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Oxidation-reduction potential of semen: what is its role in the treatment of male infertility?

Ashok Agarwal, Shubhadeep Roychoudhury, Kimberly B. Bjugstad and Chak-Lam Cho

***Abstract*:** The diagnosis of male infertility relies largely on conventional semen analysis, and its interpretation has a profound influence on subsequent management of patients. Despite poor correlation between conventional semen parameters and male fertility potential, inclusion of advanced semen quality tests to routine male infertility workup algorithms has not been widely accepted. Oxidative stress is one of the major mediators in various etiologies of male infertility; it has deleterious effects on spermatozoa, including DNA damage. Alleviation of oxidative stress constitutes a potential treatment strategy for male infertility. Measurement of seminal oxidative stress is of crucial role in the identification and monitoring of patients who may benefit from treatments. Various tests including reactive oxygen species (ROS) assay, total antioxidant capacity (TAC) assay or malondialdehyde (MDA) assay used by different laboratories have their own drawbacks. Oxidation-reduction potential (ORP) is a measure of overall balance between oxidants and antioxidants, providing a comprehensive measure of oxidative stress. The MiOXSYS™ System is a novel technology based on a galvanostatic measure of electrons; it presents static ORP (sORP) measures with static referring to the passive or current state of activity between oxidants and antioxidants. Preliminary studies have correlated sORP to poor semen qualities. It is potentially useful in prognostication of assisted reproductive techniques outcomes, screening of antioxidants either *in vivo* or during IVF cycles, identification of infertile men who may benefit from treatment of oxidative stress, and monitoring of treatment success. The simplified laboratory test requiring a small amount of semen would facilitate clinical application and research in the field. In this paper, we discuss the measurement of ORP by the MiOXSYS System as a real-time assessment of seminal oxidative stress, and argue that it is a potential valuable clinical test that should be incorporated into the male infertility workup and become an important guide to the treatment of oxidative stress-induced male infertility.

Keywords: diagnosis, male infertility, oxidation-reduction potential, oxidative stress, semen, treatment

Correspondence to:
Ashok Agarwal, PhD, HCLD
Director, Andrology Center and American Center for Reproductive Medicine, Lerner College of Medicine, Cleveland Clinic, 10681 Carnegie Avenue, Desk X11, Cleveland, OH 44195, USA
agarwaa@ccf.org

Shubhadeep Roychoudhury, PhD
American Center for Reproductive Medicine, Cleveland Clinic, Cleveland, OH, USA

Department of Life Science and Bioinformatics, Assam University, Silchar, India

Kimberly B. Bjugstad, PhD
Aytu BioScience, Inc. Englewood, CO, USA

Chak-Lam Cho, MD
Department of Surgery, Kwong Wah Hospital, Hong Kong

Introduction

Global rates of male infertility range from 2.5% to 12%. That means at least 30 million men worldwide are infertile. Africa and Eastern Europe have the highest rates [Agarwal *et al.* 2015a], whereas a calculated percentage showed that 4.5–6% of North American males are infertile [Agarwal *et al.* 2015a]. In the United States, infertility affects 9.4% of males, according to the Centers for Disease Control and Prevention [Martinez *et al.* 2012]. Males are found to be solely responsible for 20–30% of infertility cases and contribute to 50% of cases overall [Agarwal *et al.* 2015a].

Infertility can pose a wide range of sociocultural, emotional, physical and financial problems [Slade *et al.* 2007; Greil *et al.* 2010]. The management of male infertility includes assessing and identifying a patient's potential health problems. Decreased general health status has been associated with lower sperm concentration, lower total testosterone levels and higher follicle-stimulating

hormone values [Ventimiglia *et al.* 2015]. Conditions such as testicular cancer [Raman *et al.* 2005], colorectal cancer, melanoma and prostate cancer [Walsh *et al.* 2010; Eisenberg *et al.* 2013] were found to be more prevalent among infertile men as were other noncancerous disorders [Salonia *et al.* 2009]. Infertility resulting from hypogonadism has also been associated with a decline in health [Zarotsky *et al.* 2014; Aversa and Morgentaler, 2015].

Many clinicians rely on conventional semen parameters as a surrogate measure of a man's ability to father a child [Catanzariti *et al.* 2013; Esteves, 2014]. However, this approach seems to be an oversimplification of the assessment of male fertility potential due to large inter- and intra-individual variations in conventional semen parameters.

Oxidative stress has been identified as a major mediator in various etiologies of male infertility [Agarwal *et al.* 2014a]. Treatments of oxidative stress, including oral antioxidants and varicocelectomy, have been studied widely in patients with varicocele-associated male subfertility and unexplained male infertility. Current assays for seminal oxidative stress can measure reactive oxygen species (ROS) directly or indirectly, all of which have their own drawbacks.

Measurement of oxidation-reduction potential (ORP) by the MiOXSYS™ System presents a novel and comprehensive measure of seminal oxidative stress (Aytu BioScience, Inc.). It has a number of advantages over existing semen quality measures. The MiOXSYS System and measurement of ORP represent an invaluable clinical tool that obviates the need for complicated oxidative stress assays. The system facilitates wider application of oxidative stress assays in both clinical and research settings. The incorporation of ORP measurement into the armamentarium of the male infertility specialist will facilitate management of infertile couples by identifying candidates who may benefit from treatment of oxidative stress.

In this paper, we first illustrate the pitfalls of conventional semen analysis in clinical practice. We then discuss the role of oxidative stress in male infertility, potential treatment strategies, and current oxidative stress assays and their drawbacks. Finally, the superiority of ORP measurement using the MiOXSYS System is discussed.

Semen analysis

Semen analysis is the most widely used biomarker of male fertility potential [Esteves *et al.* 2012]. The results provide information on the functional status of the seminiferous tubules, epididymis and accessory sex glands [Esteves, 2014], which in turn influences the subsequent workup, treatment and outcomes [Baker *et al.* 2015]. However, semen characteristics that discriminate between infertile and fertile men are not well defined and the clinical use of conventional semen parameters is far from perfect.

Firstly, the results fall within the accepted reference ranges in up to 40% of infertile men [Moghissi and Wallach, 1983; Guzick *et al.* 2001; van der Steeg *et al.* 2011]. This is due, in part, to the fact that conventional semen analysis does not assess the diverse array of biological properties that spermatozoa express as eminent specialized cells, or account for putative sperm dysfunctions that can be assessed by sperm DNA damage or seminal oxidative stress tests [Duran *et al.* 2002; Esteves, 2014; Papillon-Smith *et al.* 2015].

Secondly, the reference values are derived from a population of fertile men and therefore do not represent the population in question: men who are unable to initiate a pregnancy. The lower reference values for 'normal' were set at the fifth percentile of the population distribution – a threshold that has no known correlation with fecundity [Joffe, 2010; Baker *et al.* 2015].

Thirdly, the World Health Organization (WHO) reference limits do not represent the distribution of fertile men across the globe [Barratt *et al.* 2011], as the group of studied men consisted of a limited population of individuals from large cities in the northern hemisphere and a small subset of men from Australia [Esteves, 2014; Papillon-Smith *et al.* 2015].

As the definition of male factor infertility remains less clear, most clinicians continue to rely on these lower reference limits of semen analysis parameters to diagnose, as well as to formulate treatment plans without investigating the underlying etiology/mechanism [Catanzariti *et al.* 2013; Esteves, 2014]. Up to 30% of men who are unable to father a biological child have a normal male infertility workup, which commonly constitutes one or two semen analyses. The male infertility evaluation must go far beyond a simple semen analysis [Esteves *et al.* 2011, 2012]. We believe

that the assessment of male fertility based on conventional semen analysis alone should not be considered adequate and must be complemented with more reliable, quantifiable, unbiased and universal functional measures of semen quality.

The American Society for Reproductive Medicine (ASRM) has acknowledged the limitations of conventional semen analysis and included sperm function testing in the assessment of infertile men [ASRM: Practice Committee of American Society for Reproductive Medicine, 2008]. The inclusion of advanced semen quality tests for the assessment of seminal oxidative stress to the male infertility workup algorithms has been recommended by experts [Zini and Sigman, 2009; Agarwal *et al.* 2013].

Oxidative stress

Oxidative stress occurs when ROS and the levels of other free radicals are greatly increased or antioxidant levels are substantially decreased such that the delicate balance between oxidants and antioxidants is disturbed [Sharma and Agarwal, 1996; Agarwal *et al.* 2012]. In other words, oxidative stress is a condition that reflects an imbalance between ROS and a biological system's ability to readily detoxify (antioxidant defense) the reactive intermediates or repair the resulting damage [Hampl *et al.* 2012; Saalu, 2010].

ROS are mostly highly reactive substances with very short halflives (atoms, molecules, or fragments of atom and molecules derived from oxygen, nitrogen, other organic compounds, or ions of transition metals such as Cu^{2+} and Fe^{2+}). These substances called radicals (or free radicals) contain at least one unpaired valence electron. They pair their unpaired electron with an electron taken from other compounds, causing oxidation. New radicals are formed from originally nonradical molecules with great oxidative ability, thus promulgating radical chain reactions [Durackova, 2014]. ROS and antioxidants significantly interfere with oxidation-reduction processes in cells and organisms, changing the redox (or oxidative) state of the cell; such states can stimulate or inhibit activities of various signal proteins, leading to the alteration of signal pathways. An oxidative milieu can lead to cell destruction by apoptosis or necrosis, and *reducing milieu* can lead to cell survival [Durackova, 2014].

The sources of ROS in semen are both intrinsic and extrinsic. Activated leukocytes (mainly polymorphonuclear leukocytes and macrophages) resulting from inflammation and infection are significant intrinsic producers of ROS in semen [Wolff, 1995; Whittington and Ford, 1999; Saleh *et al.* 2002; Potts and Pasqualotto, 2003]. Immature spermatozoa with abnormal head morphology and cytoplasmic retention are another important source [Agarwal *et al.* 2014b]. Damaged, deficient or abnormal spermatozoa as a result of impaired spermatogenesis can yield excessive ROS as well [Aitken and Clarkson, 1987; Aitken *et al.* 1989]. Sertoli cells in semen have also been shown to possess the ability to produce ROS [Hipler *et al.* 2000]. Other intrinsic etiologies include varicocele (higher grade is associated with greater amounts of ROS production), cryptorchidism, testicular torsion and ageing [Shiraishi *et al.* 2012; Ko *et al.* 2014]. Extrinsic sources such as cigarette smoking [Lavranos *et al.* 2012], alcohol consumption [Saalu, 2010], exposure to radiation [Manda *et al.* 2007; Agarwal *et al.* 2008a] and other environmental toxins [Ko *et al.* 2014] have been associated with elevated testicular and/or seminal ROS levels. Common sources of ROS in semen and their adverse effects are illustrated in Figure 1.

A delicate balance of reduction and oxidation is required for essential sperm function, including chromatin compaction in maturing spermatozoa during epididymal transit [Wright *et al.* 2014], capacitation, hyperactivation, acrosome reaction and sperm–oocyte fusion, leading to successful fertilization [de Lamirande and Gagnon, 1993; Aitken *et al.* 1995; Agarwal and Said, 2004; Kothari *et al.* 2010; Guthrie and Welch, 2012]. Supraphysiologic ROS levels can affect sperm structural and functional integrity including motility, morphology, count and viability, thereby making it one of the important etiologies of male factor infertility [Jones *et al.* 1979; Aitken, 1989; Twigg *et al.* 1998; Whittington *et al.* 1999; Pasqualotto *et al.* 2000; Agarwal *et al.* 2003, 2008b, 2014a, 2014b, 2014c; Aziz *et al.* 2004; Athayde *et al.* 2007; Desai *et al.* 2009; Chen *et al.* 2013; Vessey *et al.* 2014].

High ROS concentrations in infertile men have been associated with DNA fragmentation and poor chromatin packing [Kodama *et al.* 1997; Aitken and Krausz, 2001; Saleh and Agarwal, 2002; Aitken *et al.* 2003; Moustafa *et al.* 2004; Cocuzza *et al.* 2007; Desai *et al.* 2009; Aitken and De Iuliis, 2010; Novotny *et al.* 2013].

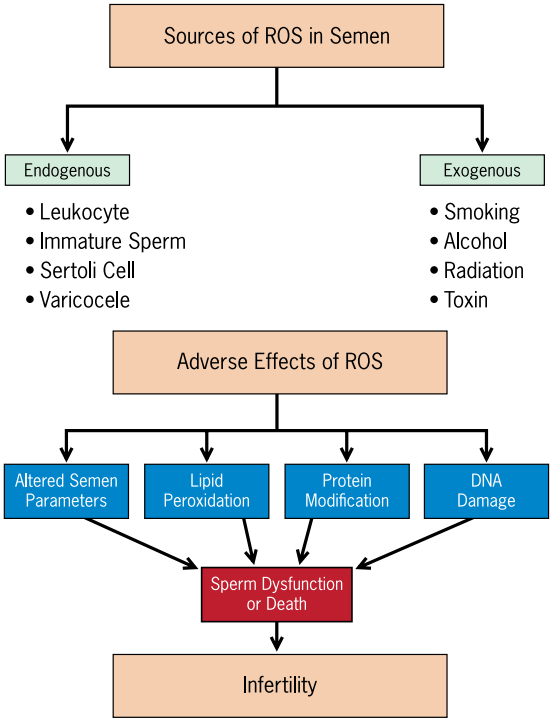

Figure 1. Common sources of excessive reactive oxygen species (ROS) in semen and their deleterious effects.

Sperm DNA damage may decrease fertilization rates, reduce implantation, impair embryonic development, and increase miscarriage/pregnancy loss and the potential for birth defects [Henkel *et al.* 2003; Ozmen *et al.* 2007; Tarozzi *et al.* 2007; Lewis and Simon, 2010; Zini *et al.* 2010; Agarwal *et al.* 2012]. ROS affect mitochondrial or nuclear DNA of sperm at an amino acid or molecular level in the form of base modification (especially guanine), attacking the phosphodiester backbones and producing base-free sites, point mutations, polymorphisms, deletions, translocations, strand breaks, and chromatin cross-links, frame shifts and even rearranging chromosomes [Agarwal *et al.* 2003, 2012; Aitken and Koppers, 2011; Gharagozloo and Aitken, 2011]. Sperm chromatin has a highly condensed and organized structure that helps to protect it from oxidative damage [Schulte *et al.* 2010], but when compaction is poor and chromatin protamination is incomplete, sperm DNA is more vulnerable to ROS. DNA damage is a contributory factor to apoptosis [Chen *et al.* 2013], and in cases of more severe damage of spermatozoa, apoptosis results in low sperm counts characteristic of idiopathic male factor infertility [Agarwal *et al.* 2003].

Alleviation of oxidative stress and potential treatment strategies

Treatment of oxidative stress-associated male infertility has been studied widely in patients with varicocele-associated subfertility and unexplained infertility. In these patients, treatment strategies such as surgical varicocele repair (varicocelectomy) and oral antioxidant therapy have been used.

Varicocelectomy

Current evidence suggests that oxidative stress is the central element contributing to infertility in men with varicocele and that varicocelectomy is beneficial for alleviating oxidative stress-associated infertility. Varicocele accounts for 19–41% of primary male infertility cases and up to 80% of secondary male infertility cases [Goldstein *et al.* 1992; Witt and Lipshultz, 1993; Madgar *et al.* 1995]. Studies demonstrate that varicocele repair is a viable option to decrease oxidative stress and restore the antioxidant defense system and, therefore, to improve fertility [Hamada *et al.* 2013]. An increase in varicocele grade is associated with an increase in seminal ROS levels and a decrease in sperm concentration [Cocuzza *et al.* 2012].

Several studies suggested that varicocelectomy reduces seminal oxidative stress in infertile men [Mostafa *et al.* 2001; Hurtado de Catalfo *et al.* 2007; Chen *et al.* 2008; Dada *et al.* 2010; Sakamoto *et al.* 2008]. Varicocelectomy performed in infertile men with clinical varicocele and high levels of seminal ROS resulted in a rapid decline in free radical levels within 1 month and a slower decline in DNA damage from pre-operative levels [Dada *et al.* 2010]. Sakamoto and colleagues reported that the excessive oxidative stress in the seminal plasma of infertile men with varicocele is reduced after varicocele repair and the procedure ameliorates sperm DNA damage after 6 months. A significant increase in sperm concentration and a reduction in nitric oxide, superoxide dismutase, 8-hydroxy-2′-deoxyguanosine level and superoxide dismutase activity were noted after varicocele repair. The percentage of apoptosis-positive sperm also decreased significantly after varicocelectomy [Sakamoto *et al.* 2008].

In subfertile patients with varicocele, varicocelectomy significantly decreased oxidative damage in sperm DNA and increased antioxidant capacity in seminal plasma [Chen *et al.* 2008]. Sperm motility, morphology and density improved in more

than 70% of postvaricocelectomy patients, whereas the incidence of 4977 base pair (bp) deletion of mitochondrial DNA and 8-hydroxy-2′-deoxyguanosine content in sperm DNA (both markers of oxidative stress) was reduced. However, levels of other markers of oxidative stress such as seminal plasma protein thiols and ascorbic acid increased in these patients after varicocelectomy [Chen *et al.* 2008].

Hurtado de Catalfo and colleagues reported that levels of liposoluble and hydrosoluble antioxidants decreased and activities of the antioxidant defense system enzymes increased in the seminal plasma and spermatozoa of infertile men with unilateral left varicocele after varicocelectomy [Hurtado de Catalfo *et al.* 2007]. Varicocelectomy was also found to reduce ROS and malondialdehyde (MDA) levels and increase antioxidant activity of seminal plasma in infertile men with varicocele [Mostafa *et al.* 2001]. MDA is formed as a byproduct as a result of the reaction between oxygen and unsaturated lipids [Ayala *et al.* 2014]. It appears to be the most mutagenic product of lipid peroxidation [Esterbauer *et al.* 1990] and is one of the frequently used indicators of overall lipid peroxidation levels. Because of the facile reaction of omega-3 and omega-6 fatty acids with thiobarbituric acid, an intensely colored chromogen fluorescent red adduct is formed and MDA can be quantified to determine the oxidative stress [Pryor, 1989]. In varicocelectomized patients, seminal plasma levels of ROS (hydrogen peroxide, nitric oxide) and MDA were significantly reduced, and levels of four out of six tested antioxidants (superoxide dismutase, catalase, glutathione peroxidase and vitamin C, but not vitamin E, albumin) were significantly increased at both 3 and 6 months postoperatively [Mostafa *et al.* 2001].

Zini and colleagues suggested that varicocelectomy can improve the disposal of residual sperm cytoplasm by the testis and/or epididymis in infertile men with varicocele. The percentage of spermatozoa with residual cytoplasm decreased, while the percentages of motile spermatozoa and normal forms increased 6 months after varicocelectomy [Zini *et al.* 1999]. They suggested that varicocelectomy reduces the potential for ROS generation by spermatozoa in infertile patients with varicocele.

Marmar and colleagues reported that spontaneous pregnancy rates were higher in infertile couples where the male partner underwent varicocelectomy than in those where the male partner did not [Marmar *et al.* 2007]. The meta-analysis indicated that varicocelectomy in infertile men with palpable lesions and at least one abnormal semen parameter improved the odds of spontaneous pregnancy in their female partners.

Onozawa and colleagues observed a higher postoperative/preoperative ratio of sperm density in higher-graded varicocele as well as a higher pregnancy rate in the partners of the varicocelectomized men than in the conservatively treated patients [Onozawa *et al.* 2002]. Another recent meta-analysis elucidated the impact of surgical varicocele repair on the pregnancy rate [Kim *et al.* 2013]. In an analysis of all seven trials included in that study, a forest plot using the random effects model showed an odds ratio of 1.90. In a subanalysis of the three studies that included patients with clinical varicocele and abnormal semen parameters, the fixed effects pooled odds ratio was higher, favoring varicocelectomy. The authors concluded that varicocelectomy is effective in men with clinical varicocele and impaired semen quality, and therefore surgical varicocele repair should be offered as the first-line treatment of clinical varicocele in subfertile men [Kim *et al.* 2013].

However, Baazeem and colleagues could not demonstrate any positive impact on spontaneous pregnancy rates after varicocele repair [Baazeem *et al.* 2011]. However, they did note that the procedure led to a reduction in sperm DNA damage and seminal oxidative stress, along with improvement in semen parameters (count and motility). Another recent review and meta-analytical study indicated that performing varicocelectomy in infertile patients with clinical varicocele prior to intracytoplasmic sperm injection (ICSI) was associated with improved pregnancy outcomes [Esteves *et al.* 2015]. Clinical pregnancy rates and live birth rates were higher in the varicocelectomized men than in the men without previous varicocelectomy. However, it has been suggested that the beneficial effect of varicocelectomy is time dependent, with greater results being achieved 6 months after surgery [Hamada *et al.* 2013]. Therefore, the contrasting results might be caused by differences in the timing of postoperative marker measurement.

Oral antioxidant therapy

In spite of the conflicting data, many urologists prescribe oral antioxidant therapy for men with clinical subfertility with or without varicocele

[Esteves and Agarwal, 2011]. The rationale for recommending oral antioxidant therapy is based on the premise that seminal oxidative stress is due in part to a deficiency in seminal antioxidants and the lack of serious side effects related to antioxidant therapy. Ideally, an oral antioxidant should reach high concentrations in the reproductive tract and restore vital elements important for spermatogenesis. Additionally, the antioxidant supplement should augment the scavenging capacity of seminal plasma and reduce levels of seminal ROS [Zini *et al.* 2009].

Oral treatment with antioxidants such as vitamins E, C, A, B complex, coenzyme Q10 (CoQ10), ubiquinol, glutathione, L-carnitine, lactoferrin, β-carotene, lycopene, pantothenic acid, α-lipoic acid, *N*-acetyl-cysteine, selenium, zinc, copper or supplements containing a combination of these antioxidants have been used with success to varying degrees [Hughes *et al.* 1998; Balercia *et al.* 2004; Greco *et al.* 2005; Piomboni *et al.* 2008; Ghanem *et al.* 2010; Wang *et al.* 2010; Zini *et al.* 2010; Moslemi and Tavanbakhsh, 2011; Chen *et al.* 2012; Safarinejad, 2012; Walczak-Jedrzejowska *et al.* 2013; Durairajanayagam *et al.* 2014; Haghighian *et al.* 2015; Thakur *et al.* 2015]. In a randomized, triple-blind, placebo-controlled clinical trial, Haghighian and colleagues observed that antioxidant therapy in the form of α-lipoic acid supplementation in infertile men improved semen parameters (sperm count, concentration and motility) and seminal levels of total antioxidant capacity (TAC) and MDA [Haghighian *et al.* 2015]. In infertile men, oral antioxidant therapy with Carni-Q-Nol (440 mg L-carnitine fumarate + 30 mg ubiquinol + 75 IU vitamin E + 12 mg vitamin C) softules twice or thrice daily decreased sperm pathology after 3 months, improved sperm density after 3 and 6 months, increased (ubiquinone + ubiquinol) and α-tocopherol, and decreased oxidative stress levels [Gvozdjakova *et al.* 2015]. Apart from improving sperm parameters, the supplementary therapy with Carni-Q-Nol resulted in a 45% pregnancy rate.

Festa and colleagues reported that oral supplementation with CoQ10 improved semen parameters and the antioxidant capacity of seminal plasma in infertile men with low-grade varicocele [Festa *et al.* 2014]. In another randomized double-blind, placebo-controlled trial of idiopathic oligoasthenoteratozoospermic men, oral CoQ10 supplementation increased seminal plasma TAC levels and thus reduced oxidative stress, but the semen parameters remained unaffected [Nadjarzadeh *et al.* 2011]. A number of other studies assessing the effects of oral antioxidant therapy in infertile men reported improved conventional semen parameters and sperm dysfunction, as well as decreased DNA fragmentation and damage [Gupta and Kumar, 2002; Greco *et al.* 2005; Chi *et al.* 2008; Piomboni *et al.* 2008; Gil-Villa *et al.* 2009; Zini *et al.* 2009; Ghanem *et al.* 2010; Wang *et al.* 2010; Agarwal and Sekhon, 2011; Moslemi and Tavanbakhsh, 2011; Chen *et al.* 2012; Safarinejad, 2012; Durairajanayagam *et al.* 2014; Kumalic and Pinter, 2014].

However, some contrasting reports exist in the literature [Donnelly *et al.* 1999; Menezo *et al.* 2007; Giustarini *et al.* 2008]. The methodological and clinical heterogeneity concerning the studied population as well as type, dosage and duration of antioxidant therapy make it difficult to compare the results and draw an unambiguous conclusion as to the optimal active dose and duration of any specific oral supplement therapy.

Moreover, antioxidant supplements are not free from potential side effects [Ko and Sabanegh, 2012; Walczak-Jedrzejowska *et al.* 2013; Ko *et al.* 2014]. Uncontrolled antioxidant therapy can be harmful to the patient as it may lead the system towards the reduced status. This paradoxical effect of antioxidants is called the 'antioxidant paradox' wherein a certain amount of antioxidants is essential for normal cell function (because cells generally function in a reduced state) on one hand, and a certain limited and localized level of ROS is also essential for sperm cell function on the other [Halliwell, 2000; Kothari *et al.* 2010]. For now, oral antioxidant supplementation continues to be a reasonable treatment option before proceeding with other more expensive treatment strategies [Ko and Sabanegh, 2014]. Further placebo-controlled, dietary-controlled, double-blind, randomized-controlled, prospective studies with standardized supplement regimens are needed to elucidate the role of antioxidant therapy in the treatment of oxidative stress and management of male infertility [Ko *et al.* 2014].

Measurement of seminal oxidative stress

The differences between the studies' results concerning the role of oxidative stress in the pathogenesis of male infertility may be partly explained by the heterogeneous test methods used to

measure oxidative stress. These tests are broadly divided into two categories based on their ability to directly or indirectly measure ROS [Hamada *et al.* 2013].

The ROS assay is the most widely utilized direct method [Zorn *et al.* 2003; Pons-Rejraji *et al.* 2009; Svobodova *et al.* 2009; Agarwal *et al.* 2014d], whereas measurement of antioxidant levels or activities using the TAC assay is less commonly used [Benedetti *et al.* 2012; Haghighian *et al.* 2015; Macanovic *et al.* 2015; Roychoudhury *et al.* 2016]. *Post hoc* damage is often measured as an indirect measure of oxidative stress. The most frequently used indirect test detects the presence of MDA, which indicates that lipid peroxidation has occurred as a result of excessive oxidative stress [Hosen *et al.* 2015; Moazamian *et al.* 2015].

The chemiluminescent ROS assay is based on the reaction between luminol and oxidizing compounds. The semen sample is incubated with a luminol reagent and the amount of light emitted is measured using a luminometer. The final chemiluminescent signal is the integrated sum of the partial signals generated by every spermatozoon [Agarwal *et al.* 2004]. It is measured in semen and the data are presented as relative light units per second per 10^6 sperm (RLU/s/10^6 sperm) [Agarwal *et al.* 2015a]. Several studies have found that ROS levels may be able to distinguish poor quality samples from good quality ones. A recent study by our group established an optimal cutoff value of 102.2 RLU/s/10^6 sperm ROS to differentiate between controls and infertile men with 76.4% sensitivity, 53.3% specificity, an 82.1% positive predictive value and a 44.5% negative predictive value [Agarwal *et al.* 2015a]. In another study, ROS levels were used by our laboratory to predict teratozoospermia-positive semen samples, achieving 63.9% sensitivity, 65.1% specificity and 61.4% area under curve [Agarwal *et al.* 2014b]. Using the same dataset, it is not surprising that morphology outpredicted the ROS cutoff, but motility also had a respectable specificity of 77.8%. An earlier study compared semen from proven donors with those that were infertile [Desai *et al.* 2009]. Again, an ROS cutoff was established, achieving 77.8% sensitivity and 82.4% specificity. These values are comparable with what has been found for predicting fertility based strictly on motility and morphology [Agarwal *et al.* 2014a]. Thus, measuring ROS might be a helpful, albeit time-consuming, way of identifying poor semen samples.

The TAC test assesses the cumulative effect of all antioxidants present within the semen based on their ability to scavenge free radicals with any specific or nonspecific mechanism(s) available [Agarwal *et al.* 2006, 2014a; Muller *et al.* 2013]. Multiple tests are available to measure TAC, although the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) is the most widely used test for seminal TAC currently. This test is also called the ABTS (2,20-azino-di-[3-ethylbenzthiazoline sulfonate]) assay, which is based on the inhibition of oxidation of ABTS to ABTS+. The capacity of the antioxidants in a given sample to prevent ABTS oxidation is proportional to their concentration. The reaction is colorimetric and data are converted into comparative units using a standard curve. A stable blue-green color is formed and the standard curve can be based on the activity of Trolox, a water-soluble vitamin E compound [Mahfouz *et al.* 2009]. It is measured in seminal plasma and the results are reported as micromoles of Trolox equivalent.

TAC levels are also used to distinguish between poor and good quality semen samples. Our laboratory standardized a colorimetric TAC assay in a kit form that was used successfully with seminal plasma [Mahfouz *et al.* 2009]. In a recent study, we established a new diagnostic cutoff TAC value of 1947 μm in seminal plasma with 63.0% specificity and 59.5% sensitivity to distinguish the prevalence of oxidative stress in infertile patients compared with healthy men [Roychoudhury *et al.* 2016]. At this cutoff value the sensitivity was 59.5% and the specificity was 63.0%.

Another measure of oxidative stress known as the ROS-TAC score can better differentiate fertile from infertile men than ROS or TAC alone [Sharma *et al.* 1999, 2001; Pasqualotto *et al.* 2008]. The ROS and TAC values from controls are used to create an index of these two variables using controls as a reference point. Infertile men who were able to initiate pregnancies had higher ROS-TAC scores than those who failed to initiate a pregnancy [Sharma *et al.* 1999].

Lipid peroxidation is a measure of the damage done to proximate lipids by free radicals. Lipid peroxidation is largely represented by the presence of a byproduct, MDA. MDA is also a mutagenic compound, so MDA-based assays can also act as indirect and *post hoc* indicators of DNA damage in semen samples [Marnett, 1999;

Table 1. Advantages and disadvantages of commonly used techniques to measure seminal oxidative stress.

Assay	Advantages	Disadvantages	Reference
ROS by chemiluminescence	• Chemiluminescence is robust • High sensitivity and specificity • Luminol measures global ROS levels – both extracellular and intracellular (superoxide anion, hydrogen peroxide, hydroxyl radical)	• Time-consuming method • Requires large and expensive equipment • Variables such as semen age, volume, repeated centrifugation, temperature control and background luminescence may interfere with measurement	Agarwal *et al.* [2004, 2015b]; Desai *et al.* [2009]; Kashou *et al.* [2013]; Vessey *et al.* [2014]
TAC	• Rapid colorimetric method • Measures total antioxidants in seminal plasma	• Does not measure enzymatic antioxidants • Length of the inhibition time is a critical aspect of the test • Requires expensive microplate readers	Miller *et al.* [1993]; Mahfouz *et al.* [2009]; Roychoudhury *et al.* [2016]
ROS-TAC score	• Better predictor compared with ROS and TAC alone	• Requires statistical modeling • Not a direct measure of ROS or TAC, rather a prediction of oxidative stress	Sharma *et al.* [2001]; Pasqualotto *et al.* [2008]
MDA (TBARS adduct by colorimetry or fluoroscopy)	• Measures lipid peroxidation • Detects MDA-TBA adduct by colorimetry or fluoroscopy	• Rigorous controls required • Non-specific test providing *post hoc* measure only	Chirico *et al.* [1993]; Marnett, [1999]; Martinez-Alfaro *et al.* [2006]; Grotto *et al.* [2007]
ORP	• Provides redox balance in real time • Measures all known and unknown oxidants and antioxidants • Less time-consuming and requires less expertise • Can be measured in semen and seminal plasma, including frozen specimens	• Affected by viscosity of the sample	Shapiro, [1972]; Rael *et al.* [2007]; Rael and Bar-Or [2014]; Agarwal *et al.* [2015b, 2016a, 2016b]

MDA, malondialdehyde; ROS, reactive oxygen species; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances.

Martinez-Alfaro *et al.* 2006]. Similar to other assays, the MDA assay is based on a color change measured by a microplate reader and converted using a standard curve.

The advantages and disadvantages of commonly used tests to measure seminal oxidative stress are presented in Table 1.

Oxidation-reduction potential: measurement and clinical utility

With the numerous ways in which 'oxidative stress' is implied by partitioned measures, it is not unreasonable to question its role in male infertility. Therefore, it is essential to develop an unbiased and universal measure of semen quality that represents the overall oxidant and antioxidant activity in a given sample.

ORP, also known as the redox potential, is a measure of the potential for electrons to move from one chemical species to another [McCord, 2000; Costantini and Verhulst, 2009]. To quench the damaging effects of oxidants, antioxidants work by donating electrons to the oxidants, thereby reducing the chances of oxidants to acquire electrons from other nearby structures and cause damage. ORP is a measure of this relationship between oxidants and antioxidants, providing a comprehensive measure of oxidative stress. Higher ORP values indicate an imbalance in the activity of oxidants relative to antioxidants, thus differentiating the degree of oxidative stress-induced male factor infertility. Monitoring ORP levels may help predict treatment efficacy in such patients as higher ORP levels are indicative of the progression of infertility. ORP represents the balance among all known and unknown contributors

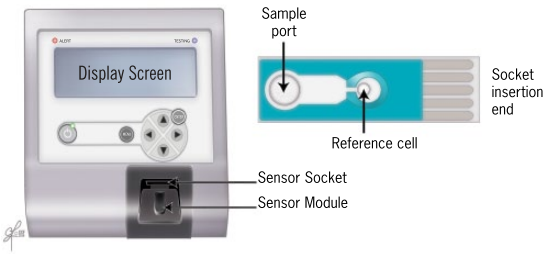

Figure 2. MiOXSYS System for measurement of oxidation-reduction potential (ORP): MiOXSYS analyzer and sensor.

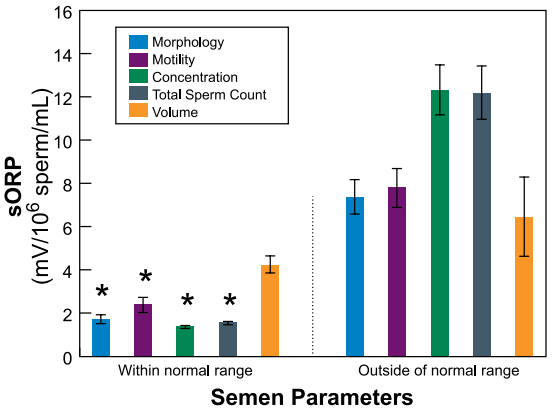

Figure 3. Comparison of sORP (mean ± SEM) measured in semen samples with semen parameters (sperm morphology, motility, concentration, total count and semen volume) within the normal range and those outside according to 2010 WHO criteria.
Total sample size, $n = 366$: morphology (within range $n = 183$, outside range $n = 183$), motility (within range $n = 219$, outside range $n = 147$), concentration (within range $n = 260$, outside range $n = 106$), total sperm count (within range $n = 264$, outside range $n = 102$), volume (within range $n = 313$, outside range $n = 53$).
Data analyzed by individual parameter using Student's *t*-test.
*Differences were statistically significant at a $p < 0.0001$.
SEM, standard error of the mean; sORP, static oxidation-reduction potential; WHO, World Health Organization.

of oxidative stress and is not limited to only a specific constituent as in ROS or TAC assays. Recently, a novel technology based on a galvanostatic measure of electrons has been developed that easily and readily measures ORP – the MiOXSYS System (Figure 2). This system has been used to assess changes in oxidative stress in trauma patients and as a function of extreme exercise [Rael *et al.* 2007, 2009; Stagos *et al.* 2015a, 2015b]. Data from the MiOXSYS System are presented in static ORP measures (sORP), with static referring to the passive or current state of activity between oxidants and antioxidants.

In the MiOXSYS System, a small volume (~30 µl) of liquefied neat semen is added to the pre-inserted sensor. The sample is wicked through the membrane to the measuring electrodes at the end. The MiOXSYS analyzer applies a low voltage current and the electron activity is measured in millivolts (mV). The entire process takes less than 4 minutes; the only preparation needed is to liquefy the semen sample – a step that is required by all oxidative stress assays. Similar to previous assays, the data are relative. Higher sORP values indicate a higher state of oxidative stress. Using sORP, our data [Agarwal *et al.* 2016a, 2016b] reinforce the impression that oxidative stress is related to poor semen quality [Zorn *et al.* 2003; Pons-Rejraji *et al.* 2009; Benedetti *et al.* 2012; Agarwal *et al.* 2014e; Macanovic *et al.* 2015]. sORP measured in semen from the male partners of infertile couples suggest that values are significantly lower in samples that have normal individual semen parameters (Figure 3). For example, semen samples that have more normal sperm morphology have significantly lower sORP values than those that have more abnormal morphology. When conventional semen parameters and sORP were analyzed in 366 men, sORP values (mean ± standard error of the mean, SEM) were lower ($p < 0.0001$) in the samples with normal semen parameters than in those with semen parameters outside the normal range (Figure 3). Volume was the only parameter that did not affect sORP values, similar to the ROS assay [Zorn *et al.* 2003]. This clearly establishes the positive association of sORP values with conventional semen parameters.

Semen samples from oligoasthenoteratozoospermic (OAT) patients have higher levels of isoprostane (a byproduct of arachidonic acid peroxidation), lower levels of catalase and lower TAC levels [Khosrowbeygi and Zarghami, 2007a, 2007b]. sORP data from 218 normozoospermic men and 69 OAT patients are presented in Figure 4; the data confirm the state of oxidative stress as the OAT patients have significantly higher ($p < 0.0001$) sORP values (mean ± SEM) than the normozoospermic men who had normal concentration, motility and morphology; this is supported by previous findings [Cavallini, 2006]. Furthermore, sORP measurements are not affected by the age of semen or seminal plasma for up to 2 hours.

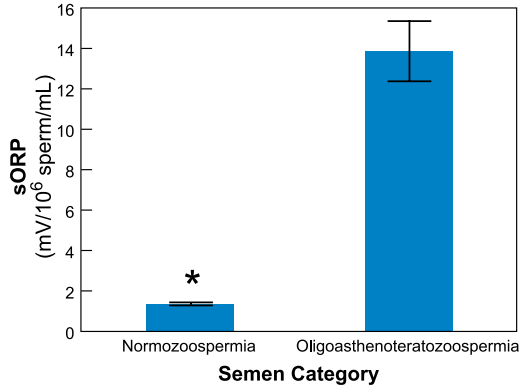

Figure 4. Comparison of sORP (mean ± SEM) in semen samples from normozoospermic and oligoasthenoteratozoospermic men. Normozoospermic semen (n = 149) have significantly lower sORP values than oligoasthenoteratozoospermic semen (n = 69). Data were analyzed by Student's t-test. *Differences were statistically significant at a $p < 0.0001$. SEM, standard error of the mean; sORP, static oxidation-reduction potential.

Application of sORP measurement in clinical practice is expected to benefit the treatment of infertile men with varicocele, infection, inflammation, spinal cord injury and severe oligozoospermia [Agarwal *et al.* 2016a]. sORP can be measured in cryopreserved semen samples as a single marker for oxidants and available antioxidant reserve, which is important in predicting the success of assisted reproductive techniques [Agarwal *et al.* 2016b]. sORP values in a given sample can be used as an initial screening tool to identify samples that are more likely to benefit from various sophisticated tests and further treatment [Agarwal *et al.* 2016b]. Assessing changes in seminal oxidative stress over time would be helpful to monitor the effect of antioxidant therapies and define effective doses and durations. Most measures of oxidative stress are inefficient and provide a single faceted view. We suggest that sORP would be a better alternative, as it is more easily applicable and comprehensive in the measurement of the overall oxidative stress in a sample.

Conclusion

ORP provides a comprehensive measure of oxidative stress by analyzing all known and unknown oxidants and antioxidants with a high sensitivity and specificity [Agarwal *et al.* 2016a, 2016b]. Compared with existing semen oxidative stress tests, sORP measurements using the portable MiOXSYS System can assess seminal oxidative stress quickly in small volumes of semen samples, and even in cryopreserved semen samples. It facilitates the wider application of oxidative stress measurement in clinical and research settings. Measurement of seminal oxidative stress is an invaluable tool to identify patients who will potentially benefit from treatment. Progress can be readily monitored and studied with the ready-to-use ORP assay kit. The measurement of sORP by the MiOXSYS System as an advanced and independent test for semen quality should find its place in the male infertility workup algorithm.

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Conflict of interest statement

Kimberly B. Bjugstad is a paid employee of Aytu BioScience, Inc.

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