Online version at http://www.minervamedica.it

Panminerva Medica 2019 June;61(2):108-17
DOI: 10.23736/S0031-0808.18.03529-2

REVIEW

HOT TOPICS IN MALE INFERTILITY

Oxidation reduction potential: a new biomarker of male infertility

Ana D. MARTINS [1, 2, 3], Ashok AGARWAL [1] *

[1]American Center for Reproductive Medicine, Department of Urology, Cleveland Clinic, Cleveland, OH, USA; [2]Department of Microscopy, Laboratory of Cell Biology, Abel Salazar Institute of Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal; [3]Unit for Multidisciplinary Research in Biomedicine, Abel Salazar Institute of Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal

*Corresponding author: Ashok Agarwal, Lerner College of Medicine at Case Western Reserve University, Andrology Center and Men's Fertility Preservation Program, American Center for Reproductive Medicine, Cleveland Clinic Foundation, 10681 Carnegie Avenue, Cleveland, OH 44195, USA.
E-mail: agarwaa@ccf.org

ABSTRACT

Oxidative stress is considered a major etiology for male infertility, more specifically idiopathic infertility. The causes of seminal oxidative stress can be intrinsic, such as varicocele or due to the presence of active leukocytes and immature germ cells. Reported external causes are smoking, alcohol or exposure to environmental toxins. Traditional methods to determine the seminal oxidative stress do not evaluate this status directly, but rather measure its components or intermediate products indirectly, instead. The major disadvantages of the traditional methods are related with time and cost as these methods are extremely time consuming and require expensive equipment, consumables and highly skilled laboratory personnel. To overcome these drawbacks, the MiOXSYS® system, a method which directly measures the oxidation-reduction potential (ORP), was developed. The evaluation of the ORP using MiOXSYS® is cost-effective, easy and quick. However, this newly introduced method to evaluate the oxidative status of semen still requires validation in different andrology laboratory settings across the world.

(*Cite this article as:* Martins AD, Agarwal A. Oxidation reduction potential: a new biomarker of male infertility. Panminerva Med 2019;61:108-17. DOI: 10.23736/S0031-0808.18.03529-2)

KEY WORDS: Male infertility - Oxidation-reduction - Oxidative stress - Semen analysis.

Infertility is defined as failure to achieve a clinical pregnancy after one year of regular and unprotected intercourse.[1] Globally, the prevalence of infertility in couples is about 15%, and is estimated between 2.5-12% of men are infertile, based on the geographic location.[1] Among the female and male factors contributing to infertility it is estimated that the male contribution ranges from 20% up to 70% to the infertility of the couple.[1] Male infertility is related to several factors, among them emotional, sociocultural and financial problems.[2] In addition, many medical conditions such as varicocele, hypogonadotropic hypogonadism, disorders in ciliary function, cystic fibrosis, infection, systemic diseases, testicular deficiency, and post testicular impairment are also associated with male infertility. Further, unhealthy lifestyle choices and associated metabolic diseases are contributing to an increase in the incidence of male fertility.[3]

The basic semen analysis according to World Health Organization (WHO) guidelines is still the cornerstone of laboratory male fertility evaluation.[4] The quality and thus the functionality of spermatozoa reflects the status of germ cells epithelium, epididymis and accessory sexual glands.[5, 6] This basic semen analysis is far from perfect. Indeed, the reference values for semen analysis as established by the WHO 2010[4] do not differentiate the fertile from infertile men. In fact, this evaluation neither takes dysfunctions such as DNA fragmentation or the oxidative state of the ejaculate, nor genetic variability of spermatozoa, into account.[7, 8] Moreover, the reference limits suggested by the WHO 2010 guidelines do not represent

the distribution of these values among fertile men[9] and are also not representative of the actual population to be evaluated (men who are unable to initiate a pregnancy) as these values are based on the evaluation of fertile men only.[10, 11]

Although there are many causes for male infertility, the etiology of male infertility is essentially idiopathic[12] and it has a prevalence of 10-20%.[13] However, one of the major causes for unexplained male infertility is oxidative stress (OS),[14] a parameter, which is currently not included in the basic semen analysis. OS is a consequence of an imbalance in the production of reactive oxygen species (ROS) and the availability of seminal antioxidant capacity.[15] On the one hand, ROS play a key role in the normal development, maturation, capacitation and acrosome reaction of spermatozoa, as well as in the fertilization process itself.[16] However, when endogenous (for example: leukocytes or immature sperm) or exogenous (for example: inflammation) sources of ROS disturb the equilibrium between oxidants and antioxidants, OS develops and can damage spermatozoa, thus leading to a decrease in sperm fertilizing potential.[17]

In light of these problems, new test systems to evaluate sperm functional disorders and anomalies are needed to improve male fertility diagnostics. In fact, the American Society for Reproductive Medicine acknowledges the limitations of basic semen analysis and has included sperm function tests, such as single-cell gel electrophoresis assay (Comet), terminal deoxynucleotide transferase–mediated dUTP nick-end labeling (TUNEL) assay or the sperm chromatin structure assay (SCSA) into the evaluation of infertile men.[18] However, measurement of OS and ROS by direct or indirect method are not included as these techniques are not properly evaluated yet, are not sensitive enough, or are too susceptible to interference.

In this review, we present an overview on the OS concepts as well as its causes and the mechanisms behind the overproduction of ROS. We discuss a new biomarker for the evaluation of OS by the measurement of oxidation-reduction potential (ORP), describe the test principle of this novel parameter and highlight the advantages and clinical significance of the assay.

Oxidative stress and spermatozoa

Reactive oxygen species

The production of ROS is essential for the homeostasis in aerobically living cells. However, sometimes the system is unable to neutralize an excessive production of ROS or exposure of cells to excessive amounts of ROS causing an imbalance between oxidants and antioxidants resulting in a state of OS. Typically, ROS are free radical oxygen derivatives. Radicals are molecules containing unpaired electrons in the outer orbit, a chemical condition, which renders these molecules electronically unstable and therefore highly reactive in order to reach stability.[18] Other strongly oxidizing molecules such as H_2O_2, though not a free radical, or nitric oxide and the peroxynitrite anion have a role in oxidation-reduction reactions in fertility.[19, 20] The main targets for ROS and oxidants are electron-rich molecules which can easily be oxidized such as polyunsaturated lip-

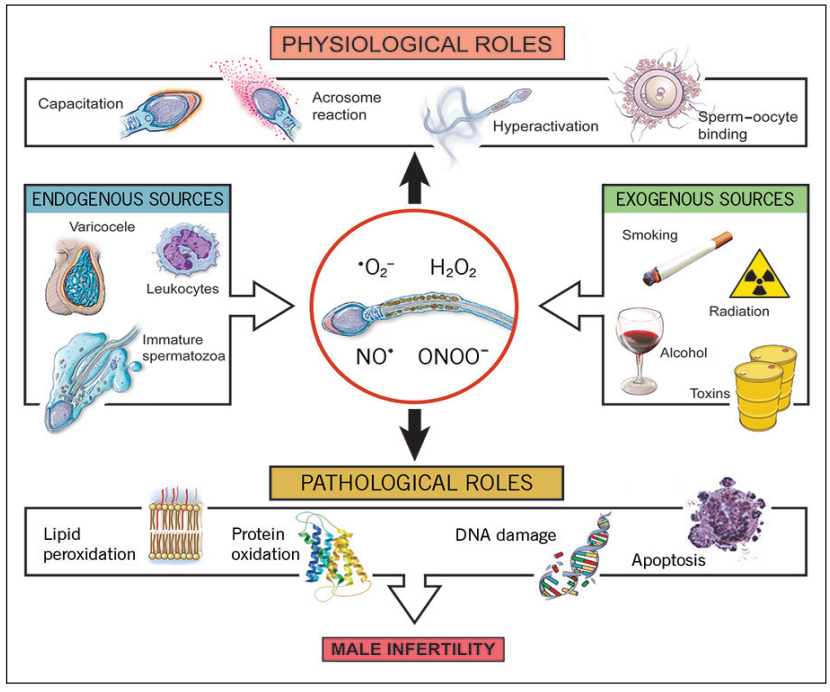

Figure 1.—The production of reactive oxygen species by the spermatozoa is essential for some physiological processes such as capacitation, acrosome reaction, hyperactivation and sperm-oocyte binding. However, when the ROS production overcome the antioxidant defenses of the spermatozoa due to endogenous or exogenous sources it can cause lipid peroxidation, protein oxidation, DNA damage or apoptosis in the male reproductive tract and lead to an infertility state.

ids in plasma membranes, carbohydrates in nucleic acids and amino acids in proteins.[21, 22] When present at physiological levels, ROS are crucial for sperm functions as these molecules trigger essential physiological events such as capacitation, acrosome reaction and oocyte fusion, while the addition of antioxidants prevented sperm cells from undergoing these events[23, 24] (Figure 1). The production of ROS in spermatozoa are derived from two different sources, namely the mitochondria via an NADH-dependent oxidase-reductase[25] or the plasma membrane through an NADPH oxidase system.[26]

Sources of reactive oxygen species

Varicocele

Varicocele is a tortuosity and dilation of the veins of the Pampiniform plexus in the spermatic cord and a major cause for male infertility.[27] Varicocele is described as a pathological cause for elevated OS and for a decrease in sperm quality. Indeed, men with varicocele have elevated OS, even when they are fertile.[28] OS in seminal plasma is reported to increase with higher grades of varicocele.[29] The mechanisms related to the increase of ROS or the decrease in antioxidant defense in case of varicoceles, however, are not well understood. A current hypothesis based on the generation of ROS due to testicular hypoxia, increase in scrotal temperature, epididymal dysfunction and accumulation of toxins.[30-34] Testicular hypoxia was investigated as a cause of OS in men with varicocele by evaluating hypoxia-inducible factor-1α. This factor was over-expressed in the internal spermatic vein and/or is related to oxidative stress.[32, 33] An increase in testicular temperature impairs spermatogenesis and consequently decreases sperm quality.[34] In fact, some studies showed an elevation in scrotal temperature of men with varicocele.[30, 31] Yet, theories on how varicocele increases temperature remain unclear.[30, 31]

Besides an improvement in sperm quality and pregnancy rates,[35-37] a decrease in seminal oxidative stress in men after a varicocelectomy is well reported, more specifically a decrease in sperm DNA damage.[38, 39]

Leukocytospermia

Leukocytes are part of the ejaculated cells in semen and present in the male reproductive tract, even in healthy men.[40] The main sources of peroxidase-positive leukocytes are the prostate and seminal vesicles.[40] According to the WHO, more than 1×10^6 per milliliter of peroxidase-positive leukocytes are regarded as leukocytospermia.[4] Leukocytes are mainly responsible for the high production of ROS,[41] which in turn are detrimental to male fertility. Yet, the effects of leukocytospermia on male fertility are controversially discussed.[42-49]

Leukocytospermia has been related with impaired capacitation and sperm fertilizing capacity. However, although many studies reported a negative effect of leukocytospermia on semen quality[42-44] or sperm DNA fragmentation,[42, 45] the connection between leukocytospermia and these processes is not well established.[46, 47] In addition, the incidence of leukocytospermia correlates only poorly with other semen parameters.[48, 49] The presence of leukocytes in semen can be a consequence of an infection, inflammation or cellular defense mechanisms in the male genital tract where leukocytes will be activated.[40] The mechanism of ROS generation in leukocytes is the same as in spermatozoa. However, in order to destroy the pathogens, leukocytes release large quantities of superoxide, which are about 1000 times more than that produced in spermatozoa.[19] Due to their immunological defense function, leukocyte contribution to the overall ROS in semen is extremely high.[40, 41] If this overwhelms the limited antioxidant capacity of spermatozoa and seminal plasma, a stage of oxidative stress will occur.[50]

Since spermatozoa are extremely prone to oxidative assaults because of their extraordinary high amount of polyunsaturated fatty acids (PUFA) in their plasma membranes, membrane lipids will be oxidized in a process named lipid peroxidation.[51] PUFAs contains more than two carbon-carbon double bonds, which are the primary site of the assault. Most PUFAs have unconjugated double bonds separated by methylene groups.[52] Chemically, double bonds adjacent to a methylene group cause that the methylene carbon-hydrogen is more susceptible to abstraction. When the abstraction occurs, the radical that is formed is stabilized by rearrangement of the double bonds, which can be then oxidized by oxygen leading to peroxyl radical, which in turn can oxidize neighboring PUFAs in a radical chain reaction. The propagation of this process depends of the antioxidant capacity of the spermatozoa.[53]

Immature spermatozoa

Besides leukocytes, immature spermatozoa are another source of ROS in the ejaculate. Here, the increase in ROS generation is linked to an increase in cytoplasmic droplets typically found in immature sperm.[54] The increase in biomarkers of cytoplasmic space and lipid peroxidation was correlated with abnormalities in spermatozoa.[55]

Environmental factors

Nowadays, many people are exposed to numerous environmental toxins as well as to cigarette smoke or excessive alcohol. The human body metabolizes alcohol and one of the products is NADH, a compound, which is responsible for the respiratory chain activity and for the increase in ROS formation. Consumption of alcohol is also associated with a state of hypoxia leading to lesions in the tissues.[56] NADH and acetaldehyde are products of alcohol metabolism. Acetaldehyde is an intermediates in this process, which, when in contact with proteins and lipids also increases ROS production.[56, 57] Moreover, due to its reactive nature by reacting with proteins and lipids, acetaldehyde is damaging the mitochondria, which consequently results in decreased ATP production.[56] NADH accumulation stimulates the activity of the respiratory chain, consuming the existing oxygen and is eventually forming ROS.

Many studies have reported low semen quality such as increase in morphologically abnormal sperm in alcoholic men.[58, 59] Smoking is another risk factor leading to excessive ROS production. Tobacco contains approximately 4000 harmful substances and cigarette smoke contains more than 7000 chemical compounds. Among these, many are known for their ability to increase ROS production,[56, 57] which is even more harmful for fertility as the presence of other byproducts in cigarette smoke such as cotinine[60-62] and hydroxycotinine that are reported in seminal plasma.[56] The effects of smoking on sperm quality are well documented with reports consistently showing decreased sperm quality *i.e.* sperm count,[58, 60, 61] motility[60, 61] and viability[60] and increased DNA damage.[62, 63]

Men are also unintentionally exposed to many other environmental toxins, such as radiation, pharmacological compounds or pollutants that can accumulate in the body and in the testes thereby increasing the ROS production. Studies have reported impairment of male fertility potential in a variety of cases. For example, pesticides and chemical fertilizers affect sperm count in farmers[64-66] and increase sperm DNA damage.[65, 67] On the other hand, phthalates, present in most plastics as plasticizer, reportedly increase DNA sperm damage[68] and reduce sperm mo-

TABLE I.—*Advantages and disadvantages of methods evaluating oxidative stress in semen.*[78-87]

Reference	Assay	Advantages	Disadvantages
Faulkner,[78] Agarwal[79]	Chemiluminescence	• High sensitivity and specificity • Evaluates intra- and extracellular ROS • Highly reproducible	• Requires large and expensive equipment • Highly time-consuming • Requires a large amount of sample • Dependent of the half-lives of the probes
Esfandiari[80]	Nitroblue Tetrazolium	• Easy to perform • Cost-effective method • Can provide the source of ROS (light microscope)	• Low specificity (can occur cross reactions with oxidoreductases) • Subjective interpretation
Dikalov[81]	Cytochrome C Reduction Test	• Detects high levels of ROS • Evaluates O_2^- released to the extracellular space	• Does not detect O_2^- intracellular • Low sensibility to detect NADPH oxidase low activity
Kohno[82]	Electron Spin Resonance	• Detects high levels of ROS • Characteristics of free radicals, formation and elimination velocities of free radicals	• Reducing agents presented in spin adduct can neutralize free radicals • If the radical reacts immediately with other molecules will not be detected
Draper[83]	Thiobarbituric Acid Assay	• Inexpensive and simple method • Can be evaluated by fluorometry or spectrophotometry • For low sperm concentrations sensitive HLPC can be used	• Requires laborious standards • Not used in clinical environment
Said,[84] Whitehead[85]	Total Antioxidant Capacity	• High sensitivity and specificity • Measures the total antioxidant capacity of the sample • Highly reproducible	• Requires large and expensive equipment • Highly time-consuming • Requires a large amount of sample • Limited to the half-lives of the probes • Requires a trained operator
Agarwal[86, 87]	MiOXSYS® system	• Snapshot of the oxidative state of the sample • Easy and simple to execute • Inexpensive • Measures fresh and frozen samples	• Affected by the viscosity of the sample • Requires further validation for outcome

tility.[69, 70] Cadmium is a heavy metal with similarities in its chemistry to the trace element zinc can be incorporated in the body where it accumulates with harmful effects of male fertility.[71-73] Radiation is also not only related to an increase in seminal ROS production, but also in low sperm motility[74, 75] and DNA integrity.[75] This includes electromagnetic radiations emitted by cell phones. Radiotherapy and chemotherapy used in cancer treatment causes azoospermia.[76, 77]

OS measurement

Since seminal OS has significant adverse effects on ejaculated spermatozoa, it is important to quantify the levels of OS in a given sample to obtain a more accurate picture of the seminal redox level and develop and optimized treatment plan for affected infertile men. The measurement of OS in the ejaculate can either be done directly by measuring OS, ROS or reactive nitrogen species, or indirectly by determining end products of lipid peroxidation (*e.g.* malondialdehyde [MDA]) or end products of ROS production, and cofactors and antioxidants. Advantages and disadvantages of the direct and indirect methods to evaluate OS are presented in Table I.[78-87]

Direct measurement of OS

Methods for the direct measurement of ROS in semen include: 1) chemiluminescence;[88] 2) nitro blue tetrazolium (NBT) test;[80] 3) cytochrome c reduction test;[81] and 4) electron spin resonance.[56]

Chemiluminescent methods are most commonly used for the measurement of ROS in semen and spermatozoa. They evaluate both intra- and extracellular ROS, but not the damage caused by the ROS.[88] The principle of these tests is based on the combination of a chemiluminescent probe with a free radical resulting in the emission of a light signal that is quantified in a luminometer. This test can use two different probes: luminol or lucigenin. Luminol is extremely sensitive at pH of 7 and reacts with the majority of ROS and the reactive radical form is generated by univalent oxidation.[78] On the other hand, lucigenin only measures the superoxide radical present in the extracellular space. The radical formed is generated by univalent reduction.[78]

The NBT test is used to evaluate neutrophil leukocyte function and quantify cellular oxidative metabolism.[89] In this test, cells are incubated with NBT, which is reduced to water insoluble formazan crystals by a cytoplasmic oxidase system (superoxide ions) which helps to transfer electrons from NADPH to NBT.[89] These formazan crystals can microscopically be evaluated. Alternatively, these crystals can be solubilized and the absorbance of the resulting purple-blue solution can be measured.[90] The results of the NBT test reflects the ROS-generating activity in the cytoplasm and can detect the cellular source of ROS in samples such as semen.[80]

The cytochrome c reduction test detects large quantities of the free radical superoxide (O_2^-) that is released into the extracellular space by the cells. The principle is based on the reduction of ferricytochrome c by O_2^- to ferrocytochrome c, a reaction that can be detected by measuring the absorbance at 550 nm.[81]

The electron spin resonance is the only method that is able to detect free radicals directly. This technique is based on the magnetic orientation and on the molecular environment of the unpaired electrons present in ROS.[56]

Indirect measurement of OS

The indirect measurement of ROS in semen includes the following methods: 1) measurement of lipid peroxidation levels;[83, 91] 2) determination of the total antioxidant capacity (TAC) (enhanced-chemiluminescence or colorimetric); and 3) determination of the ROS-TAC score.[92]

Lipid peroxidation levels can be detected by measuring the levels of MDA an end product of this process.[83] The thiobarbituric acid (TBA) assay is a method to evaluate changes in MDA levels. When TBA reacts with MDA a colored compound is formed, which can be detected by spectrophotometry or fluorometry,[83] or for low sperm MDA concentrations by highly sensitive high pressure liquid chromatography (HPLC). Alternatively, spectrofluorometric measurement of iron-based promoters can be performed.[57] Another way to evaluate lipid peroxidation is by measuring another end product such as isoprostane. This compound can be detected by commercial immunoassays (the preferred method of detection for a cost-effective analysis), generated polyclonal antibodies or mass spectrometry.[91]

The TAC assay measures the combination of antioxidant activities of all components using enhanced-chemiluminescence or colorimetric techniques.[84] The chemiluminescence principle of measuring TAC is based on the light emitted when luminol is oxidized by H_2O_2 in a reaction catalyzed by horseradish peroxidase (HRP). The continuous light emission is dependent on the production of free radicals and the radical scavenging by antioxidants.[85] This methods is generally used for the evaluation of TAC

in seminal fluid and was validated by Sharma and collaborators.[92] Contrary, the colorimetric evaluation of TAC is based on the antioxidant capacity of the sample to inhibit the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to $ABTS^{+}$ by metmyoglobin. The values obtained are then compared with a standard, which is normally Trolox, a highly potent water-soluble vitamin E derivative as radical scavenger.[93]

Finally, the ROS-TAC score is a parameter based on the measurement of ROS and TAC. This score was created in order to provide a measure derived from the levels of ROS (oxidants) produced and the antioxidant levels in a sample and is therefore a measure of the balance between oxidants and antioxidants. It minimizes the variability from individual parameters of OS.[92]

Measurement of oxidation reduction potential

Oxidation reduction potential (ORP) was used over 50 years ago to determine if the oxidant activity was sufficiently high in treated water to kill bacteria and other microbes.[94] ORP or the redox potential is a measure of the tendency of a compound A to acquire electrons from compound B whereby compound A will be reduced and compound B be oxidized. The greater the affinity for electrons, the higher the ORP of a redox pair. Hence, ORP is a reflection of the oxidative state of a chemical system, including cellular systems. Consequently, biological fluids, including semen also have an inherent ORP, which can be of clinical value as this is related to the status of biological and/or pathological processes. Thus, the ORP can provide information on the health status of a patient.[95]

Technically, ORP is a composite marker for an integrated

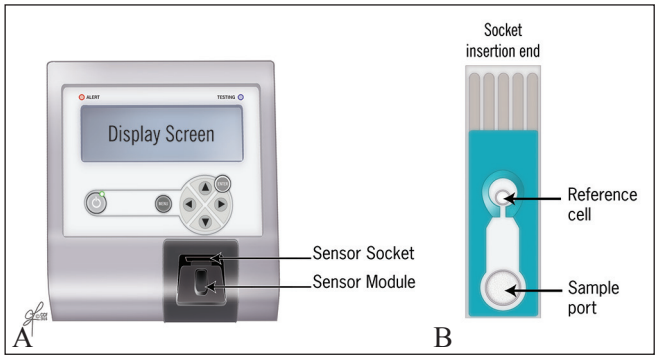

Figure 2.—MiOXSYS system. A) MiOXSYS analyzer with the sensor socket and the sensor module. B) Sensor to be inserted in the MiOXSYS analyzer with indication of the reference cell and the sample port where the sample is loaded.

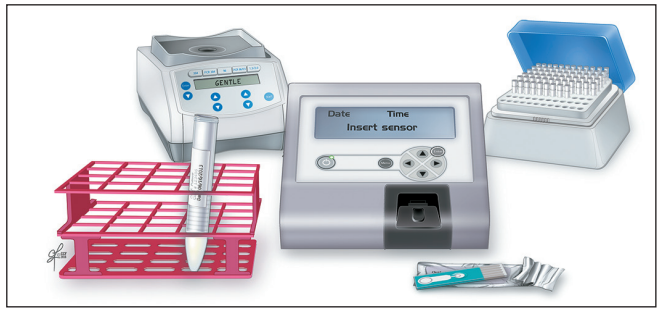

Figure 3.—MiOXSYS® analyzer clinical set-up. For the analysis of ORP, the MiOXSYS® analyzer is necessary, the small device and the sensor occupy a small space in an andrology center.

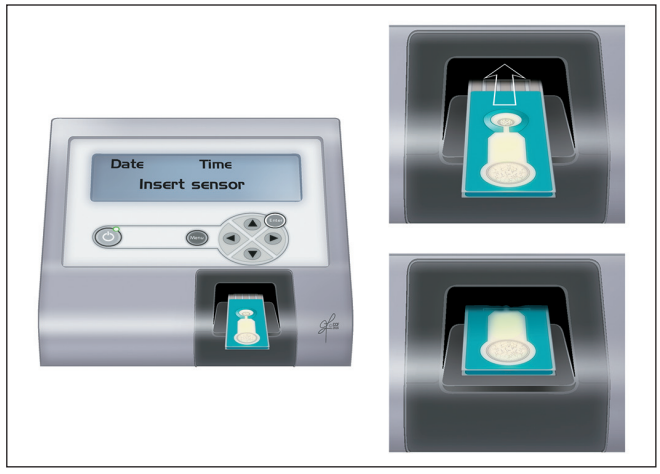

Figure 4.—The sensor should be placed on the port of the MiOXSYS® analyzer with the electrodes facing the analyzer. The sensor should be inserted by holding from side and the placing horizontal pressure on the sensor against the MiOXSYS® analyzer.

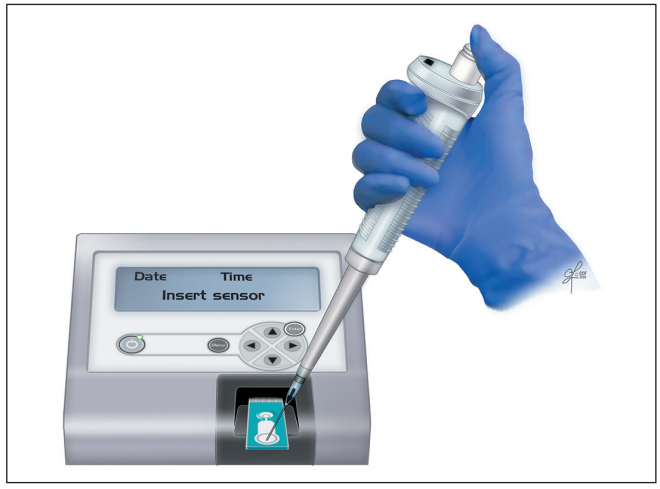

Figure 5.—A 30-µL semen sample is loaded on the sample port of the sensor strip.

evaluation of the balance between total oxidants and antioxidants in a biological fluid and provides an overall oxidative status of the body fluid of the patient.[95] However, the measurement techniques used to assess OS in a cellular system, such as semen, are based on single markers, which are not consistent. In addition, while most methods for the evaluation of OS in a cellular system are expensive, time consuming and require highly skilled technical expertise, the measurement of ORP is a simple and fast method to assess the overall oxidative status of semen.

Male infertility oxidative system (MiOXSYS®)

The development of the Male Infertility Oxidative System (MiOXSYS®) is an instrument that aims to overcome the negative aspects of other more complicated and expensive methods to evaluate OS in semen samples. MiOXSYS® is a galvanostat-based technique comprising the analyzer and a disposable sensor (Figure 2). It measures the redox potential in a rapid, simple and inexpensive way.[86] The MiOXSYS® system provides the static ORP, which represents the actual redox balance in a given sample; higher ORP is indicative of oxidative stress.[79] The advantages and disadvantages of the MiOXSYS® are summarized in Table I.

Protocol

MiOXSYS® is a simple system where a steady low voltage current is applied, and the activity of the electrons is measured in millivolts (mV). To measure the ORP in a semen or seminal plasma sample, after the analyzer is turned on, a MiOXSYS® Sensor is unwrapped (Figure 3) and placed on the port of the MiOXSYS® analyzer with the electrodes facing the MiOXSYS® Analyzer (Figure 4). Both, fresh or frozen semen or seminal plasma samples can be measured. Using a 30µL micropipette load of the sample on the application port taking care that no air bubbles are introduced, and the entire port is covered (Figure 5). Analysis will start once the sample reaches the reference cell of the sensor (Figure 2). It takes about 2 minutes for the sample to be

TABLE II.—*Clinical studies in male fertility with ORP measurements by MiOXSYS®.*[86, 87, 96-98]

Reference	Population	Findings
Agarwal[86]	• Healthy male volunteers (N.=26) • Infertile patients (N.=33)	• MiOXSYS® measured ORP in semen and seminal plasma • ORP levels are not affected by semen age
Agarwal[87]	• Proven fertile men (control) (N.=15) • Infertile men (Patient) (N.=293) (The samples were categorized in differentiate controls NZ, OZ, AZ and TZ)	• ORP levels were higher in control group compared with NZ. • ORP levels were higher in patient group compared with NZ • ORP has high predictive power for OZ patients • A cut-off of 2.59 mV/10^6 sperm from infertile men with OZ
Agarwal[98]	Two andrology centers: USA: • infertile patients (N.=194) • fertile donors (N.=51) Qatar: • infertile patients (N.=400) • fertile donors (N.=50)	• ORP levels showed no differences between both centers • In USA, a cut-off of 1.42 mV/10^6 sperm was able to differentiate between fertile and infertile (84.3% specificity and 49% sensitivity) • In Qatar, a cut-off of 2.26 mV/10^6 sperm allowed to differentiate between fertile and infertile (78% specificity and 60.8% sensitivity) • Both centers, a cut-off of 1.42 mV/10^6 sperm was able to differentiate between fertile and infertile men (74.3% specificity and 60.6% sensitivity) • Proves the reproducibility and reliability in ORP measurements
Agarwal[96]	• Healthy donors (N.=49) • Infertile patients (N.=194)	• ORP levels were higher in samples with abnormal sperm parameters • A cut-off of 1.57 mV/10^6 sperm allowed to detect at least 1 abnormal parameter (88.1% specificity and 70.4% sensitivity) • A cut-off of 2.59 mV/10^6 sperm allowed to detect OZ (91.2% specificity and 88% sensitivity)
Majzoub[97]	• Proven fertile men (N.=50) • Infertile men (N.=365)	• In infertile men the ORP values were inversely related with total sperm count, motility and morphology • ORP values were higher in samples with abnormal quality compared with normal quality • Infertile patients presented higher values of ORP when compared with fertile men • A cut-off of 1.38 mV/10^6 sperm allowed to differentiate normal from abnormal samples (87.8% specificity and 63.3% sensitivity) • A cut-off of 1.41 mV/10^6 sperm allowed to differentiate fertile from infertile men (78% specificity and 57.3% sensitivity)
	• Proven fertile men (N.=100) • Infertile men (N.=1168)	• Infertile men presented higher ORP levels compared with fertile men • ORP levels were negatively correlated with normal morphology values in infertile patients • SDF was positively correlated with ORP levels in infertile patients • A cut-off of 1.73 mV/10^6 sperm allowed to differentiate normal from abnormal morphology in sperm (72% specificity and 76% sensitivity)

AZ: asthenozoospermic; NZ: normozoospermic; ORP: oxidation-reduction potential; OZ: oligozoospermic; TZ: teratozoospermic.

read and results are displayed on the analyzer screen. Once the analysis is complete, the result will be recorded, sensor removed, and the instrument is shut down. The results are expressed as mV and are then normalized to the sperm concentration as mV/10^6 sperm/mL.[79]

Clinical relevance of OS in male fertility

The negative effect of OS on sperm quality and consequently the male fertility potential has repeatedly been described.[19, 30-34, 54] Recent reports indicate that higher ORP levels are closely related with poor semen quality.[96, 97] Male partners of fertile couples have significantly lower values when compared with normal semen parameters[97] and the ORP value allows to differentiate between fertile and infertile men. Information about the different studies performed on ORP can be found in Table II.[86, 87, 96-98]

Conclusions

The basic semen analysis remains the "cornerstone" in male fertility evaluation. However, it has a limited predictive value for fertilization to occur. Oxidative stress is implicated in the etiology of male infertility. The role of ROS in sperm was discovered long time ago. While physiological levels of ROS are necessary for normal physiological function of spermatozoa, excessive ROS will have detrimental effects. The subject is not new, but the role of ROS in spermatozoa is still a matter of debate in male fertility; mainly the effects of an imbalance between oxidants and antioxidants. The evaluation of OS in spermatozoa can be performed with several methods, of which none evaluates the oxidative status of the spermatozoa or semen directly. Furthermore, the available methods are time consuming, require expensive equipment and a trained operator. In contrast, the evaluation of the oxidative state of a semen sample using the MiOXSYS® system is cheap, timesaving, reproducible and easy. This system evaluates seminal oxidative stress in simple, fast and inexpensive way. Combinated with the MiOXSYS® system, it is an attractive alternative for the evaluation of the oxidative state of a sample in an andrological laboratory setting. ORP is not only able to distinguish normal and abnormal semen samples but is also able to differentiate fertile from infertile men.

References

1. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reprod Biol Endocrinol 2015;13:37.

2. Slade P, O'Neill C, Simpson AJ, Lashen H. The relationship between perceived stigma, disclosure patterns, support and distress in new attendees at an infertility clinic. Hum Reprod 2007;22:2309–17.

3. Vander Borght M, Wyns C. Fertility and infertility: definition and epidemiology. Clin Biochem 2018;S0009-9120(18)30220-0.

4. World Health Organization DORHaR. WHO laboratory manual for the Examination and processing of human sperm. World Health Organiz 2010.

5. Orth JM, Gunsalus GL, Lamperti AA. Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology 1988;122:787–94.

6. Comhaire FH, Mahmoud AM, Depuydt CE, Zalata AA, Christophe AB. Mechanisms and effects of male genital tract infection on sperm quality and fertilizing potential: the andrologist's viewpoint. Hum Reprod Update 1999;5:393–8.

7. Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. Hum Reprod 2002;17:3122–8.

8. Esteves SC. Clinical relevance of routine semen analysis and controversies surrounding the 2010 World Health Organization criteria for semen examination. Int Braz J Urol 2014;40:443–53.

9. Barratt CL, Mansell S, Beaton C, Tardif S, Oxenham SK. Diagnostic tools in male infertility-the question of sperm dysfunction. Asian J Androl 2011;13:53–8.

10. Baker K, Li J, Sabanegh E Jr. Analysis of semen parameters in male referrals: impact of reference limits, stratification by fertility categories, predictors of change, and comparison of normal semen parameters in subfertile couples. Fertil Steril 2015;103:59–65.e5.

11. Joffe M. Semen quality analysis and the idea of normal fertility. Asian J Androl 2010;12:79–82.

12. Hamada A, Esteves SC, Agarwal A. Unexplained male infertility: potential causes and management. Human Andrology 2011;1:2–16.

13. Esteves SC, Miyaoka R, Agarwal A. An update on the clinical assessment of the infertile male. [corrected] [corrected]. Clinics (São Paulo) 2011;66:691–700.

14. Agarwal A, Durairajanayagam D, Halabi J, Peng J, Vazquez-Levin M. Proteomics, oxidative stress and male infertility. Reprod Biomed Online 2014;29:32–58.

15. Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. Urology 1996;48:835–50.

16. Griveau JF, Le Lannou D. Reactive oxygen species and human spermatozoa: physiology and pathology. Int J Androl 1997;20:61–9.

17. Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. World J Mens Health 2014;32:1–17.

18. Practice Committee of the American Society for Reproductive Medicine. Diagnostic evaluation of the infertile male: a committee opinion. Fertil Steril 2015;103:e18–25.

19. Henkel RR. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. Asian J Androl 2011;13:43–52.

20. Wagner H, Cheng JW, Ko EY. Role of reactive oxygen species in male infertility: an updated review of literature. Arab J Urol 2017;16:35–43.

21. Agarwal A, Roychoudhury S, Bjugstad KB, Cho CL. Oxidation-reduction potential of semen: what is its role in the treatment of male infertility? Ther Adv Urol 2016;8:302–18.

22. Ochsendorf FR. Infections in the male genital tract and reactive oxygen species. Hum Reprod Update 1999;5:399–420.

23. de Lamirande E, Tsai C, Harakat A, Gagnon C. Involvement of reactive oxygen species in human sperm arcosome reaction induced by A23187, lysophosphatidylcholine, and biological fluid ultrafiltrates. J Androl 1998;19:585–94.

24. de Lamirande E, Leclerc P, Gagnon C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 1997;3:175–94.

25. Gavella M, Lipovac V. NADH-dependent oxidoreductase (diapho-

rase) activity and isozyme pattern of sperm in infertile men. Arch Androl 1992;28:135–41.

26. Aitken RJ, Buckingham DW, West KM. Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J Cell Physiol 1992;151:466–77.

27. Agarwal A, Prabakaran S, Allamaneni SS. Relationship between oxidative stress, varicocele and infertility: a meta-analysis. Reprod Biomed Online 2006;12:630–3.

28. Gul M, Bugday MS, Erel O. Thiol-disulphide homoeostasis as an oxidative stress marker in men with varicocele. Andrologia 2018;50:e12982.

29. Cocuzza M, Athayde KS, Alvarenga C, Srougi M, Hallak J. Grade 3 varicocele in fertile men: a different entity. J Urol 2012;187:1363–8.

30. Garolla A, Torino M, Miola P, Caretta N, Pizzol D, Menegazzo M, *et al.* Twenty-four-hour monitoring of scrotal temperature in obese men and men with a varicocele as a mirror of spermatogenic function. Hum Reprod 2015;30:1006–13.

31. Shiraishi K, Matsuyama H. Elevation of testicular temperature predicts testicular catch-up growth and hypotrophy after varicocelectomy and observation in adolescent varicocele. Urology 2013;82:205–9.

32. Lee JD, Jeng SY, Lee TH. Increased expression of hypoxia-inducible factor-1α in the internal spermatic vein of patients with varicocele. J Urol 2006;175:1045–8, discussion 1048.

33. Hu W, Zhou PH, Zhang XB, Xu CG, Wang W. Roles of adrenomedullin and hypoxia-inducible factor 1 alpha in patients with varicocele. Andrologia 2015;47:951–7.

34. Durairajanayagam D, Agarwal A, Ong C. Causes, effects and molecular mechanisms of testicular heat stress. Reprod Biomed Online 2015;30:14–27.

35. Practice Committee of the American Society for Reproductive Medicine; Society for Male Reproduction and Urology. Report on varicocele and infertility: a committee opinion. Fertil Steril 2014;102:1556–60.

36. Camargo M, Intasqui P, Bertolla RP. Proteomic profile of seminal plasma in adolescents and adults with treated and untreated varicocele. Asian J Androl 2016;18:194–201.

37. Esteves SC, Miyaoka R, Roque M, Agarwal A. Outcome of varicocele repair in men with nonobstructive azoospermia: systematic review and meta-analysis. Asian J Androl 2016;18:246–53.

38. Sakamoto Y, Ishikawa T, Kondo Y, Yamaguchi K, Fujisawa M. The assessment of oxidative stress in infertile patients with varicocele. BJU Int 2008;101:1547–52.

39. Alhathal N, San Gabriel M, Zini A. Beneficial effects of microsurgical varicocoelectomy on sperm maturation, DNA fragmentation, and nuclear sulfhydryl groups: a prospective trial. Andrology 2016;4:1204–8.

40. Wolff H. The biologic significance of white blood cells in semen. Fertil Steril 1995;63:1143–57.

41. Plante M, de Lamirande E, Gagnon C. Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. Fertil Steril 1994;62:387–93.

42. Alvarez JG, Sharma RK, Ollero M, Saleh RA, Lopez MC, Thomas AJ Jr, *et al.* Increased DNA damage in sperm from leukocytospermic semen samples as determined by the sperm chromatin structure assay. Fertil Steril 2002;78:319–29.

43. Wolff H, Politch JA, Martinez A, Haimovici F, Hill JA, Anderson DJ. Leukocytospermia is associated with poor semen quality. Fertil Steril 1990;53:528–36.

44. Lackner JE, Agarwal A, Mahfouz R, du Plessis SS, Schatzl G. The association between leukocytes and sperm quality is concentration dependent. Reprod Biol Endocrinol 2010;8:12.

45. Erenpreiss J, Hlevicka S, Zalkalns J, Erenpreisa J. Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. J Androl 2002;23:717–23.

46. Lackner JE, Märk I, Sator K, Huber J, Sator M. Effect of leukocytospermia on fertilization and pregnancy rates of artificial reproductive technologies. Fertil Steril 2008;90:869–71.

47. Cavagna M, Oliveira JB, Petersen CG, Mauri AL, Silva LF, Massaro FC, *et al.* The influence of leukocytospermia on the outcomes of assisted reproductive technology. Reprod Biol Endocrinol 2012;10:44.

48. Kaleli S, Oçer F, Irez T, Budak E, Aksu MF. Does leukocytospermia associate with poor semen parameters and sperm functions in male infertility? The role of different seminal leukocyte concentrations. Eur J Obstet Gynecol Reprod Biol 2000;89:185–91.

49. Tomlinson MJ, Barratt CL, Cooke ID. Prospective study of leukocytes and leukocyte subpopulations in semen suggests they are not a cause of male infertility. Fertil Steril 1993;60:1069–75.

50. Agarwal A, Mulgund A, Alshahrani S, Assidi M, Abuzenadah AM, Sharma R, *et al.* Reactive oxygen species and sperm DNA damage in infertile men presenting with low level leukocytospermia. Reprod Biol Endocrinol 2014;12:126.

51. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod 1989;41:183–97.

52. Makker K, Agarwal A, Sharma R. Oxidative stress & male infertility. Indian J Med Res 2009;129:357–67.

53. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 2003;79:829–43.

54. Tanphaichitr N, Kongmanas K, Kruevaisayawan H, Saewu A, Sugeng C, Fernandes J, *et al.* Remodeling of the plasma membrane in preparation for sperm-egg recognition: roles of acrosomal proteins. Asian J Androl 2015;17:574–82.

55. Huszar G, Vigue L. Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. J Androl 1994;15:71–7.

56. Agarwal A, Prabakaran SA. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol 2005;43:963–74.

57. Agarwal A, Majzoub A. Laboratory tests for oxidative stress. Indian J Urol 2017;33:199–206.

58. Joo KJ, Kwon YW, Myung SC, Kim TH. The effects of smoking and alcohol intake on sperm quality: light and transmission electron microscopy findings. J Int Med Res 2012;40:2327–35.

59. Goverde HJ, Dekker HS, Janssen HJ, Bastiaans BA, Rolland R, Zielhuis GA. Semen quality and frequency of smoking and alcohol consumption—an explorative study. Int J Fertil Menopausal Stud 1995;40:135–8.

60. Hamad MF, Shelko N, Kartarius S, Montenarh M, Hammadeh ME. Impact of cigarette smoking on histone (H2B) to protamine ratio in human spermatozoa and its relation to sperm parameters. Andrology 2014;2:666–77.

61. Chen HW, Kuo CT. Cotinine characterization and quality effect of sperm for smoking and nonsmoking students. Bull Environ Contam Toxicol 2007;79:11–4.

62. Shen HM, Chia SE, Ni ZY, New AL, Lee BL, Ong CN. Detection of oxidative DNA damage in human sperm and the association with cigarette smoking. Reprod Toxicol 1997;11:675–80.

63. Anifandis G, Bounartzi T, Messini CI, Dafopoulos K, Sotiriou S, Messinis IE. The impact of cigarette smoking and alcohol consumption on sperm parameters and sperm DNA fragmentation (SDF) measured by Halosperm(®). Arch Gynecol Obstet 2014;290:777–82.

64. Abell A, Ernst E, Bonde JP. High sperm density among members of organic farmers' association. Lancet 1994;343:1498.

65. Miranda-Contreras L, Gómez-Pérez R, Rojas G, Cruz I, Berrueta L, Salmen S, *et al.* Occupational exposure to organophosphate and carbamate pesticides affects sperm chromatin integrity and reproductive hormone levels among Venezuelan farm workers. J Occup Health 2013;55:195–203.

66. Pant N, Kumar R, Mathur N, Srivastava SP, Saxena DK, Gujrati VR. Chlorinated pesticide concentration in semen of fertile and infer-

tile men and correlation with sperm quality. Environ Toxicol Pharmacol 2007;23:135–9.

67. Sánchez-Peña LC, Reyes BE, López-Carrillo L, Recio R, Morán-Martínez J, Cebrián ME, *et al.* Organophosphorous pesticide exposure alters sperm chromatin structure in Mexican agricultural workers. Toxicol Appl Pharmacol 2004;196:108–13.

68. Kasahara E, Sato EF, Miyoshi M, Konaka R, Hiramoto K, Sasaki J, *et al.* Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate. Biochem J 2002;365:849–56.

69. Hauser R, Williams P, Altshul L, Calafat AM. Evidence of interaction between polychlorinated biphenyls and phthalates in relation to human sperm motility. Environ Health Perspect 2005;113:425–30.

70. Pant N, Pant A, Shukla M, Mathur N, Gupta Y, Saxena D. Environmental and experimental exposure of phthalate esters: the toxicological consequence on human sperm. Hum Exp Toxicol 2011;30:507–14.

71. Chia SE, Xu B, Ong CN, Tsakok FM, Lee ST. Effect of cadmium and cigarette smoking on human semen quality. Int J Fertil Menopausal Stud 1994;39:292–8.

72. Pant N, Upadhyay G, Pandey S, Mathur N, Saxena DK, Srivastava SP. Lead and cadmium concentration in the seminal plasma of men in the general population: correlation with sperm quality. Reprod Toxicol 2003;17:447–50.

73. Benoff S, Hauser R, Marmar JL, Hurley IR, Napolitano B, Centola GM. Cadmium concentrations in blood and seminal plasma: correlations with sperm number and motility in three male populations (infertility patients, artificial insemination donors, and unselected volunteers). Mol Med 2009;15:248–62.

74. Erogul O, Oztas E, Yildirim I, Kir T, Aydur E, Komesli G, *et al.* Effects of electromagnetic radiation from a cellular phone on human sperm motility: an in vitro study. Arch Med Res 2006;37:840–3.

75. Gorpinchenko I, Nikitin O, Banyra O, Shulyak A. The influence of direct mobile phone radiation on sperm quality. Cent European J Urol 2014;67:65–71.

76. Ash P. The influence of radiation on fertility in man. Br J Radiol 1980;53:271–8.

77. Berthelsen JG. Sperm counts and serum follicle-stimulating hormone levels before and after radiotherapy and chemotherapy in men with testicular germ cell cancer. Fertil Steril 1984;41:281–6.

78. Faulkner K, Fridovich I. Luminol and lucigenin as detectors for O2.-. Free Radic Biol Med 1993;15:447–51.

79. Agarwal A, Gupta S, Sharma R. Oxidation–Reduction Potential Measurement in Ejaculated Semen Samples. In: Agarwal A, Gupta S, Sharma R, eds. Andrological Evaluation of Male Infertility. Switzerland: Springer; 2016. p. 165–70.

80. Esfandiari N, Sharma RK, Saleh RA, Thomas AJ Jr, Agarwal A. Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. J Androl 2003;24:862–70.

81. Dikalov SI, Harrison DG. Methods for detection of mitochondrial and cellular reactive oxygen species. Antioxid Redox Signal 2014;20:372–82.

82. Kohno M. Applications of electron spin resonance spectrometry for reactive oxygen species and reactive nitrogen species research. J Clin Biochem Nutr 2010;47:1–11.

83. Draper HH, Hadley M. Malondialdehyde determination as index of lipid Peroxidation. Methods in Enzymology: Academic Press; 1990. p. 421–31.

84. Said TM, Kattal N, Sharma RK, Sikka SC, Thomas AJ Jr, Mascha E, *et al.* Enhanced chemiluminescence assay vs colorimetric assay for measurement of the total antioxidant capacity of human seminal plasma. J Androl 2003;24:676–80.

85. Whitehead TP, Thorpe GH, Maxwell SR. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. Anal Chim Acta 1992;266:265–77.

86. Agarwal A, Sharma R, Roychoudhury S, Du Plessis S, Sabanegh E. MiOXSYS: a novel method of measuring oxidation reduction potential in semen and seminal plasma. Fertil Steril 2016;106:566–573.e10.

87. Agarwal A, Henkel R, Sharma R, Tadros NN, Sabanegh E. Determination of seminal oxidation-reduction potential (ORP) as an easy and cost-effective clinical marker of male infertility. Andrologia 2018;50:e12914.

88. Agarwal A, Gupta S, Sharma R. Reactive Oxygen Species (ROS) Measurement. In: Agarwal A, Gupta S, Sharma R, eds. Andrological Evaluation of Male Infertility: A Laboratory Guide. Cham: Springer International Publishing; 2016. p. 155–63.

89. Baehner RL, Boxer LA, Davis J. The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. Blood 1976;48:309–13.

90. Choi HS, Kim JW, Cha YN, Kim C. A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. J Immunoassay Immunochem 2006;27:31–44.

91. Morrow JD. Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans. Arterioscler Thromb Vasc Biol 2005;25:279–86.

92. Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 1999;14:2801–7.

93. Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci (Lond) 1993;84:407–12.

94. Victorin K, Hellström KG, Rylander R. Redox potential measurements for determining the disinfecting power of chlorinated water. J Hyg (Lond) 1972;70:313–23.

95. Bar-Or R, Bar-Or D, Rael LT, Bjugstad KB. (WO2016085997) Determination of fertility potential from the oxidation-reduction potential of a biological sample; 2017 [Internet]. Available from: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2016085997 [cited 2018, Nov 26].

96. Agarwal A, Wang SM. Clinical Relevance of Oxidation-Reduction Potential in the Evaluation of Male Infertility. Urology 2017;104:84–9.

97. Majzoub A, Arafa M, Mahdi M, Agarwal A, Al Said S, Al-Emadi I, *et al.* Oxidation-reduction potential and sperm DNA fragmentation, and their associations with sperm morphological anomalies amongst fertile and infertile men. Arab J Urol 2018;16:87–95.

98. Agarwal A, Arafa M, Chandrakumar R, Majzoub A, AlSaid S, Elbardisi H. A multicenter study to evaluate oxidative stress by oxidation-reduction potential, a reliable and reproducible method. Andrology 2017;5:939–45.

Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Funding.—This study was supported by the American Center for Reproductive Medicine, Department of Urology, Cleveland Clinic, USA. Ana D. Martins was funded by the Fundação para a Ciência e a Tecnologia (SFRH/BD/108726/2015) and Fulbright.

Manuscript accepted: August 28, 2018. - Manuscript received: July 25, 2018.