

MiOXSYS: a novel method of measuring oxidation reduction potential in semen and seminal plasma

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Objective: To measure oxidative reduction potential (ORP) in semen and seminal plasma and to establish their reference levels.

Design: ORP levels were measured in semen and seminal plasma.

Setting: Tertiary hospital.

Subject(s): Twenty-six controls and 33 infertile men.

Intervention(s): None.

Main Outcome Measure(s): Static ORP (sORP) and capacitance ORP (cORP) were measured in semen and seminal plasma at time 0 and 120 minutes. Correlation of ORP was assessed between [1] semen and seminal plasma and [2] time 0 and 120 minutes. The association with sperm parameters was studied in (a) controls and (b) infertile patients, and a receiver operating characteristic curve was generated to establish the sORP cutoff.

Result(s): Semen sORP and cORP levels were associated with seminal plasma levels at time 0 and time 120 minutes. In controls and infertile patients, an inverse relationship of sORP levels was established with concentration and total sperm count in semen as well as seminal plasma at time 0 and 120 minutes. Classification of subjects based on sperm motility showed that subjects with abnormal motility present with poor concentration, total count, morphology, and elevated levels of semen and seminal plasma sORP at time 120 minutes. The sORP cutoff of 1.48 in semen and 2.09 in seminal plasma based on motility was able to distinguish subjects with normal semen quality from those with abnormal semen quality.

Conclusion(s): The MiOXSYS System can reliably measure ORP levels in semen and seminal plasma. ORP levels are not affected by semen age, making this new technology easy to employ in a clinical setting. (*Fertil Steril*® 2016;106:566–73. ©2016 by American Society for Reproductive Medicine.)

Key Words: Semen, seminal plasma, sperm, reactive oxygen species, oxidation reduction potential

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Routine semen analysis is the backbone of male factor infertility evaluation. However, such testing does not assess oxidative stress,

which is a major contributor to male factor infertility (1–7). Oxidative stress in sperm stems from excessive production of reactive oxygen

species (ROS). Physiological levels of ROS are necessary for optimal sperm functions such as motility, hyperactivation, capacitation, and acrosome reaction (2, 8), but when levels become too high, oxidative stress occurs. Spermatozoa and seminal leukocytes can act as potential sources of excessive ROS generation (2, 9, 10), leading to abnormal sperm parameters (1, 11). High levels of ROS are found in 25%–40% of infertile men and in 40%–88% of infertile patients with spinal cord injury (12, 13). Infertile patients

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also have low levels of antioxidants in their seminal plasma (10, 14).

Currently, a combination of independent assays, both direct and indirect, is used to measure oxidative stress (8). ROS can be measured in semen via chemiluminescence assays, and total antioxidant capacity (TAC) in seminal plasma can be assessed with a colorimetric assay. Together, the results are used to calculate a composite ROS-TAC score (10, 14, 15). Although useful, these traditional methods of measuring seminal oxidative stress are time sensitive and time consuming, making it difficult for them to be used for routine diagnostic purposes. Additionally, the tests are cumbersome, involve sophisticated instruments, require a large sample volume, and take approximately 45 minutes to complete. Therefore, there is a strong need for a method that is independent of semen age that provides results in real time.

Oxidation reduction potential (ORP) is a direct measurement of oxidative stress or redox imbalance in biological samples. As an indicator of oxidative stress, ORP has been shown to correlate well with illness and injury severity in trauma patients (16–19). Currently, there is a novel but simple technology that quickly measures ORP in small sample volumes and may provide an alternative for measuring oxidative stress in semen samples.

The aims of the study were to examine semen samples from healthy controls and infertile patients to [1] assess the ability of this novel technology to measure ORP levels in semen and seminal plasma, [2] study the effect of time on sORP levels, [3] determine the correlation of ORP with semen parameters, and [4] establish the ORP cutoff value in semen and seminal plasma.

MATERIALS AND METHODS

After approval by the Institutional Review Board, semen samples were obtained from healthy male volunteers ($n = 26$) and infertile patients ($n = 33$). All subjects provided written consent, and samples were collected and analyzed according to the 5th edition of the World Health Organization (WHO) guidelines (20). All healthy subjects presented with normozoospermia. In this group, samples that did not show any sperm in the ejaculate (azoospermic), those with round cells $>1 \times 10^6$, or those that had a positive Endtz test were excluded. The group of infertile men was composed of male factor infertility patients attending the tertiary care hospital. After liquefaction, a standard semen analysis was performed. Semen samples were subsequently divided into two fractions, and the seminal plasma was isolated from one fraction via centrifugation ($\times 300$ g, 7 minutes).

Semen Analysis

After liquefaction, a manual semen analysis was performed according to WHO guidelines (20) to determine the sperm parameters. Five microliters of the sample was used for manual evaluation of sperm concentration, motility, and round cells on wet preparation using a MicroCell counting chamber (Vitrolife) with phase optics set at $\times 20$ magnification.

Measurement of White Blood Cells

Samples with a high concentration of round cells (>5 per high power field) were examined for the presence of white blood cells, especially the granulocytes, by the peroxidase (Endtz) test (21). To conduct the Endtz test, a $20\text{-}\mu\text{L}$ well mixed aliquot of the semen sample was mixed with one volume of phosphate-buffered saline and 2 volumes of working Endtz solution in an amber colored Eppendorf tube. After 5 minutes, a drop of the aliquot was placed on a Makler chamber and examined for the presence of dark brown cells under a $\times 10$ bright field objective. Leukocytospermia was defined as the presence of $>1 \times 10^6$ white blood cells/mL according to the WHO guidelines (20).

Measurement of Sperm Morphology

For sperm morphology, air-dried smears were stained using the Diff-Quik kit (Baxter Healthcare Corporation) for assessment of sperm morphology. The morphological abnormalities were examined according to strict criteria (20). A total of 200 sperm were scored, and results were expressed as percent normal morphology.

Measurement of ORP

ORP measures the transfer of electrons from a reductant (or antioxidant) to an oxidant. ORP is measured in millivolts (mV). For the current study, ORP was measured using novel galvanostat-based technology—the MiOXSYS System (Aytu BioScience, Inc.; [Supplemental Fig. 1](#)). Briefly, $30\text{ }\mu\text{L}$ of liquefied semen or seminal plasma at room temperature was applied to the MiOXSYS sensor. The sensor was preinserted into the MiOXSYS analyzer; measurements begin automatically.

The MiOXSYS System provides two measures of oxidative stress. Static ORP (sORP), measured in millivolts, is the integrated measure of the existing balance between total oxidants and reductants in a biological system. After this initial sORP reading is recorded, the analyzer automatically applies a small current sweep to the sample, resulting in the exhaustion of all antioxidant species, providing a measure of antioxidant capacity reserve (cORP), measured in microcoulombs (μC).

Each sample was measured in triplicate, and the average values for sORP and cORP were recorded. Data were then normalized to sperm concentration to control for differences in cell numbers. Thus, data are presented as millivolts/ 10^6 sperm/milliliter for sORP and microcoulombs/ 10^6 sperm/milliliter for cORP. To assess the effects of time on ORP measures, samples were incubated at room temperature for 0 and 120 minutes before being measured.

Statistical Analysis

Data are presented as mean \pm standard error of mean (SEM) or median (25th, 75th percentile). $P < .05$ was considered statistically significant for pairwise group comparison using a Wilcoxon signed-rank test. Spearman correlations were used to measure associations between pairs of quantitative variables.

A receiver operating characteristic (ROC) curve was used to establish the cutoff, sensitivity, specificity, area under the curve (AUC), accuracy, and the ability for sORP to differentiate subjects with normal ($\geq 40\%$) and abnormal ($< 40\%$) motility.

RESULTS

Sperm parameters in controls and the patient group are shown in [Table 1](#). Significantly higher volume was seen in infertile group, whereas motility and morphology were significantly reduced in these men compared with the control group ($P < .01$). When the subjects were grouped based on normal ($\geq 40\%$) versus abnormal ($< 40\%$) motility, significantly lower concentration, total sperm count, and morphology were seen in the men with abnormal motility compared with the normal motility group ($P < .05$; [Table 1](#)).

Effect of Sample Type and Time on ORP Measurement

The comparison of sORP and cORP in the semen and seminal plasma of healthy controls and infertile patients at time 0 and 120 minutes is shown in [Supplemental Table 1](#). Compared with the control group, in the infertile patients higher ranges of sORP and cORP levels were observed in semen as well as in seminal plasma at time 0 and 120 minutes. At time 0, these levels did not differ significantly either in semen or seminal plasma. At time 120 minutes, higher mean \pm SEM or median (25th, 75th percentile; 17.39 ± 13.33 ; 0.74 [0.41, 2.89]; $P = .06$) sORP levels were seen in the semen of infertile patients compared with controls (1.04 ± 0.26 ; 0.49 [0.25, 1.32]). At this time, significantly higher levels of sORP were also seen in the seminal plasma in the infertile men (17.26 ± 13.16 ; 0.69 [0.37, 5.11]) compared with controls (1.82 ± 0.82 ; 0.45 [0.25, 1.63]; $P = .036$). The cORP values were comparable for both semen as well as seminal plasma at these time intervals.

The comparison of sORP and cORP in semen and seminal plasma of men with normal ($\geq 40\%$) and abnormal ($< 40\%$) motility at time 0 and 120 minutes is shown in [Table 2](#). In the group of men with abnormal motility, a higher range of sORP and cORP levels were observed in semen and in seminal

plasma both at time 0 and 120 minutes compared with the subjects with normal motility. These levels were significantly higher for sORP at time 120 minutes and for cORP at time 0 ([Table 2](#)).

Correlation of sORP and cORP in semen versus seminal plasma. Semen sORP levels correlated strongly with the levels in seminal plasma both at time 0 ($r = 0.956$; $P < .001$) and time 120 minutes ($r = 0.982$; $P < .001$; [Fig. 1A](#) and [1B](#)). Similarly, strong positive correlation of semen cORP levels was noted with levels in seminal plasma both at time 0 ($r = 0.991$; $P < .001$) and time 120 minutes ($r = 0.962$; $P < .001$; [Fig. 1C](#) and [1D](#)). In addition to the strong correlations, the observations also tended to be tightly bound to the line of concordance, demonstrating consistency in measurements in semen and seminal plasma.

Correlation of sORP and cORP at time 0 versus 120 minutes.

The correlation of sORP levels between time 0 and 120 minutes was highly significant in semen ($r = 0.956$; $P < .001$) and seminal plasma ($r = 0.916$; $P < .001$; [Fig. 1E](#) and [1F](#)). A similar effect of time was observed for the cORP levels. The correlation of cORP levels between time 0 and 120 minutes were highly significant in semen ($r = 0.870$; $P < .001$) and seminal plasma ($r = 0.830$; $P < .001$; [Fig. 1G](#) and [1H](#)). The observations at different time points also tended to be near the line of concordance, demonstrating consistency in measurements at 0 and 120 minutes.

Correlation of ORP Measurement in Relation to Sperm Parameters between Healthy Controls and Infertile Patients

Correlations of sORP and cORP with sperm parameters in semen and seminal plasma at time 0 and 120 minutes are shown in [Supplemental Figures 2–9](#).

Correlation of sORP in semen at time 0. In the group of healthy controls, a significantly negative correlation was seen between sORP versus concentration ($r = -0.643$; $P < .01$) and sORP versus total sperm count ($r = -0.577$; $P = .002$; [Supplemental Fig. 2A](#) and [2B](#)). In the group of infertile patients, a similar significant but stronger negative correlation was seen between sORP versus concentration

TABLE 1

Baseline semen parameters in healthy and infertile men and sORP ($mV/10^6$ sperm/mL) and cORP ($\mu C/10^6$ sperm/mL) levels as measured in men with normal ($\geq 40\%$) and abnormal ($< 40\%$) motility over time.

Parameter	Healthy controls (n = 26)	Infertile patients (n = 33)	P value	Subjects with normal ($\geq 40\%$) motility (n = 44)	Subjects with abnormal ($< 40\%$) motility (n = 15)	P value
Volume, mL	2.20 ± 0.20	3.33 ± 0.27	.004	2.63 ± 0.20	3.42 ± 0.45	.09
Concentration, $\times 10^6$ spermatozoa/mL	55.43 ± 7.97	50.91 ± 9.61	.16	61.70 ± 7.28	27.09 ± 11.03	.002
Total sperm count, $\times 10^6$ sperm	114.59 ± 17.68	153.48 ± 31.31	.98	157.92 ± 23.52	73.05 ± 25.17	.013
Motility, %	58.96 ± 1.78	44.15 ± 3.61	.003	59.30 ± 1.49	25.40 ± 3.00	<.001
Morphology, Kruger, %	7.04 ± 0.71	3.74 ± 0.65	<.001	6.49 ± 0.61	2.21 ± 0.43	<.001

Note: Values are mean \pm SEM; $P < .05$ was considered statistically significant by Wilcoxon rank sum test using pairwise group comparisons.

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

TABLE 2

sORP ($\text{mV}/10^6 \text{ sperm/mL}$) and cORP ($\mu\text{C}/10^6 \text{ sperm/mL}$) levels as measured in men with normal ($\geq 40\%$) and abnormal ($< 40\%$) motility over time.

Parameter	Time 0 min		Time 120 min		P value	
	Subjects with normal ($\geq 40\%$) motility	Subjects with abnormal ($< 40\%$) motility	P value	Subjects with normal ($\geq 40\%$) motility	Subjects with abnormal ($< 40\%$) motility	
sORP in semen	2.55 ± 0.84 0.51 (0.30, 1.88)	35.28 ± 29.18 1.56 (0.46, 6.91)	.2	2.56 ± 0.72 0.57 (0.36, 1.77)	37.60 ± 30.45 1.84 (0.80, 7.77)	.035
sORP in seminal plasma	2.61 ± 0.82 0.49 (0.31, 1.90)	34.74 ± 28.80 1.38 (0.30, 6.91)	.32	2.65 ± 0.74 0.55 (0.39, 1.72)	36.03 ± 30.31 1.59 (0.65, 7.71)	.04
cORP in semen	1.71 ± 0.86 0.67 (0.27, 1.28)	3.49 ± 0.98 1.82 (1.35, 5.68)	.009	1.54 ± 0.86 0.46 (0.17, 1.07)	2.55 ± 0.85 1.16 (0.49, 4.65)	.14
cORP in seminal plasma	1.69 ± 0.86 0.69 (0.27, 1.28)	3.36 ± 0.90 1.86 (1.44, 5.68)	.007	1.54 ± 0.86 0.36 (0.21, 1.05)	2.72 ± 0.91 1.22 (0.39, 5.32)	.14

Note: Values are mean \pm SEM, median (25th, 75th percentiles); P<.05 was considered statistically significant by Wilcoxon rank sum test using pairwise group comparisons.

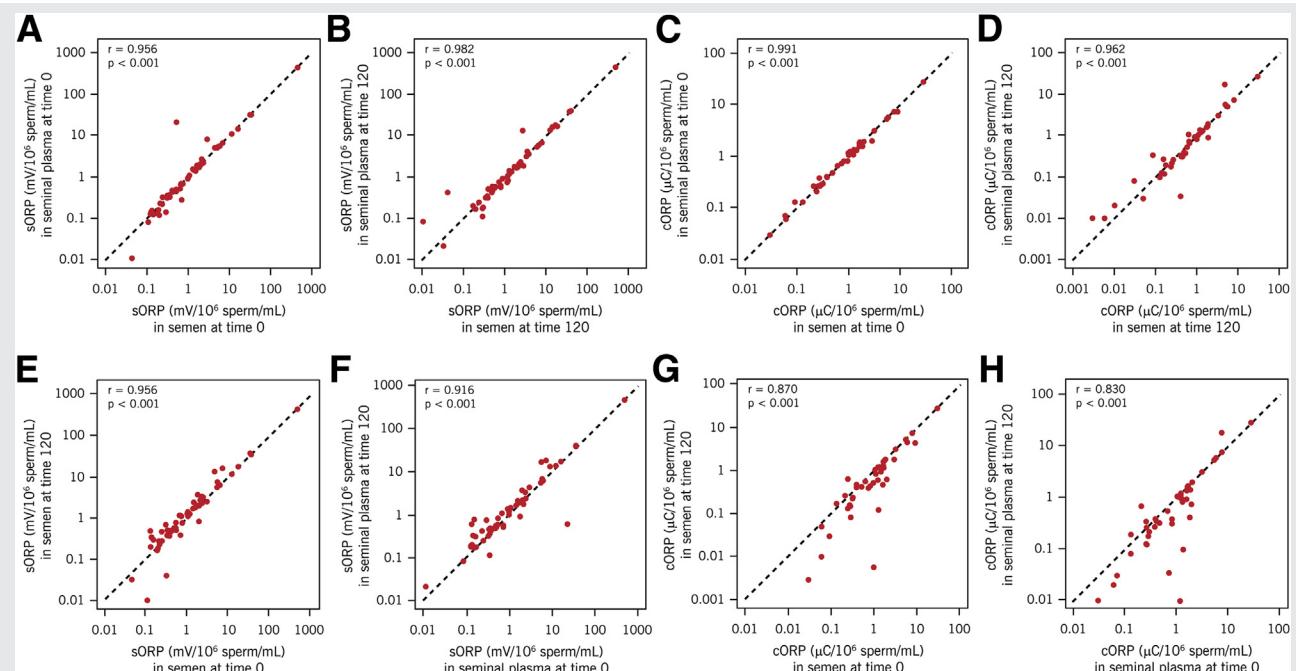
Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

($r = -0.883$; $P < .001$); sORP versus total sperm count ($r = -0.811$; $P < .001$) and also sORP versus motility ($r = -0.369$; $P = .035$; *Supplemental Fig. 2E–2G*).

Correlation of sORP in seminal plasma at time 0. In the control group, a significantly negative correlation was seen between sORP versus concentration ($r = -0.576$; $P = .02$) and sORP versus total sperm count group ($r = -0.506$; $P = .008$; *Supplemental Fig. 3A and 3B*). A similar significant but

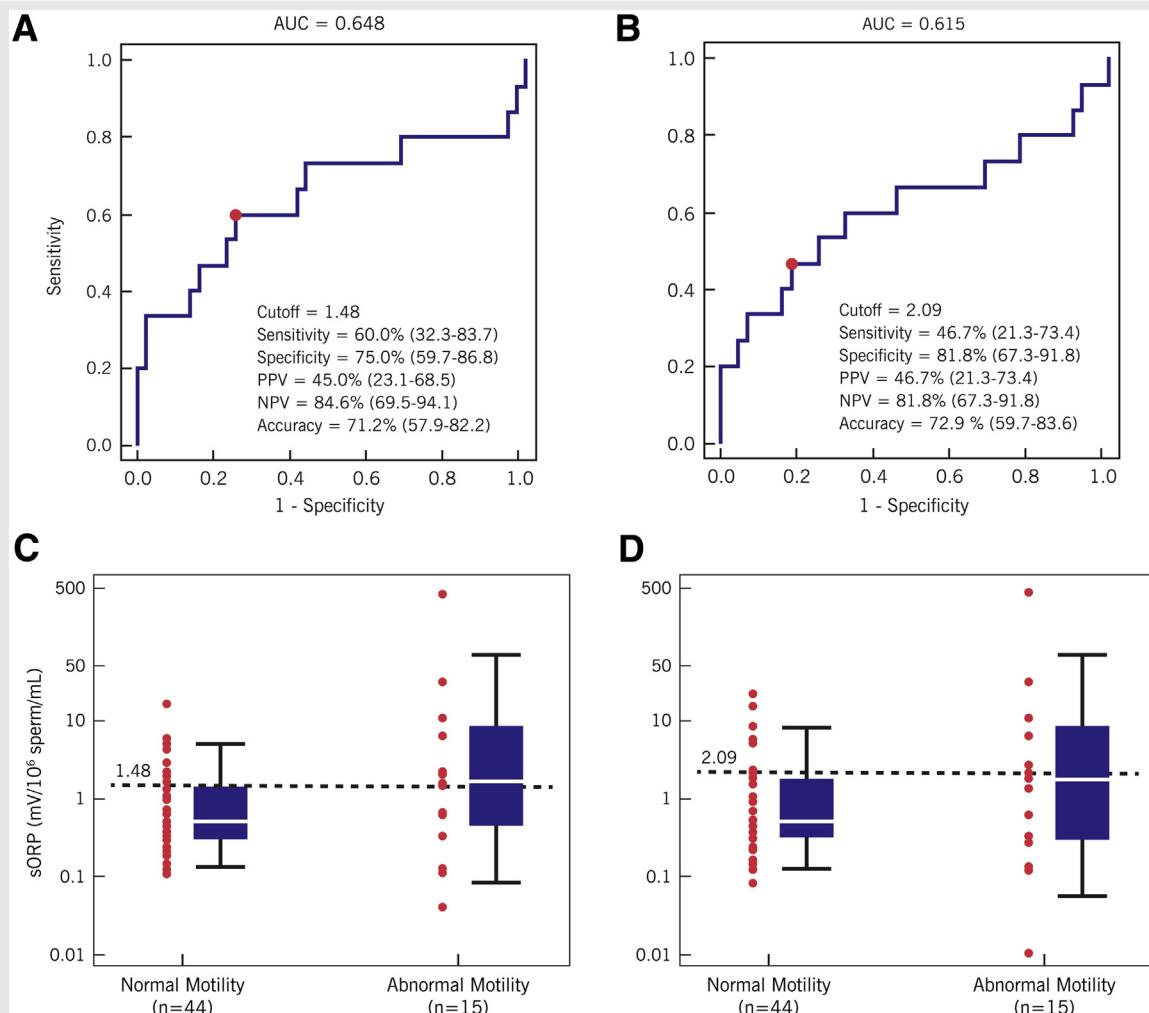
stronger negative correlation was seen in infertile patients between sORP versus concentration ($r = -0.846$; $P < .001$) and sORP versus total sperm count ($r = -0.772$; $P < .001$; *Supplemental Fig. 3E and 3F*).

Correlation of sORP in semen at time 120 minutes. A significantly negative correlation was seen in the healthy controls between sORP versus concentration ($r = -0.644$; $P < .001$) and sORP versus total sperm count ($r = -0.574$; $P = .002$;

FIGURE 1

Effect of sample type (semen or seminal plasma) and time (0 or 120 minutes) on sORP and cORP showing correlation of sORP in semen versus seminal plasma at (A) time 0 and (B) time 120 minutes; correlation of cORP in semen versus seminal plasma at (C) time 0 and (D) time 120 minutes; correlation of sORP at time 0 versus 120 minutes in (E) semen and (F) seminal plasma; correlation of cORP at time 0 versus time 120 minutes in (G) semen and (H) seminal plasma.

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FIGURE 2

ROC curve establishing the cutoff in (A) semen and (B) seminal plasma. Distribution of sORP in subjects with normal and abnormal motility in (C) semen and (D) seminal plasma, suggesting a criterion for sORP in distinguishing semen quality based on good ($\geq 40\%$) and poor ($<40\%$) motility.

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

Supplemental Fig. 4A and 4B). A similar significant but stronger negative correlation was seen between sORP versus concentration ($r = -0.882$; $P < .001$), sORP versus total sperm count ($r = -0.808$; $P < .001$), and sORP versus motility ($r = -0.446$; $P = .009$) in infertile patients (Supplemental Fig. 4E–4G).

Correlation of sORP in seminal plasma at time 120 minutes. In healthy controls, a significantly negative correlation was seen between sORP versus concentration ($r = -0.652$; $P < .001$) and sORP versus total sperm count ($r = -0.585$; $P = .002$; Supplemental Fig. 5A and 5B). A similar significant but stronger negative correlation was seen in infertile patients between sORP versus concentration ($r = -0.930$; $P < .001$), sORP versus total sperm count ($r = -0.866$; $P < .001$), and sORP versus motility ($r = -0.476$; $P = .005$; Supplemental Fig. 5E–5G).

Correlation of cORP in semen at time 0. A negative correlation was seen between cORP versus concentration ($r = -0.606$; $P = .002$), cORP versus total sperm count ($r = -0.502$; $P = .015$), and cORP versus morphology ($r = -0.418$; $P = .047$)

in healthy controls (Supplemental Fig. 6A, 6B, 6D) compared with cORP versus concentration ($r = -0.533$; $P = .020$) and cORP versus total sperm count ($r = -0.505$; $P = .029$) in infertile patients (Supplemental Fig. 6E and 6F).

Correlation of cORP in seminal plasma at time 0. A significantly negative correlation was seen between cORP versus concentration ($r = -0.610$; $P = .002$), cORP versus total sperm count ($r = -0.506$; $P = .014$), and cORP versus morphology ($r = -0.441$; $P < .035$) in healthy controls (Supplemental Fig. 7A, 7B, 7D). In the group of infertile patients, a similar significantly negative correlation was seen between cORP versus concentration ($r = -0.558$; $P = .015$) and cORP versus total sperm count ($r = -0.525$; $P = .023$; Supplemental Fig. 7E and 7F).

Correlation of cORP in semen at time 120 minutes. A significantly negative correlation was observed only in the group of healthy controls between cORP versus concentration ($r = -0.586$; $P = .003$; Supplemental Fig. 8A).

Correlation of cORP in seminal plasma at time 120 minutes. Correlations were seen between cORP versus concentration ($r = -0.515; P=.012$) and cORP versus total sperm count ($r = -0.354; P=.010$) in the control group only (Supplemental Fig. 9A and 9B).

ROC Curves and sORP Cutoff

ROC curves were generated both for semen and seminal plasma with regards to normal ($\geq 40\%$) and abnormal ($<40\%$) motility at time 0 (Fig. 2A and 2B). ROC analysis suggested a sORP cutoff of $1.48 \text{ mV}/10^6 \text{ sperm/mL}$ in semen. At this cutoff, it showed sensitivity of 60.0%, specificity of 75.0%, AUC of 0.648, and accuracy of 71.2% (Fig. 2A). In the seminal plasma, a sORP cutoff of 2.09 showed sensitivity of 46.7%, specificity of 81.8%, AUC of 0.615, and accuracy of 72.9 % (Fig. 2B).

Comparison of sORP between Normal and Abnormal Motility

We examined the distribution of normal ($\geq 40\%$) and abnormal motility ($<40\%$) groups that were below or above the sORP cutoff based on the original sperm concentration (Fig. 2C and 2D). In the group of subjects with normal motility, the median values for motility were below the sORP cutoff of 1.48. In the abnormal motility group, the median motility values were significantly above the established cutoff value, and a greater range of sORP was seen in this group (Fig. 2C). A similar trend was seen in subjects with normal and abnormal motility group for a sORP cutoff of 2.09 in seminal plasma (Fig. 2D).

DISCUSSION

Oxidative stress is an indicator of male factor infertility. Accurate assessment of oxidative stress is important for the clinical diagnosis and management of male factor infertility, especially in patients with unexplained and idiopathic infertility.

Previous methods of measuring oxidative stress markers in male factor infertility include measurement of [1] ROS by chemiluminescence assay (15, 22, 23), [2] antioxidants by colorimetric assay (14,24–28), [3] lipids by measuring oxidized products by thiobarbituric acid assay (TBARS) or 4- hydroxynonenal (4-HNE) (29, 30), [4] apoptotic markers such as annexin V (31, 32), and [5] oxidative stress modified protein alterations using proteomic tools (33, 34). These methods are antiquated, time consuming, and time sensitive and require large financial investments. More importantly, these assays measure a single marker of oxidative stress from which the overall oxidative stress in a given biological system is generalized. Individually, they provide an incomplete picture of the true oxidative stress environment and reflect only the redox status at a single point.

Unlike the assessment of single markers, assessing ORP in a biological sample provides a comprehensive measure of oxidative stress. It involves the analysis of all known and unknown oxidants and antioxidants in the given sample and does not rely on a single biomarker of oxidative stress. ORP

measures can be obtained from a variety of biological specimens, including whole blood, plasma, and urine (17–19). ORP measured in blood plasma was able to distinguish between normal controls and trauma patients in emergency departments (35–38). Imbalances in ORP have been shown to accurately reflect cellular damage resulting in progression of acute traumatic injuries and chronic diseases (16–19).

In this study, we analyzed the semen parameters according to 2010 WHO guidelines in the group of healthy controls and infertile patients. We noted significantly higher semen volume in infertile group, whereas sperm motility and morphology were significantly reduced in these men compared with the control group ($P < .01$). We also presented the subjects based on normal ($\geq 40\%$) versus abnormal ($<40\%$) motility, which revealed significantly lower values in most of the sperm parameters, such as concentration, total sperm count, motility, and morphology in the abnormal motility group compared with the normal motility group ($P < .05$). The results of this study also indicate very strong correlation of sORP and cORP with respect to the type of sample (semen or seminal plasma) as well as time. Based on these findings, it can be suggested that ORP can be measured accurately both in semen as well as in seminal plasma either immediately after liquefaction or up to 120 minutes.

Correlation studies of ORP levels with sperm parameters in the groups of healthy controls and infertile patients indicated that semen sORP has a strong negative correlation with concentration, total sperm count, and motility in infertile patients, whereas seminal plasma sORP correlated negatively with concentration and total sperm count in these men. The association of ORP with poor sperm parameters, particularly concentration and total sperm count both in healthy and infertile men, suggests the potential beneficial effect of antioxidant supplementation in these men. In the group of healthy controls, both semen and seminal plasma sORP correlated negatively with concentration and total sperm count. These correlations remained unchanged at 120 minutes of measurement. This suggests that the sORP can be measured accurately in infertile patients as well as in healthy controls irrespective of sample type (semen or seminal plasma) and time of testing up to 120 minutes of liquefaction.

Sperm motility is an important parameter in the laboratory evaluation of male factor infertility. Based on the normal and abnormal motility as defined by WHO guidelines (20), we have demonstrated higher sORP in subjects with poor motility. Furthermore, in this abnormal motility group, semen parameters (concentration, total sperm count, and morphology) were significantly poor. We therefore generated the ROC curves based on motility for both semen and seminal plasma. In the semen, at a cutoff of 1.48 for sORP, the specificity was 75% and sensitivity 60%, whereas in seminal plasma, the sORP cutoff of 2.09 showed a lower sensitivity of 46.7% and a higher specificity of 81.80%. This indicates that all samples with poor motility ($<40\%$) can be identified by the sORP values with high accuracy. In the infertile men, we observed a greater range in the sORP values as well as an overlap in the sORP values in both groups. This may

explain the low sensitivity. Including a more homogeneous control group (men of proven fertility only) and generating a ROC curve with higher sensitivity and specificity may further help predict motility and distinguish men with good semen quality from those with poor semen quality.

In an unpublished study, we compared ROS formation as measured by chemiluminescence assay with the ORP measured by the MiOXSYS System. There was no correlation between the two assays, mainly because the two measure completely different end products. While the chemiluminescence assay measures the initial amount of ROS present in a given sample, that is, the total oxidants, it does not provide any information on the amount of antioxidants or reductants present in the sample. On the other hand, the MiOXSYS System provides information on the sum total of the oxidants present and the available reductants in a sample—hence, the lack of correlation. The MiOXSYS System therefore provides a complete picture of the redox potential in a given sample.

In this study, while measuring the ORP, we overcame one of the main limitations of measuring oxidative stress, which is the use of the chemiluminescence assay for measuring ROS and the antioxidant assay for the total antioxidant activity. Although ROS measurement by the chemiluminescence assay with multiple tube luminometer is well established as a diagnostic test for screening patients with known as well as with idiopathic or unknown infertility (15), it has some limitations when compared with the measurement of redox potential using the MiOXSYS System such as [1] cost of the luminometer (ranges from \$30K), which is a deterrent for this assay being adopted for routine use; [2] sample size, which requires a minimum of 400 µL for ROS measurement, making it difficult to use in patients with low semen volume; [3] measurements, which are time sensitive and should be completed within an hour of semen collection; [4] the assay, which takes approximately 45 minutes to complete using semen samples; and [5] the fact that the assay cannot measure ROS in frozen samples.

On the other hand, with the MiOXSYS analyzer, [1] the cost is mainly related to the cost of sensor strip (ranging from \$35 to \$50), [2] the analyzer is easy to use and does not include any elaborate software or multiple steps in deriving the final results, [3] the analyzer requires a very small volume of sample about 30 µL, [4] ORP can be measured in fresh and frozen semen and seminal plasma (our unpublished work), and, lastly, [5] ORP measurements are stable for up to 120 minutes. That the ORP can be measured up to 120 minutes is important, indicating that the redox potential is not altered significantly up to this point unlike the chemiluminescence measurement, where the ROS levels are continually measured and have a very short half-life.

There were some limitations to our study: [1] While we assessed the ORP in infertile men and healthy controls, we did not classify the infertile patients based on clinical diagnoses; and [2] the study population was composed of a limited number of men with proven fertility and hence not presented as separate group. Including a group of men with proven fertility may further improve our cutoff values for sORP and also increase the sensitivity of the assay. Men who may benefit from the measurement of ORP include infertile men with varicocele, infection, spinal cord injury, severe oligozoosper-

mia, asthenozoospermia and teratozoospermia, and unexplained and idiopathic infertility.

In conclusion, our study presents some novel findings. First, the MiOXSYS System can measure ORP reliably in both semen and seminal plasma up to 120 minutes of liquefaction, demonstrating its clear advantage over existing tests of measuring individual markers of oxidative stress. Second, this makes it easier to employ this new technology for clinical use. Establishing a reference value derived from larger cohort of men in independent fertility centers is necessary for the potential use of MiOXSYS system as a diagnostic tool.

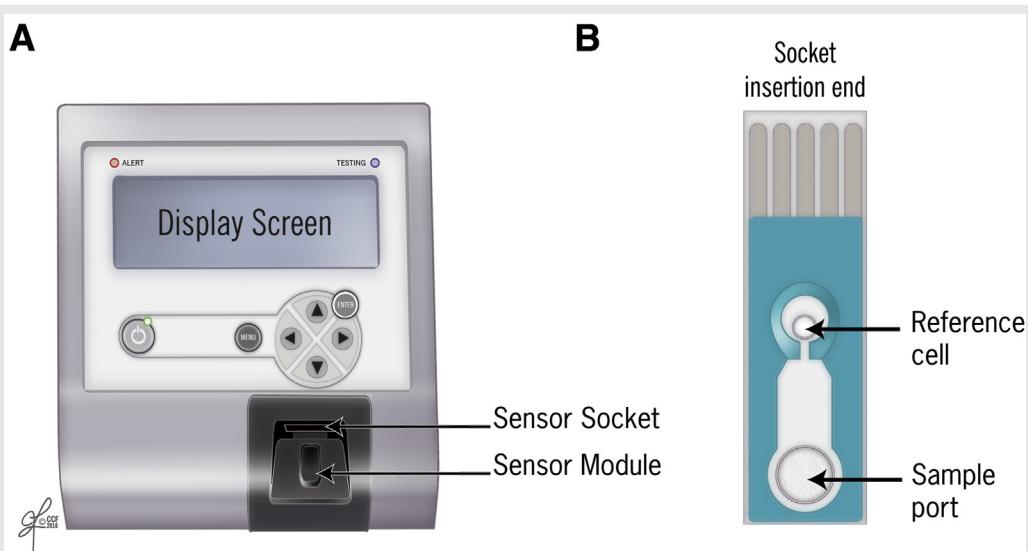
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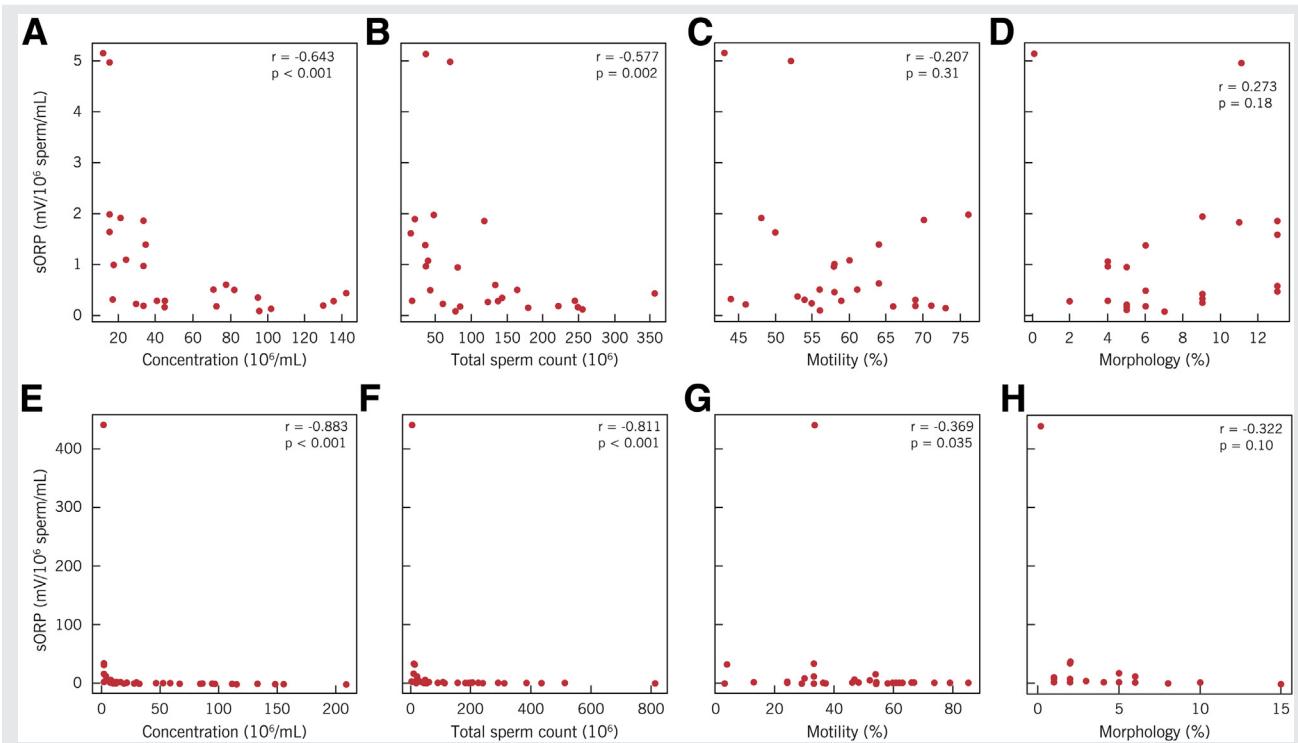
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SUPPLEMENTAL FIGURE 1



The MiOXSYS System. This device measures ORP using only a galvanostatic MiOXSYS analyzer (A) and disposable sensor strips (B).
Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

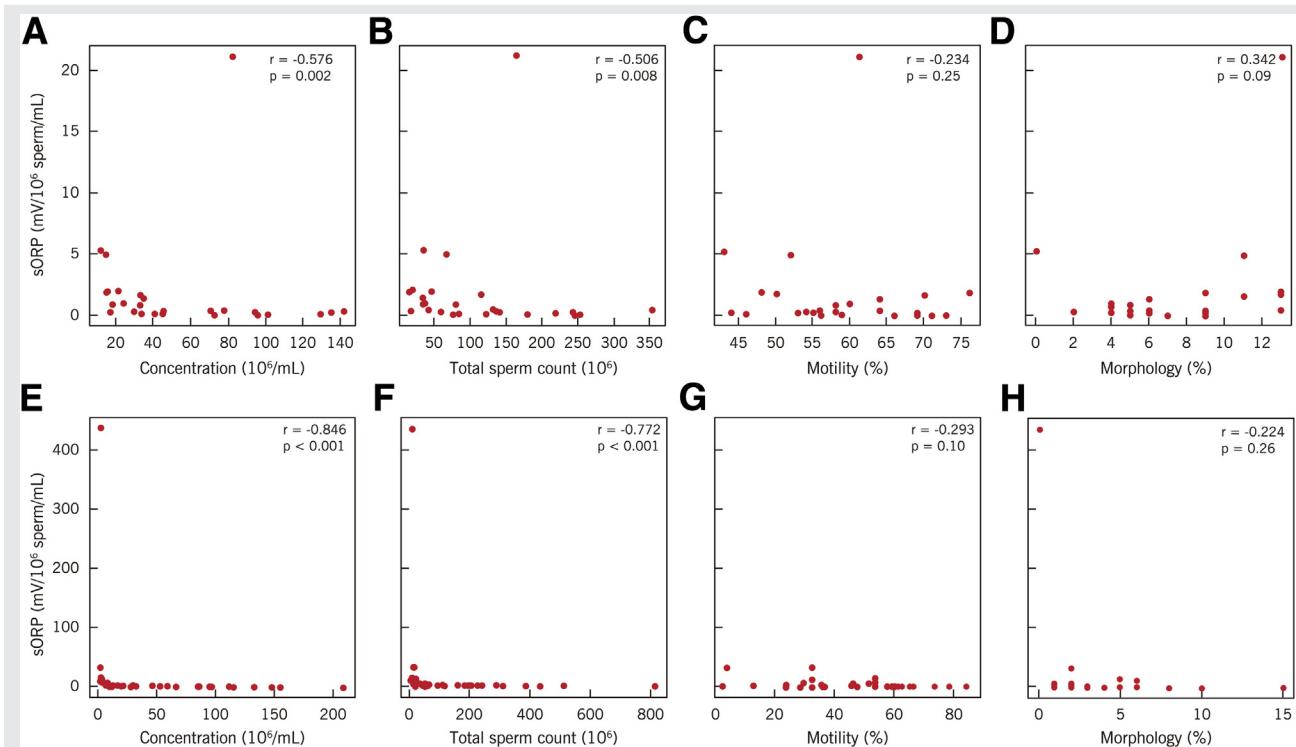
SUPPLEMENTAL FIGURE 2



Correlation of semen sORP with sperm parameters at time 0 in healthy controls (**A**, concentration; **B**, total sperm count; **C**, motility; **D**, morphology) and infertile patients (**E**, concentration; **F**, total sperm count; **G**, motility; **H**, morphology).

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

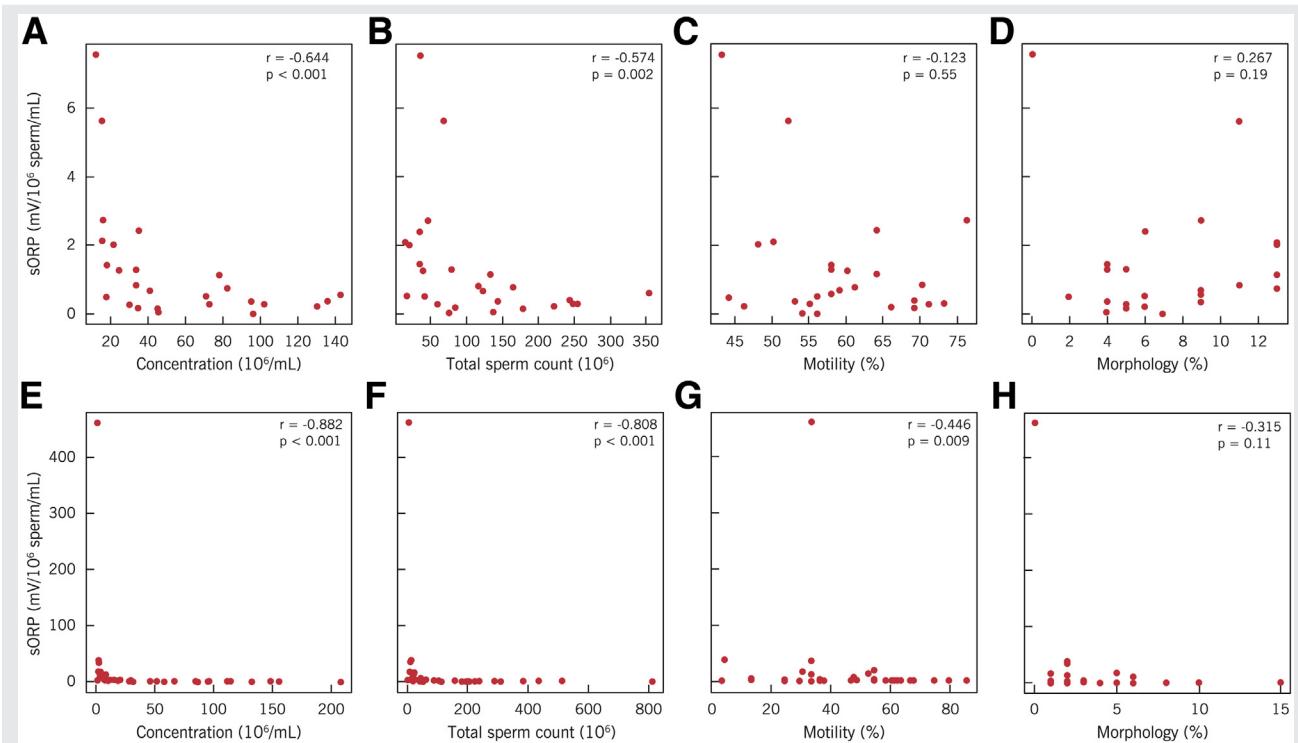
SUPPLEMENTAL FIGURE 3



Correlation of seminal plasma sORP with sperm parameters at time 0 in healthy controls (**A**, concentration; **B**, total sperm count; **C**, motility; **D**, morphology) and infertile patients (**E**, concentration; **F**, total sperm count; **G**, motility; **H**, morphology).

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

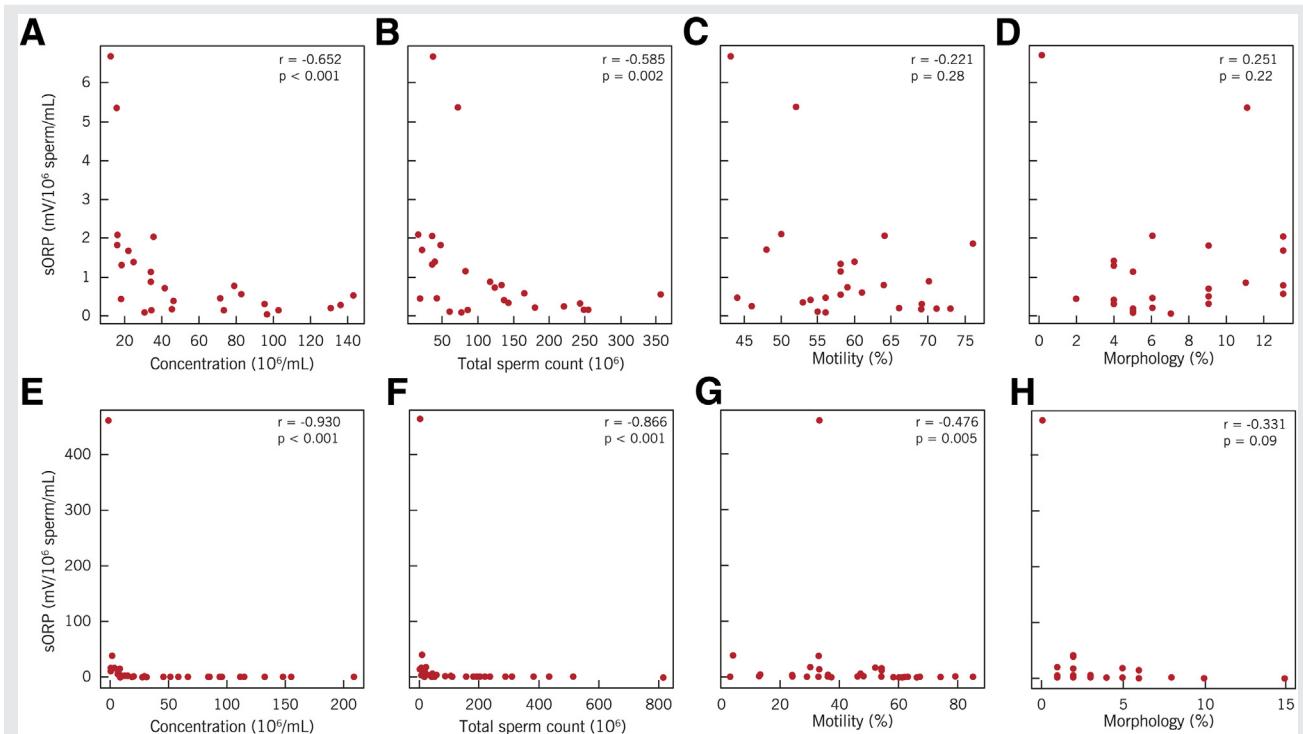
SUPPLEMENTAL FIGURE 4



Correlation of semen sORP with sperm parameters at time 120 in healthy controls (**A**, concentration; **B**, total sperm count; **C**, motility; **D**, morphology) and infertile patients (**E**, concentration; **F**, total sperm count; **G**, motility; **H**, morphology).

Agarwal. Oxidation reduction potential in seminal ejaculates. Fertil Steril 2016.

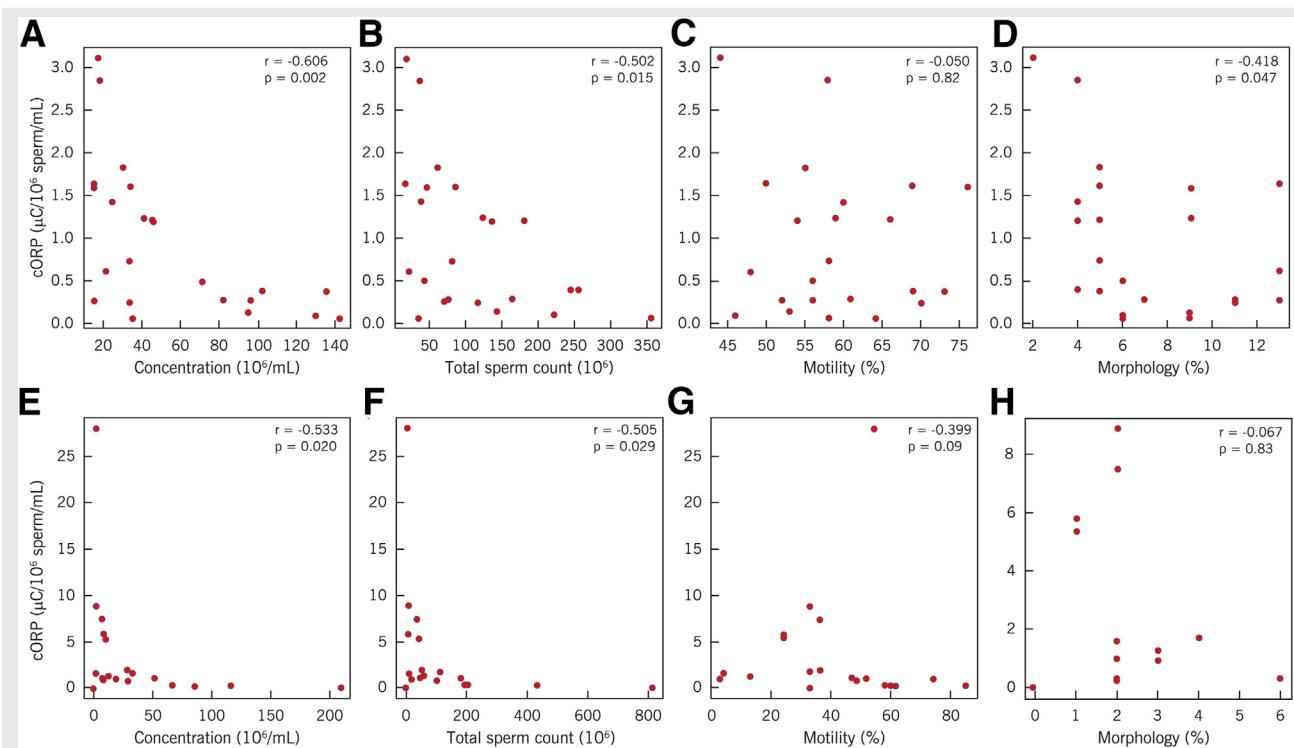
SUPPLEMENTAL FIGURE 5



Correlation of seminal plasma sORP with sperm parameters at time 120 in healthy controls (**A**, concentration; **B**, total sperm count; **C**, motility; **D**, morphology) and infertile patients (**E**, concentration; **F**, total sperm count; **G**, motility; **H**, morphology).

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

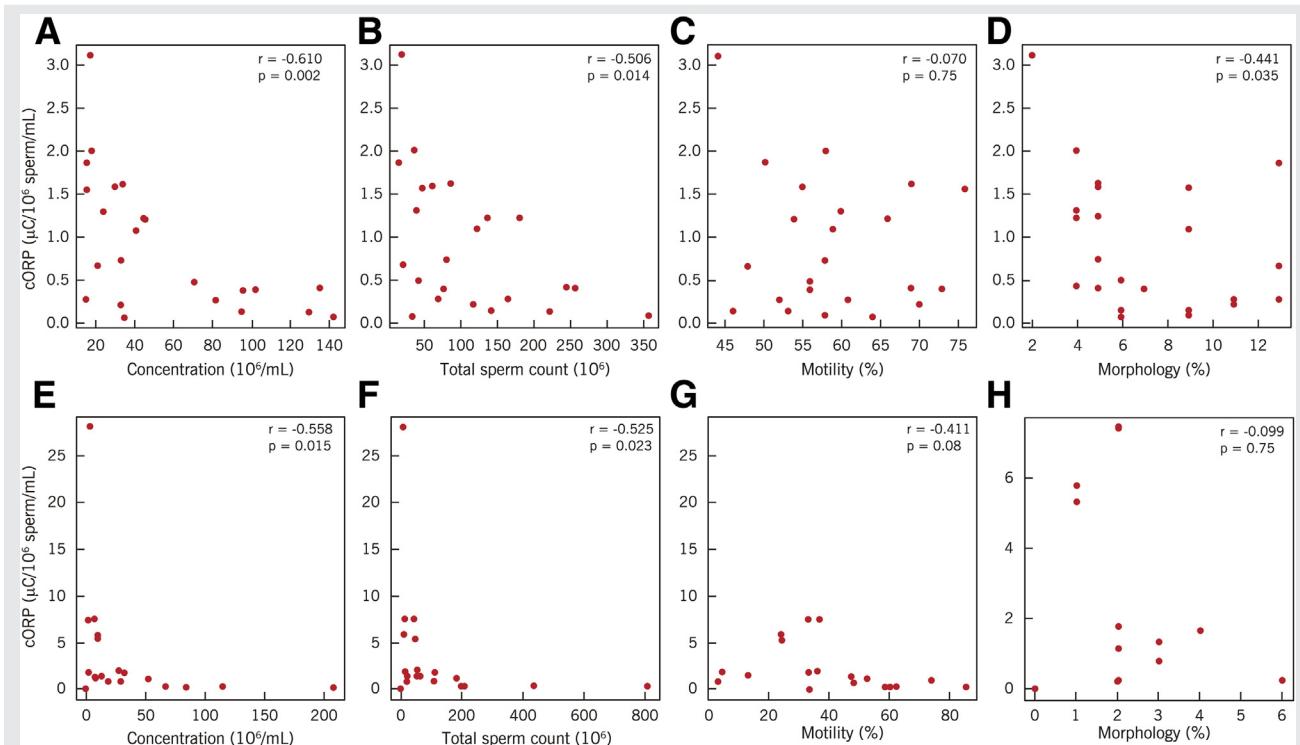
SUPPLEMENTAL FIGURE 6



Correlation of semen cORP with sperm parameters in healthy controls (A, concentration; B, total sperm count; C, motility; D, morphology) and infertile patients (E, concentration; F, total sperm count; G, motility; H, morphology).

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

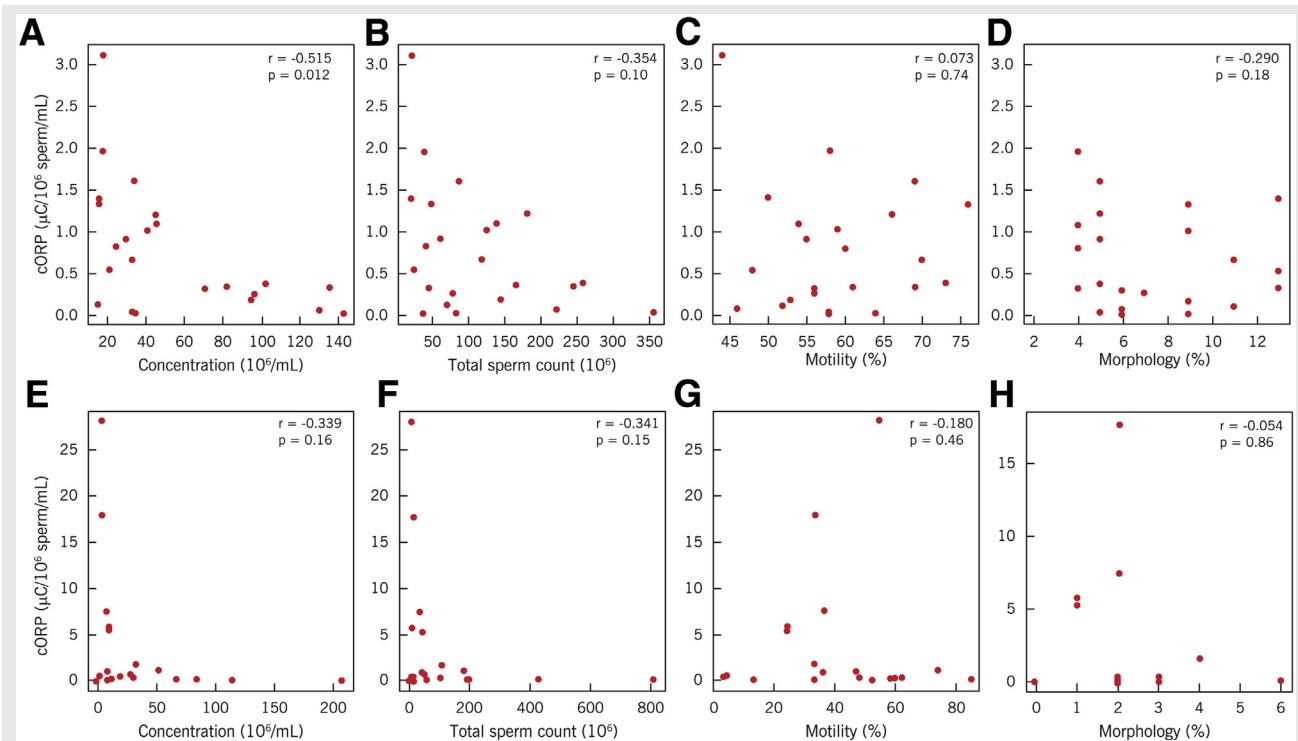
SUPPLEMENTAL FIGURE 7



Correlation of seminal plasma cORP with sperm parameters at time 0 in healthy controls (**A**, concentration; **B**, total sperm count; **C**, motility; **D**, morphology) and infertile patients (**E**, concentration; **F**, total sperm count; **G**, motility; **H**, morphology).

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

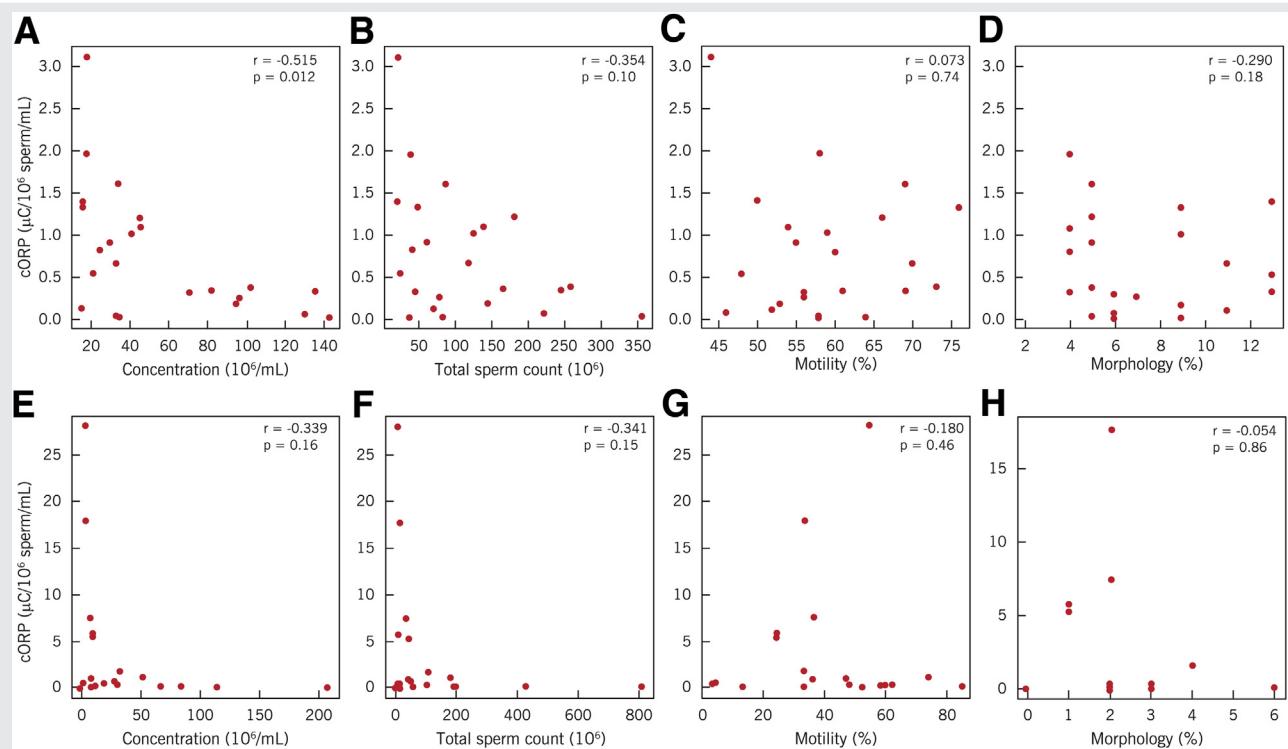
SUPPLEMENTAL FIGURE 8



Correlation of semen cORP with sperm parameters at time 120 in healthy controls (A, concentration; B, total sperm count; C, motility; D, morphology) and infertile patients (E, concentration; F, total sperm count; G, motility; H, morphology).

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

SUPPLEMENTAL FIGURE 9



Correlation of seminal plasma cORP with sperm parameters at time 120 in healthy controls (**A**, concentration; **B**, total sperm count; **C**, motility; **D**, morphology) and infertile patients (**E**, concentration; **F**, total sperm count; **G**, motility; **H**, morphology).

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.

SUPPLEMENTAL TABLE 1

sORP ($\text{mV}/10^6 \text{ sperm/mL}$) and cORP ($\mu\text{C}/10^6 \text{ sperm/mL}$) levels as measured in semen and seminal plasma of healthy and infertile men over time.

Parameter	Time 0			Time 120		
	Healthy controls	Infertile patients	P value	Healthy controls	Infertile patients	P value
sORP in semen	1.04 ± 0.26 0.49 (0.25, 1.32)	17.39 ± 13.33 0.74 (0.41, 2.89)	.10	1.29 ± 0.34 0.64 (0.28, 1.40)	19.09 ± 13.93 1.12 (0.49, 3.70)	.06
sORP in seminal plasma	1.82 ± 0.82 0.45 (0.25, 1.63)	17.26 ± 13.16 0.69 (0.37, 5.11)	.11	1.17 ± 0.31 0.58 (0.26, 1.38)	19.57 ± 13.84 0.97 (0.49, 5.94)	.036
cORP in semen	0.94 ± 0.18 0.61 (0.27, 1.50)	3.58 ± 1.49 1.08 (0.56, 3.64)	.13	0.79 ± 0.16 0.55 (0.17, 1.18)	2.99 ± 1.48 0.53 (0.23, 3.10)	.6
cORP in seminal plasma	0.90 ± 0.16 0.66 (0.26, 1.42)	3.53 ± 1.47 1.27 (0.55, 3.64)	.08	0.77 ± 0.16 0.54 (0.23, 1.15)	3.73 ± 1.68 0.41 (0.20, 3.52)	.68

Note: Values are mean ± SEM, median (25th, 75th percentiles); P<.05 was considered statistically significant by Wilcoxon rank sum test using pairwise group comparisons.

Agarwal. Oxidation reduction potential in seminal ejaculates. *Fertil Steril* 2016.