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ORIGINAL ARTICLE

Semen Analysis

Multi-center evaluation of oxidation-reduction potential by the MiOXSYS in males with abnormal semen

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According to the World Health Organization (WHO), oxidative stress (OS) is a significant contributor to male infertility. Seminal OS can be measured by a number of assays, all of which are either costly or time sensitive and/or require large semen volume and complex instrumentation. One less expensive alternative is to quantify the oxidation-reduction potential (ORP) with the MiOXSYS. In this international multi-center study, we assessed whether ORP levels measured by the MiOXSYS could distinguish semen samples that fall within the 2010 WHO normal reference values from those that do not. Semen samples were collected from 2092 patients in 9 countries; ORP was normalized to sperm concentration (mV/10⁶ sperm/ml). Only those samples with a concentration >1 × 10⁶ sperm ml⁻¹ were included. The results showed that 199 samples fell within the WHO normal reference range while the remaining 1893 samples did not meet one or more of the criteria. ORP was negatively correlated with all semen parameters ($P < 0.01$) except volume. The area under the curve for ORP was 0.765. The ORP cut-off value (1.34 mV/10⁶ sperm/ml) was able to differentiate specimens with abnormal semen parameters with 98.1% sensitivity, 40.6% specificity, 94.7% positive predictive value (PPV) and 66.6% negative predictive value (NPV). When used as an adjunct to traditional semen analysis, ORP levels may help identify altered functional status of spermatozoa caused by OS in cases of idiopathic male infertility and in male partners of couples suffering recurrent pregnancy loss, and thereby directing these men to relevant medical therapies and lifestyle modifications.

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INTRODUCTION

A physiological balance between oxidants and antioxidants (reductants) is required for important sperm functions such as testicular immunity, spermatogenesis, chromatin compaction, capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion.¹ However, excess production of reactive oxygen species (ROS) or a low concentration of seminal antioxidants drives the redox balance into a state of oxidative stress (OS). Clinically, OS in males reduces fertilization rates, impairs embryonic development, and leads to implantation failure, recurrent pregnancy loss, and poor assisted reproductive technology (ART) outcomes.¹ The WHO considers OS a significant contributor to male infertility.²

A number of assays are available to measure OS including chemiluminescence for ROS, total antioxidant capacity (TAC) for antioxidants, and the malondialdehyde (MDA) assay for *post hoc* damage from lipid peroxidation.^{3,4} While useful, these tests are difficult to incorporate into routine use because they are expensive, complex,

and time sensitive and may also require complex instrumentation, large and neat sample volumes, and extensive technical training.⁵ Additionally, these assay results do not correlate with one another and provide only a single marker of OS, either oxidant levels, antioxidant levels, or *post hoc* damage.⁶

The oxidation-reduction potential (ORP) is a measure of the overall balance between oxidants and antioxidants, which provides a comprehensive measure of the redox system and OS. Previous studies have used ORP to measure OS in the blood of patients with traumatic brain injury, stroke, metabolic syndrome, liver toxicity, sepsis, and extreme exercise habits.⁷ A novel technology based on a galvanic measure of electrons—the MiOXSYS (Aytu Bioscience, Englewood, CO, USA)—has been used to measure ORP in human semen and seminal plasma.⁸ The system overcomes many of the challenges presented by traditional OS measurement methods. It is inexpensive, requires only 30 µl of sample, and takes <4 min to complete the test.⁵ This method allows OS to be measured in real time; a high ORP level is indicative

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of OS. ORP levels have been shown to correlate negatively with semen parameters and positively with DNA damage. In one study, we used ORP to distinguish infertile men from fertile controls from two different centers.⁹

The current study aims to expand these previous findings from our group by evaluating ORP in a larger group of men with ethnic and regional differences from 9 institutions around the world. The objectives of this multicenter study were (1) to evaluate the predictability of the ORP measure using the MiOXSYS in comparison with standard semen analysis parameters (as defined by the WHO 5th edition²), (2) to evaluate the variability of all measures according to WHO criteria for the normal and abnormal semen analysis groups and (3) to verify the ORP cut-off value to distinguish men with normal semen parameters from those with abnormal semen parameters.

PARTICIPANTS AND METHODS

Study subjects

Following institutional approval by all 9 participating centers, we enrolled 2092 men who were seeking assistance from a fertility specialist. Written informed consent was obtained from all participants in the following 9 institutions: Cleveland Clinic, Cleveland, USA ($n = 502$); Hamad Medical Hospital, Doha, Qatar ($n = 1078$); Dokkyo University, Osaka, Japan ($n = 152$); The Doctor's Laboratory, London, UK ($n = 106$); VKF American Hospital, Istanbul, Turkey ($n = 100$); Sohag University, Sohag, Egypt ($n = 69$); Bezmi Alem Vakif University, Istanbul, Turkey ($n = 84$); Assam University, Silchar, India ($n = 48$); and Tulane Medical Center, New Orleans, USA ($n = 22$).

The men were divided into two groups according to the WHO 5th edition criteria: those with normal semen parameters (normozoospermia; $n = 199$) and those with abnormal semen parameters ($n = 1893$) according to the WHO 5th edition reference values. Patients were excluded if they were taking medications for chronic diseases (irritable bowel disease, colitis, or a metabolic disorder) and/or had a sperm concentration $<1 \times 10^6 \text{ ml}^{-1}$, leukocytospermia, genital tract infections, prior surgery or major trauma, or a history of infertility treatment or antioxidant supplement use. All enrolled patients were between the ages of 21 years and 45 years.

Semen analysis

Semen specimens were collected by masturbation after 2–3 days of sexual abstinence, and the sperm parameters were analyzed after complete liquefaction at 37°C for 20 min. Each sample was evaluated for both macroscopic parameters such as color, pH, ejaculate volume, the age of the sample and viscosity. An aliquot of the sample (5 μl) was examined for sperm concentration, total sperm count, sperm motility and round cell concentration. If the round cell concentration was $>1 \times 10^6 \text{ ml}^{-1}$, the sample was examined for the presence of white blood cells either by the Endtz-test using a MicroCell counting chamber (Vitrolife, San Diego, CA, USA) with phase contrast optics set at $\times 20$ or by using a CE-marked LeucoScreen™ kit (Microm, Bicester, UK) that differentiates round cells on the basis of their peroxidase content and confirmed on Papanicolaou stained slides with a cell differential count. Air-dried smears were prepared for morphological evaluation, and a minimum total of 200 sperm were scored with 4% normal morphology used as cut-off.

Measurement of oxidation-reduction potential

The ORP was measured with the MiOXSYS. A 30- μl sample was loaded into the sample port of the disposable MiOXSYS sensor within one hour of liquefaction and inserted into the MiOXSYS analyzer. The test starts immediately when the sample fills the reference cell, thereby

completing the electrochemical circuit. After a short period, the ORP values are displayed on the screen in millivolts (mV). ORP provides a “snapshot” of the current balance of the redox system. A higher ORP level indicates an imbalance in the activity of all available oxidants relative to all available antioxidants in the seminal ejaculate - a state of OS. Both absolute ORP (mV) and normalized ORP values ($\text{mV}/10^6 \text{ sperm/ml}$) were calculated.

Statistical analyses

Data were analyzed separately from each laboratory and subsequently combined and analyzed by IBM SPSS version 24 (IBM Corp., Armonk, NY, USA). Data are presented as mean $\pm$ standard deviation (s.d.). Data were tested for normal distribution with the Chi-squared test and subsequently analyzed with parametric or nonparametric tests. For group comparisons, only those samples with a concentration $>1 \times 10^6 \text{ sperm ml}^{-1}$ were included. A logistic regression model was performed on the quantitative variables to determine the predictability of identifying abnormal and normal semen quality within the patient population. Because there was a 1:10 skewness in normal versus abnormal semen analysis, a random sampling technique was performed to compensate for any dominance by site with a large patient enrollment. A backward stepwise model was also applied to further verify the logistic regression model.¹⁰ Pearson correlations were used to measure associations between pairs of quantitative variables. A receiver operating characteristic (ROC) curve was used to identify the static ORP (sORP) ($\text{mV}/10^6 \text{ sperm/ml}$) criterion that best differentiated normal and abnormal semen parameters in men. $P < 0.05$ was considered as statistically significant.

RESULTS

Of the 2092 patients examined in this study, 199 had normal semen parameters (normozoospermia) and 1893 had at least one abnormal parameter (Table 1). To examine the predictability of identifying abnormal semen quality, measures from the logistic regression model were categorized according to overall contribution and significance (Table 2). ORP ranked the highest ($\beta = 2.494$, $P = 0.001$) in terms of predicting abnormal and normal semen quality followed by progressive motility ($\beta = 1.838$, $P = 0.001$), and morphology ($\beta = 0.242$, $P = 0.001$).

To account for the dominance of patients from the Hamad Medical Hospital ($n = 1078$) in the overall study, a random sample of 110 results were removed, and a revised logistic regression analysis was performed. By using a revised sample of 1124 patients for all sites, the results were categorized according to overall contribution and significance. ORP ranked the highest ($\beta = 2.057$, $P = 0.001$) followed by progressive motility ($\beta = 1.851$, $P = 0.001$) and total motility ($\beta = 0.547$, $P = 0.001$).

ORP was negatively correlated with sperm concentration, total sperm count, total motility, progressive motility, and normal morphological forms (all $P < 0.0001$; Table 3). Of the 2092 semen samples examined in the study, 199 were normal and 1893 had at least one abnormal parameter (Table 1). Comparisons of routine semen parameters (sperm concentration, total sperm count, total motility, progressive motility, volume and normal sperm forms) and ORP levels between patients with normal and abnormal semen parameters are shown in Table 1.

ROC curves were generated from the test results of the six semen parameters and ORP ($\text{mV}/10^6 \text{ sperm/ml}$), predicting normal versus abnormal semen quality values to calculate test sensitivity, specificity, and positive and negative predictive values (Figure 1). At a cut-off value of 1.34 $\text{mV}/10^6 \text{ sperm/ml}$, ORP could be used to differentiate between normal and abnormal semen quality with 98.1% sensitivity,

Table 1: Background information on the study population with a comparison of semen parameters between the normal and abnormal groups

Semen parameters	Mean±s.d.		P	95% CI
	Normal group (n=199)	Abnormal group (n=1893)		
ORP (mV/10 ⁶ sperm/ml)	0.88±1.64	5.08±14.24	0.001	-4.97—3.43
Sperm total (10 ⁶)	231.01±171.96	108.30±147.38	0.001	92.83–152.58
Progressive motility (%)	48.08±10.87	15.81±15.29	0.001	30.28–34.25
Total motility (%)	57.19±10.44	41.26±17.16	0.001	13.97–17.88
Normal morphology (%)	6.76±3.49	4.87±7.46	0.001	1.20–2.59
Sperm concentration (10 ⁶ ml ⁻¹)	70.53±50.82	34.72±31.16	0.001	27.12–44.51
Volume (ml)	3.47±1.41	3.18±2.29	0.001	-0.09–0.68

Sperm parameters and ORP values in patients with at least one abnormal semen parameter versus patients with normal semen parameters. ORP: oxidation-reduction potential; CI: confidence interval; s.d.: standard deviation

Table 2: Logistic regression model of oxidation-reduction potential and semen analysis variables according to overall contribution and significance of predicting abnormal semen quality

Semen parameters	β	Standard error of the mean	Difference	P
ORP (mV/10 ⁶ sperm/ml)	2.494	0.672	1	0.001
Sperm total (10 ⁶)	0.290	0.135	1	0.032
Progressive motility (%)	1.838	0.152	1	0.001
Total motility (%)	0.154	0.135	1	0.254
Normal morphology (%)	0.242	0.075	1	0.001
Sperm concentration (10 ⁶ ml ⁻¹)	0.027	0.112	1	0.811
Volume (ml)	0.101	0.125	1	0.420

ORP: oxidation-reduction potential

40.6% specificity, 94.7% positive predictive value, and 66.6% negative predictive value (area under the curve [AUC] = 0.765). The distribution of patients in the normal and abnormal groups above or below the established cut-off value of 1.34 mV/10⁶ sperm/ml is depicted in **Figure 2**. The median ORP (mV/10⁶ sperm/ml) level was below the established cut-off value of 1.34 mV/10⁶ sperm/ml in the normal group. In the abnormal group, the median ORP level was above the established cut-off value, and a greater range, as well as distribution of ORP values, can be observed in this group ($P = 0.004$).

DISCUSSION

Although routine semen analysis is still used as the basis for male infertility evaluation, concerns about its technical and biological consistency and semen analysis guidelines raise questions about the reliability of this test.¹¹ High intra-individual variability is common in semen specimens owing to both biological and technical reasons, and therefore, the results of a single semen specimen may not accurately reflect a man's fertility status and cannot be used as a specific surrogate of fertility potential.⁴ Additionally, semen analysis is unable to assess many of the key aspects of the fertilization process such as capacitation, the state of the proteins required for zona pellucida binding and penetration, the ability of sperm to fertilize an egg, or any potential damage to the spermatozoa caused by OS or DNA fragmentation.^{12,13}

Approximately 30% of infertile men have a normal semen parameters. In those cases, clinicians seek advanced tests (i.e., hypo-osmotic swelling test, computer-assisted sperm analysis, antisperm antibody test, sperm penetration assay, hemizona assay, and sperm chromatin integrity test) to identify other factors.^{13,14} However, many of these tests are costly and highly technical, making them inaccessible to small laboratories, and they do not provide complete information regarding a man's fertility status.^{13,15} As such, clinicians may be quick to categorize these men with idiopathic (unexplained) infertility. Considering the limited and often inconsistent results of semen analysis and the number of couples

experiencing recurrent pregnancy loss, it is becoming more important to assess male fertility with advanced sperm function tests that are accurate, repeatable, and accessible.

It is well established that physiological levels of OS are required for sperm function. On the other hand, OS plays a key role in the pathogenesis of male infertility. Between 30% and 80% of infertile men present with OS caused by high levels of ROS or antioxidant depletion.^{16–22} Despite the prevalence of OS and the significance of its effects, clinicians do not routinely measure OS as part of a diagnostic workup due to the lack of a standardized protocol and differences between ROS reference values, given that many different tests can be used to assess OS.^{17–22} This makes it difficult to compare OS across different individuals or laboratories and to assess the significance of a sperm abnormality under a state of high OS and its impact on fertilization or clinical pregnancy rates.

The MiOXSYS overcomes many of the challenges presented by previous OS single marker tests. The system is accurate, easy to use, and inexpensive, in which it does not require capital equipment or highly trained staff. In a study conducted by our group with 51 controls and 106 infertile patients, ROC curve analysis showed that a cut-off value of 1.36 mV/10⁶ sperm/ml could distinguish infertile men from healthy men.¹²

In another previous study, we increased the sample size and applied findings to other laboratories by collaborating with a center in Doha, Qatar.¹³ In our two andrology laboratories, there was a total of 101 fertile controls and 594 infertile patients.⁹ ORP was consistent across both centers, even when other semen parameters were not, and the ROC curve analysis generated an ORP cut-off value of 1.42 mV/10⁶ sperm/ml to distinguish between healthy and infertile men.⁹

In the current study, we increased our sample size by enrolling men from 9 international centers and also measured ORP in conjunction with traditional semen analysis. We were able to detect abnormal semen quality with a 98.1% sensitivity, 40.6% specificity, 94.7% positive predictive value, and 66.6% negative predictive value. The cut-off value of the ORP measure was 1.34 mV/10⁶ sperm/ml and is consistent with previous research.⁵

Using an ORP test in conjunction with the standard semen analysis resulted in slightly higher predictability ($R^2 = 0.538$ versus $R^2 = 0.526$) than performing standard semen analysis without ORP. This is a current limitation of the current study and can be addressed in the future by assessing clinical pregnancy as an endpoint. Despite this, ORP was able to distinguish abnormal semen samples from normal samples reliably.

In our multi-center study, the high positive and negative predictive values suggest that ORP levels measured by the MiOXSYS can identify abnormal and normal sperm quality especially well. This predictive ability is also reflected in the results of the logistic regression model

in which ORP contributed greater than any other semen parameters in determining abnormal and normal semen quality.

ORP correlated with most of the semen parameters (sperm concentration, motility, total sperm count, progressive motility, and normal morphology) and body mass index (BMI). Although both measures (ORP and concentration) were significant in the logistic regression model, the contribution ($\beta = 2.494$) of ORP in the logistic regression model was greater than that of concentration ($\beta = 0.027$), suggesting that each measure is unique and influences the diagnosis differently. The negative correlation between ORP and concentration may be expected given that ORP is normalized to concentration, and as evidenced in previous studies, ORP can predict oligozoospermia with high sensitivity and specificity, suggesting that OS may play a key pathologic role in patients with reduced sperm concentration.^{9,21}

One of the main advantages of the current study was that it included patients from all parts of the world, including those who were omitted from the development of the WHO 5th edition reference guidelines (Qatar, Japan, Turkey, Egypt, and India). This ensured that the ORP measurement cut-off values applied to multiple ethnic and geographical

populations remained stable across laboratories in different countries where other semen parameters are not.^{9,11} Significant differences in semen parameters were found between the centers for samples that met the normal reference range of the WHO 5th edition guidelines and those that did not. Significant ($P < 0.001$) differences were found in progressive motility, total motility, and the morphology value for each center. Sperm concentration and semen volume were significantly different between centers that did not meet WHO criteria. Both ORP and total sperm count were nonsignificant. Although significant differences between the parameters were present, ORP levels for fertile and infertile patients did not differ significantly between the participating centers within the study (Table 4).

The limitations of our study include the following: (1) infertile patients were classified based on their clinical diagnosis of abnormal and normal semen parameters, but not by specific sperm abnormalities (*i.e.*, oligasthenoteratozoospermia versus isolated teratozoospermia); (2) the study did not balance the number of healthy controls with proven fertility and infertile patients, but to account for the dominance of patients in the overall study, a random sample of 110 results were removed and a revised logistic regression analysis was performed; and (3) while semen parameters are an important part of the assessment of the infertile male, the gold standard is the ability to determine

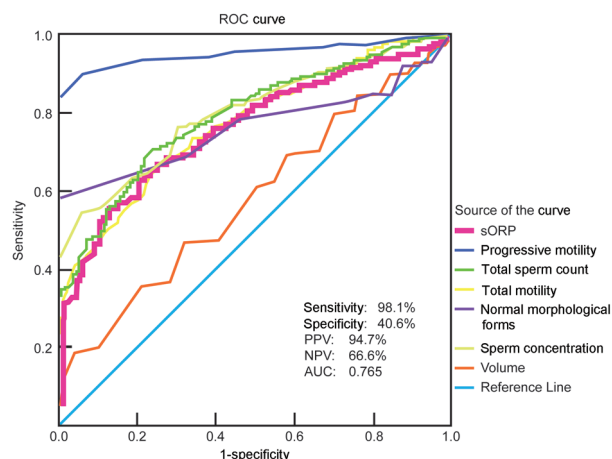


Figure 1: A ROC curve was used to identify the sORP ($\text{mV}/10^6$ sperm/ml) criterion, *i.e.*, cut-off, sensitivity, and specificity, PPV, NPV, and AUC in conjunction with semen parameters that best predicted the normal and abnormal semen parameters. sORP: static oxidation-reduction potential; ROC: receiver operating characteristic; PPV: positive predictive value; NPV: negative predictive value; AUC: area under the curve.

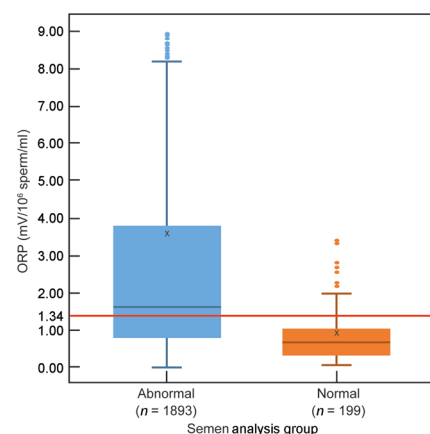


Figure 2: Distribution of ORP in patients with at least one abnormal sperm parameter versus patients with normal sperm parameters, showing the established cut-off value of $1.34 \text{ mV}/10^6$ sperm/ml. ORP: oxidation-reduction potential.

Table 3: Correlation of oxidation-reduction potential with sperm parameters in all patients ($n=1893$)

sORP	Sperm total (10^6)	Progressive motility (%)	Total motility (%)	Normal morphology (%)	Sperm concentration (10^6 ml^{-1})	Volume (ml)
Pearson correlation	-0.188**	-0.185**	-0.200**	-0.111**	-0.287**	-0.011
<i>P</i> (two-tailed)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.678

sORP: static oxidation-reduction potential

Table 4: Sperm parameters and oxidation-reduction potential values ($\text{mV}/10^6$ sperm/ml) in infertile patients ($n=2092$) with at least one abnormal sperm parameter for combined dataset

Criteria	Total sperm count (10^6)	Progressive motility (%)	Total motility (%)	Normal morphology (%)	Sperm concentration (10^6 ml^{-1})	Volume (ml)	ORP
WHO (5 th) meets ($n=199$)							
<i>P</i>	0.191	0.001	0.001	0.001	0.245	0.393	0.955
Difference of means values	157.60–288.19	34.37–57.69	41.75–77.66	4.9–12.06	51.06–83.62	2.95–3.82	0.46–1.14
WHO (5 th) fails ($n=1893$)							
<i>P</i>	0.126	0.001	0.001	0.001	0.001	0.001	0.002
Difference of means values	98.71–139.75	12.18–51.54	37.53–58.80	1.38–4.83	31.60–51.05	2.75–3.97	2.73–4.64

Inter-rater reliability between participating centers was analyzed for the infertile patients. ORP: oxidation-reduction potential; WHO: World Health Organization

the reproductive outcome. Finally, pregnancy outcomes were not prospectively measured in the infertile group.

CONCLUSION

ORP represents an advanced sperm function measure for OS that can reliably predict sperm abnormalities under a state of high OS. A reference value of 1.34 mV/10⁶ sperm/ml for ORP provided the greatest predictability when identifying abnormal/normal semen quality among 2092 patients with male infertility. Abnormal ORP levels can be useful in identifying altered sperm functional status, especially in cases of idiopathic infertility and/or in male partners of couples who have suffered recurrent pregnancy loss. Ongoing studies are underway to further understand the clinical role of this new and reproducible measure of OS in ART. Similar to sperm DNA fragmentation studies, evaluation of the ORP measure will be closely assessed for fertility success in accordance with fertilization and embryo development rates.

AUTHOR CONTRIBUTIONS

Study design was planned by A Agarwal. A Agarwal, MA, HO, SH, AK, BB, RS, A Armagan, SR and SS were involved in analysis and interpretation of the data. Manuscript was written by A Agarwal and reviewed by MKPS, MA, HO, SH, AK, BB, A Armagan, SR and SS. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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