Diagnostic application of oxidation-reduction potential assay for measurement of oxidative stress: clinical utility in male factor infertility

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KEY MESSAGE
We report a standardized protocol for measuring oxidative stress (as oxidative reduction potential; ORP) in semen using the MiOXSYS System. A reference value of 1.36 mV/10^6 sperm/ml ORP in semen could distinguish normal men (controls) from male factor infertility patients. Our results confirm the reproducibility of the test for use in a clinical setting.

ABSTRACT
The objectives of this study were to: (i) describe a protocol measuring the oxidation-reduction potential (ORP) by MiOXSYS System as an alternative method of seminal oxidative stress (OS) testing; (ii) establish a reference value for static ORP (sORP) to distinguish between controls and male factor infertility patients; (iii) evaluate intra-observer and inter-observer reliability; and (iv) examine association of sORP with sperm parameters predictive of male factor infertility. Elevated levels of sORP were seen in infertile patients (6.22 ± 1.10 mV/10^6 sperm/ml) compared with controls (1.59 ± 0.29 mV/10^6 sperm/ml) (P = 0.004). A sORP cut-off value 1.36 mV/10^6 sperm/ml identified normal semen and abnormal semen quality with a sensitivity 69.6%, specificity 83.1%, positive predictive value 85.3% and negative predictive value 65.9%. The test demonstrated strong intra-observer (CV 8.39%) and inter-observer reliability (correlations >0.97). Higher sORP levels were associated with poor sperm parameters across the fertility status of subjects. Negative correlations were noted with sperm parameters (concentration, total sperm count, motility and morphology) indicating these male infertility parameters are related to OS. In conclusion, the introduction of ORP as a novel clinical test for assessment of OS will help clinicians to better diagnose and manage male factor infertility patients.

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Introduction

Male infertility is a relatively common medical condition affecting up to 12% of men globally [Agarwal et al., 2015a]. The Centers for Disease Control and Prevention (CDC) report a 9.4% infertility rate among men in the USA [Martinez et al., 2012]. Male partners are found to be solely responsible for 20–30% of infertility cases and contribute to roughly 50% of cases overall [Agarwal et al., 2015b]. Many clinicians rely on conventional semen analyses as a surrogate measure of a man’s ability to father a child [Bjornsdal et al., 2016; Catanzariti et al., 2013; Esteves, 2014]. However, this approach seems to be an oversimplification of the assessment of male fertility potential due to large intra- and inter-individual variations in conventional semen parameters and is unable to precisely predict the likelihood of pregnancy. Advanced tests of sperm function have been proposed as alternative methods that can enhance the diagnostic accuracy of male infertility particularly in cases of unexplained infertility, one or more abnormal semen parameters, recurrent pregnancy loss or failure of intrauterine insemination [American Urological Association, 2012].

Oxidative stress (OS) resulting from a number of endogenous and exogenous stressors is believed to play a central role in the pathogenesis of male infertility [Agarwal et al., 2014a, 2014b, 2014c, 2014d, 2014e, 2015a; Benedetti et al., 2012; Gvozdjakova et al., 2015; Hosen et al., 2015; Ko et al., 2014; Roychoudhury et al., 2016; Zorn et al., 2003]. Reactive oxygen species (ROS) are oxygen-based, chemically reactive molecules that are kept at an equilibrium with antioxidants in a system called the redox system [Agarwal et al., 2012; Sharma and Agarwal, 1996]. A delicate balance in the redox system is required for essential physiologic functions of the spermatozoa such as chromatin compaction in maturing spermatozoa, capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion [de Lamirande and Gagnon, 1993; Du Plessis et al., 2015; Guthrie and Welch, 2012; Kothari et al., 2010; Wright et al., 2014].

When excessive amounts of ROS are produced, or when antioxidant activity fails, this redox system is disrupted, resulting in a state of OS, which can result in lipid peroxidation, DNA damage and aggravated apoptosis. Studies have shown that infertile men are more likely to have higher concentrations of ROS [de Lamirande and Gagnon, 1995] and lower concentrations of antioxidants in their seminal plasma [Roychoudhury et al., 2016; Sharma et al., 1999]. The World Health Organization (WHO) has also acknowledged OS as an important parameter that plays a significant role in male infertility, and thus its assessment and management are critical for patient care [World Health Organization, 2010].

Currently available assays for OS measure only known or a discrete quantity of oxidants [ROS by chemiluminescence assay], antioxidants (total antioxidant capacity [TAC] assay) or post-hoc damage (MDA assay) [Agarwal et al., 2014a, 2014b; Moazamian et al., 2015; Roychoudhury et al., 2016; Svobodova et al., 2009]. Such tests are also tedious, time consuming and require special technical skills and large sample volumes. Since OS describes a state of the redox system in which the activity of the oxidants exceeds the capabilities of the antioxidants to quench them [McCord, 2000], a measure that comprises all known and unknown oxidants and all antioxidant activity in a semen sample would be the best indicator to describe its role in male infertility.

Oxidation-reduction potential (ORP) is a measure of the relationship between oxidants and antioxidants that provides a comprehensive measure of the redox system and thus of OS. After major traumas [Rael et al., 2007, 2009a, 2009b] or strenuous exercise in athletes [Stagos et al., 2015], elevated ORP levels in blood plasma are correlated with inflammation and injury severity. Blood ORP levels are also correlated with organ dysfunction, particularly liver toxicity [Bar-Or et al., 2009]. The ORP test is novel in the area of infertility. Recently, a novel technology based on a galvanostatic measure of electrons – the MiOXSYS System – has been developed that easily and readily measures ORP in semen [Agarwal et al., 2016a, 2016b, 2016c].

The goals of this study were to: (a) describe a protocol for measuring ORP using the MiOXSYS System as an alternative method of seminal OS testing; (b) establish a reference value for static ORP (sORP) to distinguish between controls and male factor infertility patients; (c) evaluate the intra-observer and inter-observer reliability of the test; and (d) examine the association of sORP with sperm parameters predictive of male factor infertility.

Materials and methods

Subjects

In this prospective case-control study, semen samples were obtained from 51 healthy controls with proven and unproven fertility and 106 infertile patients between August 2015 and March 2016. The control group was composed of 51 healthy men based only on the normal semen parameters according to the WHO 5th edition guidelines [World Health Organization, 2010]. Participants with proven (n = 15) and unproven fertility (n = 36) were included in this group. They were recruited from our existing pool of healthy donors. Enrolment criteria were absence of any comorbid medical condition, absence of genitourinary disease [such as history of testicular injury or infection, undescended testes, sexually transmitted disease, varicocele and vasal reconstruction] and absence of occupational and habitual activities associated with a higher OS potential [such as smoking, drinking more than two alcoholic beverages per week, and exposure to excessive heat, radiation and chemicals such as bleach, benzene and pesticides].

The infertile group was composed of patients presenting to the male infertility unit complaining of primary or secondary infertility. The exclusion criteria for both patients and controls were: presence of azoospermia on semen analysis, evidence of obstructive pathology or ejaculatory dysfunction suggested by a low semen volume with/without an acidic pH and incomplete semen collection.

The study population was divided according to the results of the semen analysis into an abnormal semen group and a normal semen group. The abnormal semen group had a semen volume <1.5 ml, and/or sperm concentration <15 × 10⁶ sperm/ml, and/or total sperm count <39 × 10⁹ sperm, and/or total motility <40%, and/or normal morphology <4%. In the normal semen group, these parameters fell within the 2010 WHO normal reference ranges.

Semen analysis

Semen specimens were collected by masturbation after 48 to 72 h of sexual abstinence, and the sperm parameters were analysed after complete liquefaction at 37°C for 20 min. Each sample was evaluated for both macroscopic parameters such as colour, pH, ejaculate volume, semen age (from collection to analysis) 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 x 10⁶/ml, the aliquot was examined for the presence of white blood cells using the Endtz or the peroxidase test (World Health Organization, 2010). Air dried smears were prepared for morphological evaluation; 4% normal morphology was used as a cut-off.

### Measurement of oxidation-reduction potential

Oxidation reduction potential was measured using novel galvanostat-based technology (MiOXSYS™ System; Aytu Bioscience, Englewood, CO, USA). Briefly, a disposable MiOXSYS sensor is inserted into the MiOXSYS Analyzer. A 30 μL sample suspension is loaded on the sample port of the sensor. The test starts when the sample fills the reference electrode, thereby completing the electrochemical circuit. The test started when the sample filled the reference electrode; thereby completing the electrochemical circuit. After a short period, the sORP values, in millivolts (mV), were displayed on the screen (Agarwal et al., 2016a). The MiOXSYS System measures sORP, which is a ‘snapshot’ of the current balance of the redox system. A higher sORP level indicates an imbalance in the activity of all available oxidants relative to all available antioxidants in the seminal ejaculate – a state of OS.

### Intra-observer reliability

Three semen samples were measured three times by three experienced observers using the same analyser for all measures. The reliability of an individual observer was assessed by calculating the coefficient of variation (%CV) for each sample, where the mean sORP and standard deviation (SD) for each of the three samples were calculated from the three replicates. The %CV for each sample was then generated. The average %CV across all three samples within an individual observer was calculated as an indicator of individual intra-observer reliability. Overall intra-observer reliability was determined by the average %CV of all three observers.

### Inter-observer reliability

Ten semen samples were measured four times (replicates A–D) by three experienced observers (Observers 1–3) for each sample in order to calculate the extent to which there was agreement between observers. This was determined by the difference between observer means, and correlations among observers. ANOVA was used to verify whether the average sORP [mV/10⁶ sperm/ml] for each observer was significantly different from any other observer (P < 0.05). Pearson’s r correlations between observers were used to verify whether the sORP [mV/10⁶ sperm/ml] values for each sample were consistent across observers. The %CV was calculated from the mean sORP [mV/10⁶ sperm/ml] and SD for each observer.

### Statistical methods

Comparisons of groups were performed using Fisher’s exact test or chi-squared test for categorical variables such as frequency distribution of sperm parameters, Wilcoxon’s rank sum test was used for group comparisons with respect to quantitative variables such as volume, sperm concentration, total sperm count, percentage motility, sperm morphology and sORP [mV/10⁶ sperm/ml] between the groups. Relationships between sORP and sperm parameters were determined using Spearman’s correlation. A receiver operating characteristic (ROC) curve was used to identify the sORP [mV/10⁶ sperm/ml] criterion (cut-off, sensitivity, and specificity, positive predictive value, negative predictive value, accuracy and area under curve [AUC]) that best predicted the normal and abnormal semen parameters as well as differentiated normal healthy controls from male factor infertility patients. Tests were performed at a significance level of P < 0.05. Boxplots were used to demonstrate the distributions of sORP [mV/10⁶ sperm/ml] variants within different groups using the median, quartiles, and the minimum and the maximum ranges. Results are reported as mean ± standard error, and tests were performed at a significance level P < 0.05. Analyses were performed using R version 2.15.1 [www.r-project.org].

### Results

Background information on the study population is presented in Table 1 along with a comparison of semen parameters between the control and infertile groups. The participants in the control group were selected on the basis of normal semen parameters according to the WHO 5th edition guidelines. Concentration, total sperm count, motility and morphology were significantly lower in the infertile men than in the control group (P < 0.001). None of the participants in the control group was positive for the Endtz test, whereas in the patient group, 26% of the subjects had Endtz >0. The mean sORP [mV/10⁶ sperm/ml] in the

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls (n = 51)</th>
<th>Patients (n = 106)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>2.59 ± 0.17</td>
<td>3.06 ± 0.17</td>
<td>NS</td>
</tr>
<tr>
<td>Concentration (×10⁴ sperm/ml)</td>
<td>55.19 ± 5.15</td>
<td>39.02 ± 4.45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total sperm count (×10⁶ sperm)</td>
<td>133.42 ± 14.24</td>
<td>109.56 ± 14.14</td>
<td>0.001</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>58.9 ± 1.39</td>
<td>42.6 ± 1.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leukocytospermia</td>
<td>0 ± 0</td>
<td>1.4 ± 1.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Morphology (%)</td>
<td>7.39 ± 0.46</td>
<td>4.00 ± 0.31</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; P < 0.05 was considered statistically significant by Wilcoxon rank sum test for pairwise group comparisons. NS = not statistically significant.
semen of the infertile patients was $6.22 \pm 1.10$ mV/10$^6$ sperm/ml whereas that of the control group was $1.59 \pm 0.29$ mV/10$^6$ sperm/ml ($P = 0.004$). Out of the 157 samples, 65 were found to have normal semen parameters and 92 were found to have abnormal semen parameters. ROC curve analysis of the sORP (mV/10$^6$ sperm/ml) test results predicting normal versus abnormal semen quality values was performed to calculate test sensitivity, specificity, and positive and negative predictive values. A cut-off value of 1.36 mV/10$^6$ sperm/ml sORP in semen could facilitate better diagnosis of OS in patients with male factor infertility (Figure 1). At this cut-off point, the sensitivity was 69.6%, specificity 83.1%, positive predictive value 85.3% and negative predictive value 65.9%. The accuracy of the test was 75.2% (area under the curve [AUC] = 0.770).

The distribution of subjects in the control and infertile groups above or below the established cut-off value of 1.36 mV/10$^6$ sperm/ml is depicted in Figure 2. The median sORP (mV/10$^6$ sperm/ml) level was below the established cut-off value of $1.36$ mV/10$^6$ sperm/ml in the control group, whereas it was above this cut-off in the group of infertile patients ($P = 0.004$). In men undergoing evaluation for male factor infertility ($n = 106$), 44.3% (47/106) presented with sperm concentration <15 x 10$^6$/ml in the seminal ejaculates. The median sORP (mV/10$^6$ sperm/ml) values for the patient group were higher than that of the control group in all of the subgroups but especially so when the infertile subgroups were compared with only the proven fertility group. The distribution of sORP (mV/10$^6$ sperm/ml) within different subgroups of controls and infertile patients was also assessed and is presented in Figure 3. Within the control group, the sORP (mV/10$^6$ sperm/ml) levels were similar between the men with proven fertility and those with unproven fertility. Compared with the control group,
reliability was based on 27 sORP measurements made by three experienced observers. The %CV for the three observers tested were: Observer 1 = 7.98%, Observer 2 = 5.72% and Observer 3 = 11.96%, with an average %CV of 8.39%, suggesting a strong level of intra-observer reliability. On average, the difference between any two replicates for any one observer was less than 0.1 mV/10^6 sperm/ml (Figure 4A).

For assessing the inter-observer reliability, a total of 120 measurements of sORP (mV/10^6 sperm/ml) were taken by three experienced observers from 10 semen samples measured in four replicates. Overall, there was no significant difference between the sORP (mV/10^6 sperm/ml) values obtained across observers [F(2,62) = 1.22; Figure 4B]. Correlations between observers exceeded 0.97 in all cases. The %CV across observers was 3.61%. Based on these three indicators, there was high agreement in sORP (mV/10^6 sperm/ml) values obtained between observers for the same samples, indicating a high inter-observer reliability.

The association of sORP (mV/10^6 sperm/ml) with semen variables was examined in order to establish the ability of the ORP test to predict the diagnosis of OS. The correlation of sORP with overall sperm parameters is presented in Figure 5A–D. A strong negative correlation of sORP (mV/10^6 sperm/ml) was seen with all major parameters: concentration \( r = -0.823; P < 0.001 \), total sperm count \( r = -0.728; P < 0.001 \), motility \( r = -0.485; P < 0.001 \), and morphology \( r = -0.238; P < 0.001 \). The correlation of sORP levels (mV/10^6 sperm/ml) in controls and patients is given in Figures 6 and 7 (A–D).

Discussion

Oxidation-reduction potential in biological systems has been described as an integrated measure of the balance between total oxidants [i.e. oxidized thiols, superoxide radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide, peroxynitrite and transition metal ions] and total reductants [i.e. free thiols, ascorbate, α-tocopherol, β-carotene and uric acid]. It is a measure of the transfer of electrons from a reductant (or antioxidant) to an oxidant (McCord, 2000). The MiOXSYS system provides an estimation of the static oxidation-reduction potential (sORP). It represents the integrated measure of the existing balance between total oxidants and reductants in a biological system as described by us in our recent publication (Agarwal et al., 2016).
In the field of infertility, where OS plays an important role, the measure of sORP as an indicator of semen quality is novel. The MiOXSYS System uses a small amount (30 \( \mu l \)) of semen and provides results in real time (less than 5 min). Equipment standardization and validation controls available as internal controls further facilitate easy handling and operation. Measurement of sORP potentially offers the andrology and IVF laboratories that are routinely involved in the diagnosis of male factor infertility with an alternative to current measures of OS. ORP test is able to assess OS directly in semen samples, thus potentially making it a better indicator of OS in male infertility than currently used single markers such as ROS, TAC or MDA. This test also overcomes many of the limitations of these other assays, which are tedious and time consuming, require special technical skills, and need large sample volumes (Agarwal et al., 2016a).

No correlation was seen between sperm concentration and sORP values if the sORP was not normalized, which further emphasizes the importance of normalization in biological samples such as seminal ejaculate. It is important to normalize the absolute values of sORP with the sperm concentration for a number of reasons. sORP is based not only on the number of spermatozoa but also on the quality of spermatozoa. Spermatozoa are the principal source of ROS production in semen (Fisher and Aitken, 1997), which is mostly caused by NADPH oxidase – an enzyme complex that is contained in the cell membrane – and the mitochondria, which leak electrons from the respiratory chain. Spermatozoa with abnormal morphology, mainly those with cytoplasmic residue (which indicates their immaturity and reduced fertility potential), produce higher amounts of ROS than spermatozoa with normal structure (Gomez et al., 1996).

Spermatozoa are deficient in enzymatic antioxidants due to a lack of cytoplasm. However, they do possess small molecules [e.g. glutathione and cysteine] and protein-based buffers [e.g. thioredoxin] that help them regulate ambient redox potentials in the various intracellular compartments, influence the status of redox-sensitive macromolecules and protect against OS (O’Flaherty, 2014a, 2014b).

Pluschkell and Flickinger (1996) demonstrated that the redox potential of the culture medium is directly proportional to the number of viable cells (Pluschkell and Flickinger, 1996). Human semen is a live cell suspension and is equivalent to cells in culture medium where the physiological status of the cell affects the ORP of the extracellular medium (Jones, 2006). Thus it is not merely the concentration of the spermatozoa but the quality of spermatozoa (morphologically immature, abnormal with poor motility) that regulates the availability of the antioxidants and ultimately the ORP of the extracellular fluid, i.e. seminal plasma. Thus, two samples with an equal number of spermatozoa at different physiological states (e.g. under heat stress and ambient temperature) and oxidative stress will have a different ORP.
ORP is a measure of the dynamic exchange between the sperms’ release of oxidants and the local environment’s (seminal plasma) capacity to modulate the effects of those oxidants via its antioxidant content. More spermatozoa will inherently produce more oxidants, but the unknown here is the highly individualized antioxidant content of the seminal plasma. Because of this unknown, it is important to normalize against sperm concentration so that different semen samples from different donors, infertile men, aetiological conditions and geographical locations (countries) can be compared directly. Because we are interested in levels of OS, which is the ratio of oxidants to antioxidants, measuring the antioxidant concentration alone will only provide one small part of a larger picture. We have recently shown in our proteomic studies that high concentrations of ROS and the resulting oxidative stress in semen samples (i.e. both spermatozoa and seminal plasma) alter the differential expression of proteins that are involved in energy metabolism, sperm motility, acrosome reaction, apoptosis and other important functions of the spermatozoa (Agarwal et al., 2016; Ayaz et al., 2015).

In addition to the operator, other variables that may seem to be of less importance may also influence the final test result[s] e.g. the MiOXSYS sensor, viscosity of the sample, centrifugation speed and liquefaction time. Therefore, it is imperative that a detailed quality control is in place to provide reproducible results. Establishing inter- and intra-observer and inter- and intra-assay variability are standard quality control measures that must be performed. This is important in order to validate any given new test/instrument irrespective of its ease of use and simplicity. This is especially important when a system is used in a clinical setting to report patient results. Seminal ejaculate shows a significant variation in sperm concentration and absolute value does not provide a clear and accurate picture of the cellular contribution of spermatozoa to OS – in this case to sORP. A sORP cut-off value of 1.36 mV/10^6 sperm/ml was capable of predicting abnormality in semen quality with a sensitivity of 69.6%, a specificity of 83.1%, positive predictive value of 85.3% and a negative predictive value of 65.9%. The accuracy of the test was 75.2% (AUC = 0.770). For any diagnostic test to be considered valid for clinical use, it should combine high sensitivity, specificity, accuracy and positive and negative predictive values. Provided a given test has a high sensitivity, it will correctly identify subjects with the condition of interest, i.e. infertility as in this case. In contrast, highly specific tests correctly identify subjects who do not have this condition of interest. Although predictive values are influenced by sensitivity and specificity, they depend on the prevalence of the condition of interest in the general population. For instance, if the prevalence of infertility among young men is low, the positive predictive value will be low despite a high sensitivity and specificity. If prevalence of the given con-

Figure 6 – Correlation of static oxidation-reduction potential (sORP) with sperm parameters in normal healthy controls (A: concentration, B: total sperm count, C: motility, D: morphology).
dition is high, the positive predictive value will be high if a highly specific test cut-off point is chosen. Therefore, given the high sensitivity and predictive value of our preferred seminal sORP (mV/10^6 sperm/ml) cut-off of 1.36 mV/10^6 sperm/ml, we believe that ORP testing will be clinically useful in identifying OS in men at risk for infertility that would otherwise go undetected with a routine semen analysis. Furthermore, ORP represents an easier and more complete measure of OS in comparison to ROS, TAC, and MDA assays.

All participants in the control group from this study – those with proven or unproven fertility – were enrolled based on the WHO 5th edition criteria of normal sperm concentration, motility and morphology. Participants in the patient group were enrolled irrespective of their sperm concentration unless they were azoospermic or had extremely low sperm concentration (<1 × 10^6/mL). Oxidative stress is an established marker in the pathology of male infertility, and semen parameters such as motility and morphology are affected by OS. More importantly, we have also demonstrated that this association is not always true and that ROS is an independent marker of sperm quality (Agarwal et al., 2006). The distribution of sORP levels between the controls and the patient groups signifies the importance of OS in the pathophysiology of male infertility. Men with infertility had a significantly higher sORP value than men in the control group. Assessing sORP in men seeking fertility should help the clinician in the decision-making process.

In conclusion, this study has standardized the ORP test in semen using the MiOXSYS System as an alternative method for measuring OS and distinguishing normal men (controls) from male factor infertility patients. As a first step, this protocol will enable other andrology and IVF laboratories to confirm the validity of our findings. Secondly, this study proposed a preferred reference value above which the sORP levels in semen are abnormal and may result in OS. The results of the present study establish a new diagnostic cut-off value of 1.36 mV/10^6 sperm/ml sORP in semen to distinguish between normal men (controls) and patients with male factor infertility. Thirdly, precision is very important to any new technology and implies that the results are reproducible and can be performed by any operator.

In this study, the presence of white blood cells was not eliminated as this was not the main goal of the study. We agree that even a small concentration of white blood cells – especially activated polymorphonuclear granulocytes – contribute to ROS production as has been demonstrated in our earlier work (Sharma et al., 2001). Although the WHO 5th edition does not advocate lowering the cut-off of the WBC concentration in the ejaculate and has not changed the definition of leukocytospermia, it may be important to completely eliminate the presence of WBC in the seminal ejaculate using CD45 beads to rule out the formation of ROS even by a few active granulocytes prior to analysing the samples.

Figure 7 – Correlation of static oxidation-reduction potential (sORP) with sperm parameters in infertile patients (A: concentration, B: total sperm count, C: motility, D: morphology).
in a reliable fashion. The results of the intra- and inter-observer reliability experiments in this study confirmed the reproducibility of the test for use in a clinical setting. Lastly, examining the association of sORP with semen parameters in normal controls and infertile men indicated that sperm parameters related to male infertility are under a state of OS. A blinded randomized trial will further strengthen the information obtained in this study.

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