Predictive value of oxidative stress testing in semen for sperm DNA fragmentation assessed by sperm chromatin dispersion test

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Abstract

Background: Although standard semen analysis is a cornerstone of male infertility investigation, it has significant limitations. Seminal oxidative stress and DNA damage have shown potential to better predict male fertility potential and provide improved diagnostic and management strategies.

Objective: The aim of this study was to investigate whether seminal oxidation-reduction potential (ORP), a new parameter to measure oxidative stress directly, can accurately predict the percentage of sperm DNA fragmentation (SDF) and thereby serve as a surrogate marker in the evaluation of male infertility.

Materials and methods: ORP was evaluated in 3968 patients using the MiOXSYS system and SDF in 1147 patients using the Halosperm G2 test kit. Both parameters were analyzed in 1068 patients, along with seminal analysis, according to WHO guidelines 2010 (5th edition).

Results: SDF correlated positively with seminal ORP normalized for sperm concentration \( n = 1068; r = .218; P < .0001 \) as well as with ORP normalized for the motile sperm concentration (motORP; \( n = 1068; r = .387; P < .0001 \)). MotORP can significantly \( P < .0001 \) better predict SDF than ORP normalized for the sperm concentration (area under the curve (AUC): 0.719 vs 0.623) (specificity: 71.5%; sensitivity: 61.9%; PPV: 47.1%; NPV: 82.1%). Moreover, motORP can significantly \( P < .0001 \) better predict normozoospermia with high sensitivity (82.75%) and specificity (68.5%).

Discussion: Unlike other oxidative stress (OS) markers, ORP provides a global vision of the redox balance in semen. Moreover, SDF can also be induced by mechanisms different from OS, which could explain why its predictive power was low. The higher predictive power of motORP reflects the impact of seminal ROS on motility rather than inherent DNA breaks present in immature and aborted apoptotic spermatozoa.

Conclusion: The evaluation of motORP seems a more promising parameter than ORP normalized for sperm concentration for the prediction of SDF and normozoospermia. However, even if ORP and SDF are inter-related, they measure independent sperm functions and one test cannot replace the other in the evaluation of sperm function defects.
1 | INTRODUCTION

Globally, approximately 15% to 20% of couples are suffering from infertility, of which up to 50% of the cases can be attributed to male factor infertility. Therefore, it is imperative to develop new strategies not only to investigate sperm dysfunction parameters, but also to understand the sperm cell’s contribution to the fertilization process. Here, seminal oxidative stress and DNA damage have shown potential to better predict the male fertility potential and provide improved diagnostic and management strategies.

In men affected by infertility, 30% to 85% of these cases can be attributed to seminal oxidative stress, in which the levels of reactive oxygen species (ROS) are in excess in relation to the reducing effects of antioxidants (reductants). ROS are a group of highly reactive oxygen derivatives, many of which have a free, unpaired electron in the outer orbital. All aerobic cells generate ROS through mitochondrial complexes I and III during oxidative phosphorylation. Around 1% to 2% of the inhaled oxygen is not used for the synthesis of ATP and leaks from the normal mitochondrial electron transfer chain to the generation of superoxide anion. Aerobic cells have adapted to low level of ROS. In fact, these molecules are essential to trigger important physiological functions, including the regulation of essential cellular signaling events and gene transcription, spermy activation, capacitation, and acrosome reaction.

Excessive endogenous and exogenous ROS levels are detrimental to cells through increasing oxidative stress above physiological levels. This is an important common mechanism involved in the pathophysiology of several diseases, including obesity and metabolic syndrome, diabetes, vascular diseases, neurodegenerative diseases, and malignancy. Importantly, oxidative stress further negatively affects male semen quality and fertility. A negative association with seminal parameters, such as total/progressive motility, sperm concentration, morphology, and vitality, has been repeatedly reported.

Excessive ROS can impair male fertility by targeting and damaging numerous cellular components, causing sperm dysfunction through multiple molecular mechanisms. Particularly, OS provokes the generation of oxidized DNA adducts, such as 8-hydroxy-2-deoxyguanosine (8OHdG) within the DNA, leading to single- or double-strand breaks. In addition, ROS activate caspases and nuclease involved in the apoptotic pathways, thereby indirectly causing sperm DNA fragmentation (SDF) through a mechanism of abortive apoptosis.

Currently, the investigation of oxidative stress is based on the detection of intracellular ROS through chemiluminescence assay, total antioxidant capacity (TAC), quantification of the end products of lipid peroxidation (eg, malondialdehyde27 or DNA damage (8OHdG)28 with strong correlations being reported between these different OS markers, and increased SDF in semen samples of infertile men. Furthermore, SDF negatively affects sperm fertility potential and embryo development in natural conception and assisted reproductive techniques (ART). In addition, evidence suggests that the assessment of oxidative stress could be important for infertile patients, who could take advantage of antioxidants supplementation or modification of the lifestyle (eg, smoking, exposure to pollutants, varicocoele, high BMI).

Recently, a novel, galvanostat-based technique to measure OS was introduced that determines the balance between oxidants and reductants (redox potential) in semen, termed the oxidation-reduction potential (ORP). However, further studies investigating the relationship of ORP to sperm parameters, including SDF, are required. Considering the close relationship between oxidative stress and SDF, the aim of this study was to investigate whether seminal ORP, as a direct measure for oxidative stress, can accurately predict the SDF and thereby serve as a surrogate marker in the evaluation of male infertility.

2 | MATERIALS AND METHODS

2.1 | Study subjects and ethical clearance

This retrospective cross-sectional study analyzed a total of 4,068 men attending Hamad Medical Center, Doha, Qatar, for fertility diagnosis from January to July 2018. ORP and SDF were evaluated in 3968 and 1147 patients, respectively Figure 1. Both parameters were analyzed in 1068 patients, along with seminal analysis, according to WHO guidelines 2010 (5th edition). Patients receiving antioxidant, hormonal therapy or antibiotics, as well as patients with testicular malignancy, receiving chemotherapy or radiotherapy were excluded from this study. Semen samples were collected after 2-5 days of sexual abstinence and evaluated according to WHO guidelines.
presented as mV/10^6 spermatozoa/mL. An ORP value of 1.34 mV/10^6 subsequently normalized to the sperm concentration, hence, data are analyzed. The results are provided in milli Volts (mV) and serve as a cutoff value to subdivide patients in low (≤1.34 mV/10^6 spermatozoa/mL) or high (>1.34 mV/10^6 spermatozoa/mL), reported by Agarwal et al.38

Hamad Medical Center, Doha, Qatar, and all patients signed an informed consent.

2.2 Measurement of oxidation-reduction potential

ORP was determined in semen specimens using the galvanostat-based technology MiOXSYS system (Aytu BioScience, Englewood, CO) as previously described.37 In brief, 30 µL of each sample was loaded on the sensor and analyzed. The results are provided in milli Volts (mV) and subsequently normalized to the sperm concentration, hence, data are presented as mV/10^6 spermatozoa/mL. An ORP value of 1.34 mV/10^6 spermatozoa/mL, reported by Agarwal et al.38 to discriminate semen samples with abnormal semen parameters, was considered as a clinical cutoff value to subdivide patients in low (≤1.34 mV/10^6 spermatozoa/mL) and high (>1.34 mV/10^6 spermatozoa/mL) ORP groups.38 We calculated the motile sperm fraction (sperm count/mL × total motility). Additional statistical analyses were conducted by normalizing ORP to the motile sperm fraction (motORP: mV/10^6 motile spermatozoa/mL).

2.3 Measurement of sperm DNA fragmentation

The percentage of sperm DNA fragmentation (SDF) was analyzed in semen specimens using the Halosperm G2 test kit (Halotech DNA SL). Briefly, each sperm sample was diluted in PBS in order to obtain a final concentration of 20 × 10^6 spermatozoa/mL. The washed samples were mixed with agarose (6.5%) in a 1:1 ratio and then loaded on the sample well. After 5 min at 4°C, the samples were incubated with the acid solution provided in the kit for 7 min at room temperature and subsequently with the lysis solution for 20 min. Then, the slides were washed with distilled water and dehydrated with 70% ethanol. Staining was performed by incubating the slides in eosin and thiazine-based solutions for 7 min each. Once air-dried, slides were stored in the dark until evaluation with conventional bright-field microscopy. Spermatozoa show a halo of DNA loops when DNA is not fragmented while, on the contrary, in case of DNA fragmentation, the size of halo is dramatically reduced.39 A recent survey published by Majzoub et al.40 reported that 61.2% of questioned specialists with demonstrated clinical experience in the field of infertility considered a SDF cutoff equal to 30%.40 Therefore, patients were divided into two subgroups: low (≤30%) and high (>30%) SDF.

2.4 Statistical analysis

Statistical analysis was performed using MedCalc Statistical Software version v19.0.3 (MedCalc Software bvba, Ostend, Belgium). Following testing for normal distribution using the chi-squared test, Mann-Whitney test and Fisher’s exact test were performed to compare groups according to quantitative and categorical variables, respectively. Spearman’s rank correlation was used to investigate the relationship between ORP and the percentage of DNA-fragmented spermatozoa. Receiver operator characteristic (ROC) analyses were used to determine whether ORP normalized for sperm concentration and motile sperm concentration, respectively, could predict the rate of SDF and the condition of normozoospermia, according to the WHO guidelines (2010).36 The following parameters were reported: the area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), and the negative predictive value (NPV).

For calculations, both, SDF and ORP, patients were subdivided into groups, namely: low (≤30%) or high (>30%) SDF and low (≤1.34 mV/10^6 spermatozoa/mL) or high (>1.34 mV/10^6 spermatozoa/mL) ORP, according to the cutoff values reported in the literature (SDF ≤ 30%; ORP ≤ 1.34 mV/10^6 spermatozoa/mL).40,41 A P-value of less than .05 was considered significant.

3 RESULTS

3.1 Statistical correlations between sperm DNA fragmentation and ORP

The summary statistics for ORP values and SDF were determined in 3968 and 1147 patients, respectively, and depicted in Table 1. Those patients having both tests performed on the same specimen (n = 1068) were included in the statistical analysis. Demographic and semen parameters are reported in Table 2. Overall, SDF correlated significantly positively with seminal ORP normalized for sperm concentration (r = .218, P < .0001) as well as with the ORP normalized for motile sperm concentration (motORP) (r = .387, P < .0001) (Figure 2). The correlation of SDF with motORP was

| TABLE 1 The levels of sperm DNA fragmentation (SDF) and oxidation-reduction potential (ORP) in the cohorts. Data are represented as mean ± SD values and median (interquartile: IQ range) |
|---|---|---|
| n | 1147 | 3968 |
| Mean ± SD | 29.8 ± 19.5 | 5.24 ± 10.9 |
| Median (IQ range) | 25 (15-38.75) | 1.84 (0.99-4.38) |
significantly \((P < .0001)\) stronger than for ORP normalized for sperm concentration. ORP normalized to sperm count and motORP was strongly and positively correlated \((r = .917, P < .0001)\).

Grouping of patients in high/low SDF and high/low ORP and subsequent analysis with Fisher’s exact test resulted in the identification of distinct \((P < .0001)\) categories of 28.8% of the patients showing low ORP with low SDF, 21.5% showing high ORP with high SDF, 41.7% high ORP with low SDF, and 8.0% low ORP with high SDF.

When ORP values normalized for sperm concentration were analyzed in the SDF subgroups (high/low), patients with high SDF showed significantly \((P < .0001)\) higher (mean: 4.1 mV/10^6 spermatozoa/mL) ORP values than patients with low SDF (mean: 2.5 mV/10^6 spermatozoa/mL) (Figure 3A). In the same way, when the SDF was analyzed in the ORP subgroups (High/Low), patients with low ORP values showed significantly \((P < .0001)\) lower (mean: 25.1%) SDF than patients with high ORP (mean: 30.9%) (Figure 3B).

### 3.2 | ROC analysis

A receiver operating characteristic (ROC) curve was generated to determine whether ORP could predict patients with high SDF. Although the area under the curve (AUC) of 0.623 is not very high and lies relatively close to the random guess line, an ORP equal to 1.77 mV/10^6 spermatozoa/mL can significantly \((P < .0001)\) predict high SDF with a specificity of 56.3%, a sensitivity of 63.5%, a positive predictive value (PPV) of 37.3%, and a negative predictive value (NPV) of 79.1%. A second ROC curve was generated considering the motORP to predict the rate of SDF. In this case, a cutoff value of 4.96 mV/10^6 motile spermatozoa/mL showed a better prediction of SDF than ORP (AUC: 0.719) with a specificity of 71.5%, a sensitivity of 61.9%, a PPV of 47.1%, and a NPV of 82.1% (Figure 4). A significant difference between the two curves was observed \((P < .0001)\).
The power of ORP and motORP in predicting the condition of normozoospermia was evaluated. When ORP was used, the ROC curve showed AUC = 0.623; specificity = 56.3%; sensitivity = 63.5%; PPV = 37.3%; and NPV = 79.1%. When motORP was used, the ROC curve showed AUC = 0.719; specificity = 71.5%; sensitivity = 61.9%; PPV = 47.1%; and NPV = 82.1%. Comparison of ROC curves between ORP and SDF showed significant difference (P < .0001).

The power of ORP and motORP in predicting the condition of normozoospermia was evaluated. When ORP was used, the ROC curve showed AUC = 0.771; sensitivity = 57.33%; specificity = 78.6%; PPV = 16.8%; and NPV = 96.1%. When motORP was used, the ROC curve showed AUC = 0.826; specificity = 68.5%; sensitivity = 82.7%; PPV = 16.5%; and NPV = 98.1%. ROC curves differed significantly between ORP and SDF (P < .0001).

FIGURE 4 Comparison between ROC curves. The ORP (in blue) and motORP (in red) variables were used to predict the rate of SDF. When ORP was used, the ROC curve showed AUC = 0.623; specificity = 56.3%; sensitivity = 63.5%; PPV = 37.3%; and NPV = 79.1%. When motORP was used, the ROC curve showed AUC = 0.719; specificity = 71.5%; sensitivity = 61.9%; PPV = 47.1%; and NPV = 82.1%. Comparison of ROC curves between ORP and SDF showed significant difference (P < .0001).

FIGURE 5 Comparison between ROC curves. The ORP (in blue) and motORP (in red) were used to predict normozoospermia. When ORP was used, the ROC curve showed AUC = 0.771; sensitivity = 57.33%; specificity = 78.6%; PPV = 16.8%; and NPV = 96.1%. When motORP was used, the ROC curve showed AUC = 0.826; specificity = 68.5%; sensitivity = 82.7%; PPV = 16.5%; and NPV = 98.1%. ROC curves differed significantly between ORP and SDF (P < .0001).

**DISCUSSION**

In up to 40% of cases of male infertility, seminal parameters are altered with no apparent reason leading to idiopathic infertility. It is estimated that around 80% of cases of idiopathic male infertility, alongside with 30% to 40% of known or unexplained infertility cases, are mediated by oxidative stress. Globally, oxidative stress affects the reproductive potential of approximately 56 million people. Therefore, an assessment of oxidative stress in clinical management of male infertility is imperative.

Considering that spermatozoa have limited repair mechanisms for DNA damage, oxidative stress has a severe impact on sperm DNA integrity, leading to sperm DNA fragmentation. A positive correlation between SDF and all markers of oxidative stress has been widely described in fertile and infertile men. However, the relationship between ORP, a new composite marker of oxidative stress based on the evaluation of global redox potential, and SDF is still unexplored. The results of this study show a weak, yet significant, positive association between SDF and ORP. Previously,
Majzoub et al reported a positive correlation between SDF and ORP in a cohort of 1168 infertile patients, while Homa et al observed a correlation between SDF and seminal ROS levels, but no correlation was observed between SDF and ORP in a small group of 47 patients with unknown fertility. In the latter study, the small sample size may be the explanation for these apparently contrasting results.

Since oxidative stress severely impacts on sperm DNA integrity, the use of ORP as a predictive factor of SDF was investigated. This study reports that, although ORP levels were significantly correlated with high SDF, ORP can only predict abnormal SDF at a relatively low power. Unlike the other OS markers, which provided information only about one aspect of oxidants/antioxidants systems, ORP provides a global vision of the redox balance in the semen. In addition, SDF can also be induced by mechanisms different from OS, which could explain why the predictive power of the ROC curve analysis is low (although significant).

The induction of apoptosis during spermatogenesis, an incorrect chromatin remodeling or influence of environmental toxicants and specific therapies can all affect sperm DNA integrity. Additionally, meiosis I involves the deliberate induction of double-strand breaks, which triggers homologous recombination, thus helping to ensure normal chromosome segregation. Sertoli cells can fail in eliminating apoptotic sperm cells with poor DNA integrity, so that they can be found in the ejaculate. Furthermore, mistakes in crossing over or in the protamination process can lead to the formation of unrepaired DNA nicks before spermiation, impacting on sperm DNA integrity and consequentially on male reproductive potential. Therefore, other mechanisms different from oxidative stress could contribute to induce sperm DNA fragmentation. This explanation would correspond with the argumentation by Henkel et al who showed a better correlation of SDF with sperm intracellular ROS than with ROS produced by leukocytes. Sperm nuclear DNA is generally well protected against oxidative assaults, and it is thus reasonable to think that extracellular OS would only affect the sperm nuclear DNA if the amount of extracellular ROS is relatively high. Therefore, it is plausible that a portion of the acquired DNA fragmentation might be a natural process of elimination of defective spermatozoa produced during meiosis, which takes place in testis and accounts for DNA breaks found in dead ejaculated spermatozoa, whereas oxidative stress is responsible for inducing SDF because of systemic stress conditions during the transit of spermatozoa through the male genital tracts, accounting for DNA breaks observed in viable spermatozoa of the ejaculate.

When the ORP was normalized to the motile sperm concentration, its power in predicting SDF markedly increased, basically reflecting the impact of seminal ROS on motility rather than inherent DNA breaks present in immature and aborted apoptotic spermatozoa, which are not phagocytosed and appear in the ejaculate. This is not surprising because sperm motility is the first parameter to be affected by oxidative stress. The disturbance of a physiological mitochondrial membrane potential, in fact, is associated with oxidative stress condition. It results in an increased synthesis of ROS and a reduced production rate of ATP, with consequent impairment of sperm motility. Therefore, the motORP may represent a predictor relevant to the affected sperm motility and DNA integrity through ORP assessment as both parameters undergo oxidative stress-induced damages. At the same time, its high sensitivity in predicting normozoospermic condition suggests the motORP as a new marker for the evaluation of male infertility.

In conclusion, the evaluation of motORP seems a more promising parameter than ORP normalizing for sperm concentration for the prediction of SDF and normozoospermia. However, even if ORP and SDF are inter-related, they measure independent sperm functions and one test cannot replace the other in the evaluation of sperm function defects. Based on these results, we conclude that both ORP and SDF tests should be incorporated in routine clinical practice for a comprehensive evaluation of male infertility.

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CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

AUTHORS CONTRIBUTION
H. Elbardisi and A. Majzoub involved in the research design, acquired the data, revised the manuscript, and approved the final version of the manuscript. R. Finelli analyzed the data, wrote the manuscript, and approved the final version of the manuscript. A. Agarwal involved in the research design, analyzed the data and revised the manuscript, and approved the final version of the manuscript. R. Henkel conceived the study, involved in the research design, analyzed the data, wrote and revised the manuscript, and approved the final version of the manuscript. M. Arafa involved in the research design, analyzed the data, wrote the manuscript, and revised the manuscript, and approved the final version of the manuscript.

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