fertil Steril. 1995; 64:p. 868-70.
12. Sies, H.: Strategies of antioxidant defense. Eur J Biochem. 1993; 215:p. 213-9.
13. Halliwell, B.: How to characterize a biological antioxidant. Free Radic Res Commun. 1990; 9:p. 1-32.
14. Buettner, G.R.: The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. Arch Biochem Biophys. 1993; 300:p. 535-43.
15. Irshad, M. and P.S. Chaudhuri: Oxidant-antioxidant system: role and significance in human body. Indian J Exp Biol. 2002; 40:p. 1233-9.

16. Ochsendorf, F.R.: Infections in the male genital tract and reactive oxygen species. Hum Reprod Update. 1999; 5:p. 399-420.
17. Sharma, R.K., F.F. Pasqualotto, D.R. Nelson, A.J. Thomas, Jr. and A. Agarwal: The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod. 1999; 14:p. 2801-7.
18. Aitken, R.J. and H.W. Baker: Seminal leukocytes: passengers, terrorists or good samaritans? Hum Reprod. 1995; 10:p. 1736-9.
19. Pasqualotto, F.F., R.K. Sharma, H. Kobayashi, D.R. Nelson, A.J. Thomas, Jr. and A. Agarwal: Oxidative stress in normospermic men undergoing infertility evaluation. J Androl. 2001; 22:p. 316-22.
20. Sharma, R.K. and A. Agarwal: Role of reactive oxygen species in male infertility. Urology. 1996; 48:p. 835-50.
21. Potts, J.M. and F.F. Pasqualotto: Seminal oxidative stress in patients with chronic prostatitis. Andrologia. 2003; 35:p. 304-8.
22. Agarwal, A., T.M. Said, M.A. Bedaiwy, J. Banerjee and J.G. Alvarez: Oxidative stress in an assisted reproductive techniques setting. Fertil Steril. 2006; 86:p. 503-12.
23. Potts, J.M., R. Sharma, F. Pasqualotto, D. Nelson, G. Hall and A. Agarwal: Association of ureaplasma urealyticum with abnormal reactive oxygen species levels and absence of leukocytospermia. J Urol. 2000; 163:p. 1775-8.
24. Gavella, M. and V. Lipovac: NADH-dependent oxidoreductase (diaphorase) activity and isozyme pattern of sperm in infertile men. Arch Androl. 1992; 28:p. 135-41.
25. Pasqualotto, F.F., R.K. Sharma, J.M. Potts, D.R. Nelson, A.J. Thomas and A. Agarwal: Seminal oxidative stress in patients with chronic prostatitis. Urology. 2000; 55:p. 881-5.
26. Wymann, M.P., V. von Tscharner, D.A. Deranleau and M. Baggiolini: Chemiluminescence detection of H_2O_2 produced by human neutrophils during the respiratory burst. Anal Biochem. 1987; 165:p. 371-8.
27. Alvarez, J.G., J.C. Touchstone, L. Blasco and B.T. Storey: Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J Androl. 1987; 8:p. 338-48.
28. McKinney, K.A., S.E. Lewis and W. Thompson: Reactive oxygen species generation in human sperm: luminol and lucigenin chemiluminescence probes. Arch Androl. 1996; 36:p. 119-25.
29. Aitken, R.J., D.W. Buckingham and K.M. West: Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J Cell Physiol. 1992; 151:p. 466-77.
30. Aitken, R.J., J.S. Clarkson and S. Fishel: Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod. 1989; 41:p. 183-97.
31. Lenzi, A., M. Picardo, L. Gandini, F. Lombardo, O. Terminali, S. Passi and F. Dondero: Glutathione treatment of dyspermia: effect on the lipoperoxidation process. Hum Reprod. 1994; 9:p. 2044-50.
32. Kessopoulou, E., H.J. Powers, K.K. Sharma, M.J. Pearson, J.M. Russell, I.D. Cooke and C.L. Barratt: A double-blind randomized placebo cross-over controlled

trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. Fertil Steril. 1995; 64:p. 825-31.
33. Thiele, J.J., H.J. Friesleben, J. Fuchs and F.R. Ochsendorf: Ascorbic acid and urate in human seminal plasma: determination and interrelationships with chemiluminescence in washed semen. Hum Reprod. 1995; 10:p. 110-5.
34. Rajasekaran, M., W.J. Hellstrom, R.K. Naz and S.C. Sikka: Oxidative stress and interleukins in seminal plasma during leukocytospermia. Fertil Steril. 1995; 64:p. 166-71.
35. Nissen, H.P. and H.W. Kreysel: Superoxide dismutase in human semen. Klin Wochenschr. 1983; 61:p. 63-5.
36. Li, T.K.: The glutathione and thiol content of mammalian spermatozoa and seminal plasma. Biol Reprod. 1975; 12:p. 641-6.
37. Buch, J.P., T.F. Kolon, N. Maulik, D.L. Kreutzer and D.K. Das: Cytokines stimulate lipid membrane peroxidation of human sperm. Fertil Steril. 1994; 62:p. 186-8.
38. Agarwal, A., S.S. Allamaneni and T.M. Said: Chemiluminescence technique for measuring reactive oxygen species. Reprod Biomed Online. 2004; 9:p. 466-8.
39. Berthold, F., K. Herick and R.M. Siewe: Luminometer design and low light detection. Methods Enzymol. 2000; 305:p. 62-87.
40. Kobayashi, H., E. Gil-Guzman, A.M. Mahran, Rakesh, D.R. Nelson, A.J. Thomas, Jr. and A. Agarwal: Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. J Androl. 2001; 22:p. 568-74.
41. Allamaneni, S.S., A. Agarwal, K.P. Nallella, R.K. Sharma, A.J. Thomas, Jr. and S.C. Sikka: Characterization of oxidative stress status by evaluation of reactive oxygen species levels in whole semen and isolated spermatozoa. Fertil Steril. 2005; 83:p. 800-3.
42. Allamaneni, S.S., A. Agarwal, S. Rama, P. Ranganathan and R.K. Sharma: Comparative study on density gradients and swim-up preparation techniques utilizing neat and cryopreserved spermatozoa. Asian J Androl. 2005; 7:p. 86-92.
43. Saleh, R.A. and A. Agarwal: Oxidative stress and male infertility: from research bench to clinical practice. J Androl. 2002; 23:p. 737-52.
44. Saleh, R.A., A. Agarwal, E. Kandirali, R.K. Sharma, A.J. Thomas, E.A. Nada, D.P. Evenson and J.G. Alvarez: Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. Fertil Steril. 2002; 78:p. 1215-24.
45. Aitken, R.J. and D. Buckingham: Enhanced detection of reactive oxygen species produced by human spermatozoa with 7-dimethyl amino-naphthalin-1, 2-dicarbonic acid hydrazide. Int J Androl. 1992; 15:p. 211-9.
46. Bass, D.A., J.W. Parce, L.R. Dechatelet, P. Szejda, M.C. Seeds and M. Thomas: Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol. 1983; 130:p. 1910-7.
47. Rothe, G. and G. Valet: Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. J Leukoc Biol. 1990; 47:p. 440-8.
48. Henkel, R., E. Kierspel, M. Hajimohammad, T. Stalf, C. Hoogendijk, C. Mehnert, R. Menkveld, W.B. Schill, and T.F. Kruger: DNA fragmentation of spermatozoa and assisted reproduction technology. Reprod Biomed Online. 2003; 7:p. 477-84.

49. Lissak, A., Z. Wiener-Megnazi, A.Z. Reznick, S. Shnizer, D. Ishai, B. Grach, S. Lahav-Baratz, H. Shiloh, M. Koifman, and M. Dirnfeld: Oxidative stress indices in seminal plasma, as measured by the thermochemiluminescence assay, correlate with sperm parameters. Fertil Steril. 2004; 81 Suppl 1:p. 792-7.
50. Wang, A.W., H. Zhang, I. Ikemoto, D.J. Anderson and K.R. Loughlin: Reactive oxygen species generation by seminal cells during cryopreservation. Urology. 1997; 49:p. 921-5.
51. Mazzilli, F., T. Rossi, L. Sabatini, F.M. Pulcinelli, S. Rapone, F. Dondero and P.P. Gazzaniga: Human sperm cryopreservation and reactive oxygen species (ROS) production. Acta Eur Fertil. 1995; 26:p. 145-8.
52. Helbock, H.J., K.B. Beckman, M.K. Shigenaga, P.B. Walter, A.A. Woodall, H.C. Yeo and B.N. Ames: DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc Natl Acad Sci U S A. 1998; 95:p. 288-93.
53. Donnelly, E.T., N. McClure and S.E. Lewis: Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. Fertil Steril. 2001; 76:p. 892-900.
54. Donnelly, E.T., E.K. Steele, N. McClure and S.E. Lewis: Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod. 2001; 16:p. 1191-9.
55. Anzar, M., L. He, M.M. Buhr, T.G. Kroetsch and K.P. Pauls: Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod. 2002; 66:p. 354-60.
56. Hammadeh, M.E., A.S. Askari, T. Georg, P. Rosenbaum and W. Schmidt: Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. Int J Androl. 1999; 22:p. 155-62.
57. O'Connell, M., N. McClure and S.E. Lewis: The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod. 2002; 17:p. 704-9.
58. Thompson-Cree, M.E., N. McClure, E.T. Donnelly, K.E. Steele and S.E. Lewis: Effects of cryopreservation on testicular sperm nuclear DNA fragmentation and its relationship with assisted conception outcome following ICSI with testicular spermatozoa. Reprod Biomed Online. 2003; 7:p. 449-55.
59. Baumber, J., B.A. Ball, J.J. Linfor and S.A. Meyers: Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J Androl. 2003; 24:p. 621-8.
60. Askari, H.A., J.H. Check, N. Peymer and A. Bollendorf: Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Arch Androl. 1994; 33:p. 11-5.
61. Nasr-Esfahani, M.H., J.R. Aitken and M.H. Johnson: Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. Development. 1990; 109:p. 501-7.
62. Yang, H.W., K.J. Hwang, H.C. Kwon, H.S. Kim, K.W. Choi and K.S. Oh: Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. Hum Reprod. 1998; 13:p. 998-1002.

63. Wang, Y., R.K. Sharma, T. Falcone, J. Goldberg and A. Agarwal: Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. Fertil Steril. 1997; 68:p. 826-30.
64. Attaran, M., E. Pasqualotto, T. Falcone, J.M. Goldberg, K.F. Miller, A. Agarwal and R.K. Sharma: The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int J Fertil Womens Med. 2000; 45:p. 314-20.
65. Pasqualotto, E.B., A. Agarwal, R.K. Sharma, V.M. Izzo, J.A. Pinotti, N.J. Joshi and B.I. Rose: Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertil Steril. 2004; 81:p. 973-6.
66. Das, S., R. Chattopadhyay, S. Ghosh, S. Ghosh, S.K. Goswami, B.N. Chakravarty and K. Chaudhury: Reactive oxygen species level in follicular fluid--embryo quality marker in IVF? Hum Reprod. 2006; 21:p. 2403-7.
67. Padron, O.F., N.L. Brackett, R.K. Sharma, C.M. Lynne, A.J. Thomas, Jr. and A. Agarwal: Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. Fertil Steril. 1997; 67:p. 1115-20.
68. Agarwal, A. and T.M. Said: Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. BJU Int. 2005; 95:p. 503-7.
69. Allamaneni, S.S., C.K. Naughton, R.K. Sharma, A.J. Thomas, Jr. and A. Agarwal: Increased seminal reactive oxygen species levels in patients with varicoceles correlate with varicocele grade but not with testis size. Fertil Steril. 2004; 82:p. 1684-6.
70. Daitch, J.A., M.A. Bedaiwy, E.B. Pasqualotto, B.N. Hendin, J. Hallak, T. Falcone, A.J. Thomas, Jr., D.R. Nelson, and A. Agarwal: Varicocelectomy improves intrauterine insemination success rates in men with varicocele. J Urol. 2001; 165:p. 1510-3.
71. de Lamirande, E., B.E. Leduc, A. Iwasaki, M. Hassouna and C. Gagnon: Increased reactive oxygen species formation in semen of patients with spinal cord injury. Fertil Steril. 1995; 63:p. 637-42.
72. Saleh, R.A., A. Agarwal, R.K. Sharma, T.M. Said, S.C. Sikka and A.J. Thomas, Jr.: Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil Steril. 2003; 80:p. 1431-6.
73. Agarwal, A. and S.S. Allamaneni: Role of free radicals in female reproductive diseases and assisted reproduction. Reprod Biomed Online. 2004; 9:p. 338-47.
74. Gupta, S., A. Agarwal, N. Krajcir and J.G. Alvarez: Role of oxidative stress in endometriosis. Reprod Biomed Online. 2006; 13:p. 126-34.
75. Bedaiwy, M.A. and T. Falcone: Peritoneal fluid environment in endometriosis. Clinicopathological implications. Minerva Ginecol. 2003; 55:p. 333-45.
76. Agarwal, A., S. Gupta and R.K. Sharma: Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 2005; 3:p. 28.
77. Agarwal, A., K.P. Nallella, S.S. Allamaneni and T.M. Said: Role of antioxidants in treatment of male infertility: an overview of the literature. Reprod Biomed Online. 2004; 8:p. 616-27.
78. Gupta, S., A. Agarwal, L. Sekhon, N. Krajcir, M. Cocuzza and T. Falcone: Serum and peritoneal abnormalities in endometriosis: potential use as diagnostic markers. Minerva Ginecol. 2006; 58:p. 527-51.
79. Agarwal, A., S.S. Allamaneni, K.P. Nallella, A.T. George and E. Mascha: Correlation of reactive oxygen species levels with the fertilization rate after in vitro fertilization: a qualified meta-analysis. Fertil Steril. 2005; 84:p. 228-31.

80. Steele, E.K., N. McClure, R.J. Maxwell and S.E. Lewis: A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. Mol Hum Reprod. 1999; 5:p. 831-5.
81. Sakkas, D., O. Moffatt, G.C. Manicardi, E. Mariethoz, N. Tarozzi and D. Bizzaro: Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. Biol Reprod. 2002; 66:p. 1061-7.
82. Chen, M.J. and A. Bongso: Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. Hum Reprod. 1999; 14:p. 759-64.
83. Geva, E., J.B. Lessing, L. Lerner-Geva and A. Amit: Free radicals, antioxidants and human spermatozoa: clinical implications. Hum Reprod. 1998; 13:p. 1422-4.
84. Kattera, S. and C. Chen: Short coincubation of gametes in in vitro fertilization improves implantation and pregnancy rates: a prospective, randomized, controlled study. Fertil Steril. 2003; 80:p. 1017-21.