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INVITED REVIEW

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Evaluation of seminal oxidation-reduction potential in male infertility

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Abstract

The role of oxidative stress in male infertility has been broadly recognised, and the search for a new marker to determine the redox environment in semen has gained considerable interest. Oxidation–reduction potential (ORP) or redox potential, is a measure of the electron transfer from antioxidants to oxidants and provides information on the redox balance. In this review, the benefits of ORP as a new oxidative stress marker, the protocol for its evaluation and the importance of its measurement in the context of male infertility are discussed. In association with the standard semen analysis, seminal ORP has been analysed to evaluate semen quality and male fertility status. However, further studies are required to establish its use in assisted reproductive techniques (ART) practice.

KEYWORDS

male infertility, oxidation–reduction potential, oxidative stress

1 | BACKGROUND

Oxidation–reduction potential (ORP) or redox potential is a measure of the tendency of a molecule to lose or gain electrons. The ORP levels of a system determine the transfer of electrons from lower ORP-valued molecules (reductants) to higher ORP-valued molecules (oxidants). Currently, ORP testing is widely used in the evaluation of the global redox status in human biological fluids, such as urine (Cao, He, Bai, & Liu, 2016) and plasma (Polson, Villalba, & Freeman, 2018). In the case of traumatic brain injury, ORP is used as a marker to assess the severity of the injury (Bjugstad et al., 2016). The ORP measurement has a higher power in predicting severity and survival outcomes than other tools usually used in this pathological condition, such as the Injury Severity Score, Abbreviated Injury Scale and Glasgow Coma Scale (Bjugstad et al., 2016). Cao et al. (2016) reported higher ORP levels in urine samples of diabetic patients compared to normal healthy men. In athletes, ORP levels were evaluated in blood plasma to assess their redox status (Stagos et al., 2015). Further, Polson et al. (2018) optimised the procedure for measuring ORP levels in plasma collected from heparinised blood samples (Polson et al., 2018). Recently, the evaluation of ORP has been introduced to measure oxidative stress in seminal fluid of men with infertility (Agarwal, Sharma, Roychoudhury, Du Plessis, & Sabanegh, 2016).

Oxidative stress has been correlated with high levels of reactive oxygen species (ROS) and poor sperm quality in infertile men (Agarwal, Rana, et al., 2018; Gharagozloo & Aitken, 2011; Saleh & Agarwal, 2002). An estimate of 30%–80% of infertile men are diagnosed with seminal oxidative stress, accounting for more than 55 million people worldwide (Agarwal, Parekh, et al., 2019). The condition of these infertile men having poor semen quality and high levels of oxidative stress has been described as MOSI (Male Oxidative Stress Infertility; Agarwal, Parekh, et al., 2019). At physiological concentrations, ROS are intimately involved in the regulation of several sperm functions such as capacitation, acrosome reaction, hyperactivation and membrane fusion between spermatozoa and oocyte (Aitken, 2017; Du Plessis, Agarwal, Halabi, & Tvrda, 2015). However, ROS can lead to cellular damage and infertility if the concentration overwhelms the seminal antioxidants. Sperm motility is mainly affected by oxidative stress as mitochondrial activity and

ATP generation is affected by the increased levels of ROS (Wang, Sharma, Gupta, et al., 2003). Mitochondrial membrane potential (MMP) is a direct marker of the cellular energy status and mitochondrial functionality (Marchetti, 2002). It negatively correlates with the ROS levels and is affected by oxidative stress (Wang, Sharma, Gupta, et al., 2003). Further, ROS has a significant negative impact on sperm morphology by altering the acrosome structure. El-Taieb, Ali, and Nada (2015) reported an increased number of acrosome abnormalities (e.g. acrosome swelling and inclusions) in patients with high levels of malondialdehyde, a well-known marker of lipid peroxidation (El-Taieb et al., 2015). Seminal oxidative stress has also been associated with the induction of cellular apoptosis, resulting in a reduced number of viable spermatozoa and increased DNA damage (Mahfouz et al., 2010; Wang, Sharma, Sikka, et al., 2003). A high rate of sperm DNA damage can trigger the apoptotic process, as spermatozoa lack base repair mechanisms (Aitken, De Iuliis, Gibb, & Baker, 2012). Hence, the evaluation of oxidative stress provides an insight into the semen quality and male fertility potential.

Currently, several methods are used for the laboratory evaluation of oxidative stress in semen samples (Agarwal, Qiu, & Sharma, 2018); these are categorised as direct and indirect tests (Table 1). The direct tests measure the levels of ROS in semen samples, whereas the indirect tests evaluate the amount of individual or global antioxidants or oxidised products such as malondialdehyde and the modified base 8-hydroxy-2′-deoxyguanosine (8-OHdG). The chemiluminescence assay is one of the widely used direct tests to measure ROS levels using a luminol probe that emits photons (Agarwal, Ahmad, & Sharma, 2015; Khan et al., 2014). Luminol can detect both intra- and extracellular ROS, but cannot discriminate between the different types of ROS present in semen samples. Furthermore, it is a time-consuming technique and requires a minimum of 800 µl of semen samples. Frozen semen, azoospermic or hyperviscous samples are not suitable for chemiluminescence assays, thus limiting their diagnostic potential (Aitken, Baker, & O'Bryan, 2004). Redox homeostasis is established by the fine balance between the oxidants and reductants. Hence, measurement of only either oxidants or reductants does not provide enough evidence to support the diagnosis of oxidative stress. There is a need to identify a new marker of oxidative stress to fully determine the redox potential status in semen. The ORP testing was introduced to measure the global balance of the redox potential in semen samples (Agarwal, Roychoudhury, Sharma, et al., 2016). A strong correlation has been observed between seminal ORP and various sperm parameters such as concentration, motility and morphology, indicating ORP as a marker of seminal oxidative stress (Agarwal, Panner Selvam, et al., 2019). Furthermore, ORP has been shown to be a feasible adjunct test to conventional semen analysis (Agarwal, Panner Selvam, et al., 2019; Agarwal, Sharma, et al., 2016; Agarwal & Wang, 2017; Arafa et al., 2018; Douglas, Parekh, Kahn, Henkel, & Agarwal, 2019).

2 | TECHNOLOGY AVAILABLE TO MEASURE ORP

2.1 | MiOXSYS analyser

The analysis of oxidative stress is carried out in conjunction with the standard semen analysis to identify the aetiology associated with poor semen quality and male infertility. The MiOXSYS analyser (Male Infertility Oxidative System; Aytu BioScience; Figure 1a) is an ultrahigh impedance electrometer, which evaluates the ORP levels in semen sample by measuring the electron transfer from the antioxidants to the oxidants (Agarwal, Sharma, et al., 2016). Unlike other techniques, ORP measurement by MiOXSYS is an easily performed technique that does not require a specific personnel training or sample processing. Moreover, it analyses ORP in a small amount of both fresh or frozen samples (30 µl) in <4 min, with stable results for up to 120 min after collection of the semen samples (Agarwal, Sharma, et al., 2016). Hence, it is recommended to freeze the sample if it cannot be evaluated within 120 min after liquefaction.

2.2 | Other probes and analysers

The ORP sensors work similarly to the pH sensors. They detect the ORP of the solution by measuring the difference in redox potential between a working electrode and a reference electrode made up of gold or platinum. The manufacturer Sensorex Inc developed several types of sensors, depending on its application and the resistance to increasing temperature (up to 100°C; https://sensorex.com/). ORP sensors can be wirelessly connected to an android tablet or phone (Camlab) to allow the storage and retrieval of previous results (https://www.camlab.co.uk/). Other sensors can simultaneously detect ORP, temperature and pH as well as other water

TABLE 1 Most commonly used tests to evaluate oxidative stress in the seminal plasma

Direct tests	Indirect tests
Chemiluminescence	Myeloperoxidase or Endtz test
Nitro blue tetrazolium (NBT)	Lipid peroxidation—malondialdehyde levels
Cytochrome c reduction test	Evaluation of inflammatory mediators (chemokines and interleukins)
Fluorescein isothiocyanate (FITC)-labelled lectins	Antioxidants, micronutrients, vitamins (vitamin E and vitamin C)
Electron spin resonance (ESR)	Total antioxidant capacity (TAC)
Oxidation–reduction potential (ORP)	DNA damage—8-hydroxy-2′-deoxyguanosine levels

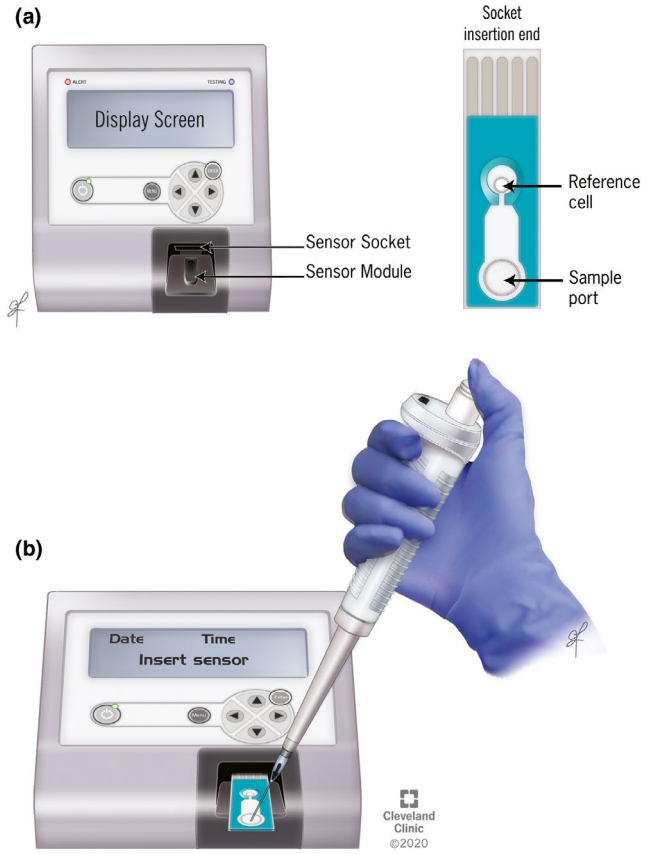

FIGURE 1 (a) MiOXSYS analyser and sensor; (b) Loading of semen sample into the application port, filling the reference electrode

characteristics such as dissolved oxygen, conductivity, salinity, ammonium, nitrate and chloride (Xylem Inc, https://www.xylem.com/en-us/). In the medical field, the type of analyser varies in relation to the sample that is analysed, with the electrodes being smaller when the sample volume to be analysed is less. Pluschkell and Flickinger measured ORP in cell cultures using a Thermo Scientific Orion analyser (Pluschkell & Flickinger, 1995), while the RedoxSYS Diagnostic System (Luoxis Diagnostics, which then merged into Aytu BioScience), the MiOXSYS precursor, was used to measure ORP in human blood plasma (Bjugstad et al., 2016; Polson et al., 2018; Stagos et al., 2015).

3 | MEASUREMENT OF ORP USING MIOXSYS

3.1 | Principle

The electrons transferred from the antioxidants (electron donators) to oxidants (electron acceptors) are measured by the MiOXSYS analyser based on the Nernst equation:

$$E_{(ORP)} = E^{\circ} - RT/nF \ln\left([\text{Red}] / [\text{Ox}]\right)$$

where E = standard reduction potential; R = the universal gas constant; T = the absolute temperature; n = number of moles of exchanged electrons; F = Faraday constant; Red = concentration of reduced species; and Ox = concentration of oxidised species.

3.2 | Sample collection and preparation

3.2.1 | Fresh or neat semen

Samples collected after a sexual abstinence of 2–7 days are kept in the incubator at 37°C for 20 min. After complete liquefaction, semen analysis is carried out to assess both macroscopic characteristics such as pH, volume and colour, and microscopic characteristics such as concentration, motility and morphology (WHO, 2010). A small aliquot of semen sample (30 µl) is analysed using the MiOXSYS.

3.2.2 | Frozen semen

The ORP measurement can be performed using frozen semen samples (Agarwal, Sharma, et al., 2018). Sample freezing does not influence the ORP values. Initially, semen samples should be thawed at 37°C before testing and vortexed to ensure proper mixing of the sample. Then, a small aliquot of sample (30 µl) is used for ORP measurement.

3.3 | Instrumentation and analysis

The MiOXSYS sensor contains three electrodes: Ag/AgCl reference electrode and two working electrodes made of platinum. A small amount of sample (30 µl) is loaded on the sensor application port (Figure 1b). The sample flows through the sensor and fills the reference cell. It is very important to fully cover the application port to avoid the formation of any air bubbles. Once the sensor is introduced into the analyser, the electrochemical circuit is closed, and the measurement starts. The MiOXSYS analyser detects the voltage between the reference and the working electrodes every 0.5 s interval, and the final ORP is calculated after ~120 s as the average of the last 10 s (or the last 20 readings) of the run. Results are expressed in millivolt (mV) as static ORP (sORP).

3.4 | Calculation and interpretation of results

The ORP is influenced by the concentration of cells, and it is inversely proportional to the viability of cells (Pluschkell & Flickinger, 1995). Therefore, the ORP must be normalised against the sperm concentration to obtain a value, which is representative of the semen. ORP levels are influenced by both the quantity (concentration) and quality (morphology) of the cells (Pluschkell &

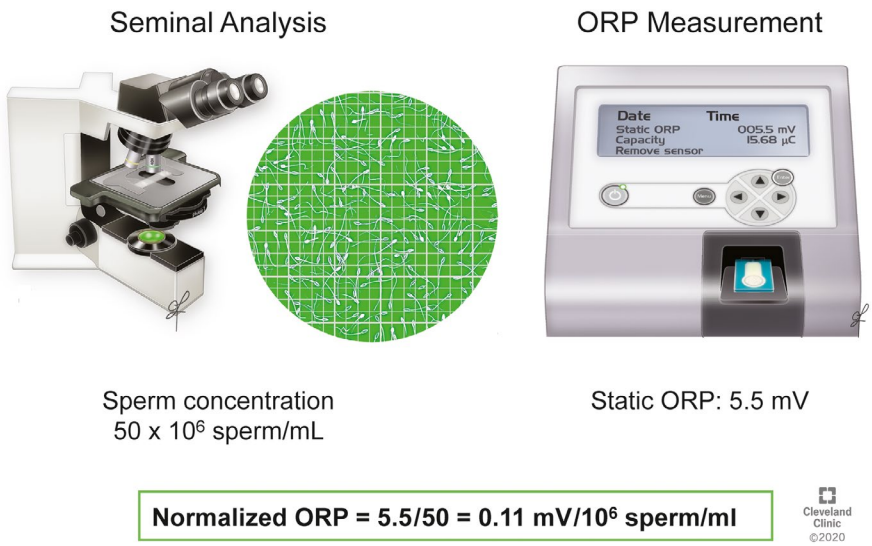

FIGURE 2 ORP value displayed on the analyser is normalised with the sperm concentration. Results are expressed as mV/10^6 spermatozoa/ml

Flickinger, 1995). In semen sample, the presence of immature or abnormal spermatozoa can reduce the availability of antioxidants as well as increase the generation of ROS (Agarwal, Hamada, & Esteves, 2012). Hence, the normalisation process for ORP is crucial to distinguish the oxidative status between samples with the same concentration but with varying sperm quality. The normalised ORP is expressed as mV/10^6 spermatozoa/ml (Figure 2).

Seminal parameters can influence the efficiency of MiOXSYS to detect ORP. For instance, a sample characterised by poor liquefaction or high viscosity shows resistance in flow from the application port to the reference cell, making the analysis challenging. Moreover, the necessity to normalise the ORP with sperm concentration limits its use with azoospermic samples. In addition, temperature variation may influence the analysis, as the optimal working temperature is between 5 and 45°C. Hence, it is important to thaw the frozen semen samples to 37°C before testing for ORP using MiOXSYS.

3.5 | Quality control

Quality control (QC) is an essential step carried out by each laboratory to ensure both precision and accuracy of the results. The WHO guidelines strongly recommend the necessity of performing QC in andrology practice to guarantee the robustness of the analysis and minimise false results (WHO, 2010). The QC for the MiOXSYS analyser is carried out using two aqueous solution matrices provided by the manufacturer having known low (33–70 mV) and high (91–117 mV) ORP values. The QC checks of the instrument are performed prior to the testing of semen sample to ensure the device is functioning optimally. QC should also be done routinely to verify each new lot of sensors. Calibration of the instrument should be done before analysing the sample using a calibration verification key (CVK) provided by the manufacturer. Both sides of the CVK (side A: 99–101 mV and side B: 295.8–304.2 mV) are analysed, and the values must fall between the acceptance limits; otherwise, maintenance should be requested.

4 | CLINICAL RELEVANCE OF ORP MEASUREMENT

4.1 | ORP and male infertility

In a clinical andrology setting, ORP has been utilised as a reliable marker of oxidative stress in human semen samples (Agarwal, Roychoudhury, Bjugstad, & Cho, 2016). Several studies have demonstrated the clinical relevance of ORP testing in the evaluation of male infertility (Agarwal et al., 2017; Agarwal, Henkel, Sharma, Tadros, & Sabanegh, 2018; Agarwal, Sharma, et al., 2016; Agarwal & Wang, 2017). In a preliminary study, Agarwal et al. reported a negative correlation between semen parameters, such as concentration and motility, and ORP levels in semen and in seminal plasma. Further reports have confirmed the negative association between sperm concentration, motility, total motile sperm count and morphology with seminal ORP levels (Agarwal et al., 2017; Agarwal, Henkel, et al., 2018; Agarwal, Roychoudhury, Sharma, et al., 2016; Agarwal, Sharma, et al., 2016; Agarwal & Wang, 2017; Majzoub et al., 2018, 2019). In comparison with a chemiluminescence assay that measures ROS levels, seminal ORP shows a stronger negative correlation with sperm motility and morphology, suggesting ORP as a more reliable marker of oxidative stress than ROS in a clinical setting (Homa et al., 2019). However, although leucocytes are the main source of ROS production (Henkel, 2011), no correlation has been reported between seminal ORP and leucocyte concentration in semen samples (data from our laboratory).

Studies conducted to predict the ability of ORP in discriminating semen samples in men with abnormal parameters based on their fertility status from normal or fertile samples, respectively, are shown in Table 2. Agarwal et al. reported higher levels of seminal ORP in infertile patients than in fertile men and proposed an ORP cut-off ≥1.36 mV/10^6 spermatozoa/ml to discriminate patients based on semen quality (Agarwal, Henkel, et al., 2018; Agarwal, Roychoudhury, Sharma, et al., 2016; Agarwal & Wang, 2017; Arafa et al., 2018). A multicentre study involving larger cohorts of patients

TABLE 2 Ability of oxidation–reduction potential (ORP) to detect semen samples with abnormal parameters and the corresponding ORP cut-off values reported in different pathological conditions related to male infertility according to the fertility status from normal or fertile samples

Study	Patients	Pathological indication	ORP cut-off values (mV/10^6 sperm/ml)	Sensitivity (%)	Specificity (%)	PPV (%)	*p*-value
Agarwal, Sharma, et al. (2016)	• 33 infertile men • 26 healthy donors	Able to detect asthenozoospermia using semen sample	1.48	60.0	75.0	45.0	NA
		Able to detect asthenozoospermia using seminal plasma	2.09	46.7	81.8	46.7	NA
Agarwal, Roychoudhury, Sharma, et al. (2016)	• 106 infertile men • 51 healthy controls with proven and unproven fertility	Able to discriminate infertile patients from healthy men with normal semen parameters	1.36	69.6	83.1	85.3	NA
Agarwal and Wang (2017)	• 194 infertile men • 49 healthy men with proven (n = 15) and unproven (n = 34) fertility	Able to detect at least one abnormal sperm parameter	1.57	70.4	88.1	95.5	<.001
		Able to detect oligozoospermia	2.59	88.0	91.2	90.0	NA
Agarwal et al. (2017)	• 194 (USA) + 400 (Qatar) infertile men • 51 (USA) + 50 (Qatar) fertile donors	Able to discriminate fertile from infertile samples	1.42	60.6	74.3	93.3	NA
Arafa et al. (2018)	• 365 infertile men • 50 fertile donors	Abnormality from a neat semen sample	1.38	63.3	87.8	97.6	<.0001
		Able to discriminate between infertile and fertile semen samples	1.41	57.3	78.0	95.0	<.001
Majzoub et al. (2018)	• 1,168 infertile men • 100 fertile donors	Able to detect teratozoospermia	1.73	76.0	72.0	69.2	NA
Agarwal, Henkel, et al. (2018)	• 293 infertile men • 15 fertile donors	Able to discriminate between fertile and infertile samples	2.63	40.4	93.3	99.2	.0400
		Able to detect teratozoospermia	1.42	65.7	65.3	64.3	<.0001
		Able to detect oligozoospermia	2.63	81.5	92.7	89.1	<.0001
		Able to detect asthenozoospermia	5.2	46.9	86.3	75.3	<.0001
		Able to detect samples with any two semen abnormalities	2.7	64.6	83.9	75.7	<.0001
		Able to detect OAT	5.3	69.0	87.4	58.8	<.0001
Agarwal, Panner Selvam, et al. (2019)	• 1,893 abnormal semen samples • 199 normozoospermic samples	Abnormality from a neat semen sample	1.34	98.1	40.6	94.7	NA

Abbreviations: NA, not available; OAT, oligoasthenoteratozoospermia; ORP, oxidation–reduction potential; PPV, positive predictive value.

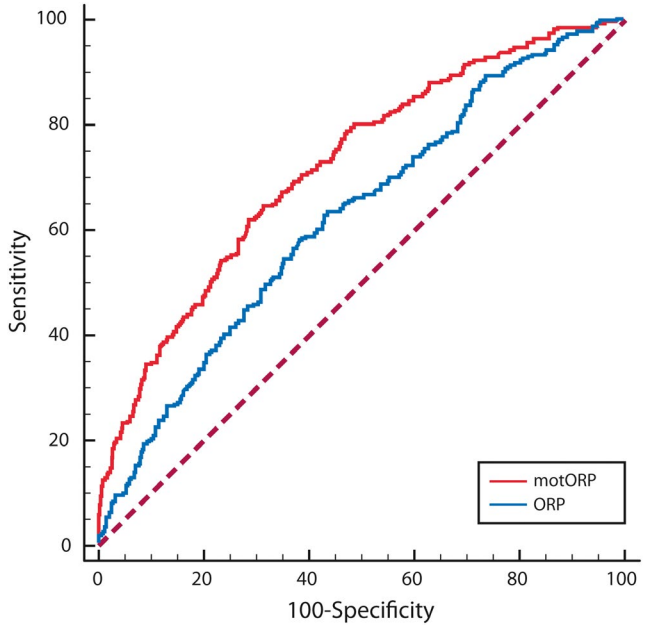

FIGURE 3 Comparison between ROC curves. The ORP (in blue) and motORP (in red) are used to predict the rate of SDF (Elbardisi et al., 2019)

with abnormal semen quality (n = 1,893) and normozoospermic men (n = 199) from nine different institutions around the world suggested a more appropriate cut-off value of 1.34 mV/10^6 spermatozoa/ml to discriminate samples based on semen quality (Agarwal, Panner Selvam, et al., 2019). As ORP measurement is a more stable parameter compared to standard semen analysis, ORP is therefore a more reliable parameter in the male infertility workup of patients with different ethnicity (Agarwal, Panner Selvam, et al., 2019).

Seminal ORP was also measured in varicocele patients to discriminate patients based on their fertility status (Arafa et al., 2018). In varicocele patients, oxidative stress plays an important role as an aetiological factor (Agarwal et al., 2012). Varicocele patients with poor semen quality had higher levels of ORP than healthy men of proven or unproven fertility (Agarwal, Roychoudhury, Sharma, et al., 2016; Samanta et al., 2018). A significant negative association has been reported between ORP and testicular volume in varicocele condition (r = −.386; p < .0001; Arafa, Henkel, Agarwal, Majzoub, & Elbardisi, 2019). However, further studies are required to investigate the role of ORP to predict the presence and the severity of varicocele.

As oxidative stress can damage sperm DNA, the association between ORP and sperm DNA fragmentation (SDF) has been investigated. Infertile patients show higher levels of SDF when compared with a fertile control (Santi, Spaggiari, & Simoni, 2018). SDF and ORP showed a weak positive correlation (r = .218, p < .0001; Arafa et al., 2019; Elbardisi et al., 2019; Majzoub et al., 2018) and an ORP value of 1.77 mV/10^6 spermatozoa/ml, can be used to predict SDF with moderate sensitivity (63.5%) and specificity (56.3%) (Elbardisi et al., 2019). Whereas, the specificity of the assay increased to 71.5% when the ORP was normalised with the motile sperm fraction (motORP; Figure 3; Elbardisi et al., 2019). Among all the sperm parameters, total motility is directly affected by oxidative stress. Hence, the use of motORP rather than the ORP normalised against sperm concentration has been suggested to be a better predictor of SDF. However, the predictive power of motORP is still moderate, probably due to the fact that sperm DNA damage can be due to several conditions other than oxidative stress, such as exposure to toxicants, medical therapies as well as defective chromatin compaction and induction of apoptosis (Morris, 2002; Muratori et al., 2015; Sakkas, Seli, Bizzaro, Tarozzi, & Manicardi, 2003).

ORP is an extracellular marker, while SDF is an intracellular marker of semen quality. Oxidative stress cannot cause immediate damage to the sperm DNA; rather, it is induced due to continuous exposure to ROS over a period of time. This may explain why ORP can hardly predict SDF. Therefore, even if inter-related, SDF and ORP seem to measure independent sperm functions and cannot replace each other in the evaluation of male fertility potential (Elbardisi et al., 2019).

4.2 | ORP and in vitro fertilization

Oxidative stress has adverse effects on the in vitro fertilisation process. As such, the redox state of the reagents and culture media used in the assisted reproductive techniques (ART) are critical for the development of embryo. The shifting of the redox balance in medium towards a more oxidative or reductive environment may result in poor reproductive outcomes (Du Plessis, Makker, Desai, & Agarwal, 2008; Esteves, Roque, & Agarwal, 2015; Henkel, 2011). In a recent study, the ORP levels were measured by the MiOXSYS device in 10 different culture media used in ART practice to either wash, freeze or culture cells (Panner Selvam, Henkel, Sharma, & Agarwal, 2018). The authors reported a lower levels of ORP for the sperm cryopreservation and ART culture media analysed in comparison with sperm wash medium; particularly, ORP was lower in a one-step medium than in sequential culture media. This suggests that the one-step medium provides a better capacity to counterbalance oxidative stress, which may be correlated with higher rate of blastocyst formation in one-step medium (Sfontouris et al., 2016). Furthermore, in embryo culture systems, the ORP levels were adjusted using a combination of antioxidants to adjust the ORP levels similar to that of follicular fluid from oocyte donors. Adjustment of ORP levels in culture medium resulted in increased blastocyst formation and ongoing pregnancy rates (Maldonado Rosas et al., 2019).

Similarly, TYB (test yolk buffer) freezing medium showed a lower ORP value due to the presence of antioxidants (Panner Selvam et al., 2018). During cryopreservation, there is an increased production of ROS (Zribi et al., 2010); hence, the use of a low ORP freezing medium may help in maintaining the redox balance. Using a mouse ICSI (intracytoplasmic sperm injection) model, ORP has been used to examine the effect of sperm exposure to polyvinyl pyrrolidone (PVP) and hyaluronic acid (HA) frequently used in ART practice (Roychoudhury et al., 2018). Spermatozoa selected by a

PVP-containing medium showed a lower ORP value than using a hyaluronic acid-containing medium, which suggests an antioxidant action of PVP. Nakamura et al. (2018) suggested that in vivo intrauterine ORP measurement at cycle days 9–10 could predict pregnancy after frozen-thawed embryo transfer, with significantly lower ORP levels in the pregnant group (Nakamura et al., 2018). A prospective pilot study reported a seminal ORP cut-off equal to 1.36 mV/10^6 spermatozoa/ml to predict the fertilisation and blastulation rates in a cohort of 50 patients undergoing ICSI (Morris, Siebert, Agarwal, & Henkel, 2019). However, these studies are still preliminary, and more evidence are needed to assess the role of ORP in evaluating clinical parameters in ART practices.

5 | CONCLUSION

Seminal ORP represents a valid marker for the evaluation of global redox status. MiOXSYS is a reliable, user-friendly device, with great potential to be used in clinical andrology settings. In association with the standard semen analysis, ORP has already been tested to evaluate semen quality and male fertility status, while further studies are necessary before considering its utility in ART practice.

TAKE-HOME MESSAGE

- ORP provides an evaluation of the global redox status, unlike other techniques which assess only the oxidants or the antioxidants.
- MiOXSYS is a simple and user-friendly device which can measure ORP in both fresh and frozen samples.
- ORP has been validated in clinical andrology practice as a complementary tool to standard semen analysis for the evaluation of semen quality and male fertility potential.

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