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Seminal oxidation–reduction potential levels are not influenced by the presence of leucocytospermia

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Abstract

Oxidative stress (OS) is characterised by an excessive amount of reactive oxygen species (ROS) which negatively affect sperm functions. In this study, the influence of leucocytes on seminal oxidation–reduction potential (ORP) and sperm DNA fragmentation (SDF) was investigated in 1,068 men. Seminal leucocyte concentration did not correlate with SDF, unadjusted ORP, ORP normalised for sperm concentration (sORP), ORP normalised for total motile sperm concentration (motORP) or total motile sperm count (TMSC-ORP). Although receiver operator characteristic (ROC) curve analyses show that leucocytospermia does not predict high sORP values (>1.34 mV/10^6 spermatozoa/ml), the motORP (AUC: 0.666) and TMSC-ORP (AUC: 0.683) predict the rate of leucocytospermia significantly (p = .0195 and p = .0085 respectively). Moreover, SDF can significantly predict leucocytospermia (AUC: 0.679; p = .011) and vice versa (AUC: 0.657, p = .0298). Our data confirm the association between OS and SDF. In conclusion, motORP and TMSC-ORP may be better predictive factors of leucocytospermia, probably because sperm motility, included in motORP and TMSC-ORP calculation, is the first seminal parameter to be affected by OS. Although all these parameters are indicative of OS, ORP values, SDF and leucocytospermia should be considered independently for the evaluation of redox seminal status, as they probe distinct seminal features.

KEYWORDS
leucocytospermia, male infertility, oxidation–reduction potential, oxidative stress, sperm DNA damage

1 | INTRODUCTION

Globally, infertility remains a prevalent and escalating health condition with the male factor contributing partially or wholly to approximately half of the infertile couples (Inhorn & Patrizio, 2015), of which oxidative stress (OS) is a major contributor. OS is defined as an excessive amount of reactive oxygen species (ROS) as compared to the available amount of seminal antioxidants (Agarwal, Panner Selvam, et al., 2019; Henkel et al., 2003). Consequently, the physiological mechanisms in maintaining the redox equilibrium, which play a crucial role for sperm functions and the fertilisation process

(Henkel et al., 2003), are totally overwhelmed. As a result, OS may lead to male infertility due to the reaction between oxidants and any cellular components, hence causing an increased rate of lipid peroxidation, loss of protein function and sperm DNA damage. Currently, it is estimated that OS is the causative factor in 30%–80% of the male infertility cases (Agarwal, Prabakaran, & Allamaneni, 2006; Iwasaki & Gagnon, 1992; Ochsendorf et al., 1994; Shekarriz, Thomas, & Agarwal, 1995; Zini, Lamirande, & Gagnon, 1993).

Leucocytes represent a major source of seminal ROS as they are thought to produce about 1,000 times more ROS than spermatozoa, when they are activated in response to a proper stimulus (Plante,

Lamirande, & Gagnon, 1994). According to the WHO guidelines, leucocytospermia is defined as a seminal leucocyte concentration higher than 1×10^6 white blood cells (WBC)/ml semen (WHO, 2010) and is associated with inflammatory and infectious conditions, as well as lifestyle habits, such as smoking, alcohol and abuse of drugs (Sandoval, Raburn, & Muasher, 2013). Leucocytospermia has been shown to have a negative effect on sperm parameters (Aziz, Agarwal, Lewis-Jones, Sharma, & Thomas, 2004; Moskovtsev, Willis, White, & Mullen, 2007; Omu, Al-Qattan, Al-Abdul-Hadi, Fatinikun, & Fernandes, 1999; Thomas et al., 1997).

The leucocyte count correlates positively with seminal ROS levels, suggesting OS as a mediator for functional sperm abnormalities (Henkel et al., 2003). Activated leucocytes, in fact, can release much higher amounts of ROS than spermatozoa (de Lamirande & Gagnon, 1995; Plante et al., 1994), which explains why leucocyte counts, even lower than 1×10^6 WBC/ml, can significantly impair sperm motility (Thomas et al., 1997), morphology (Aghazarian, Stancik, Pflüger, & Lackner, 2013) and chromatin integrity (Erenpreiss, Hlevicka, Zalkalns, & Erenpreisa, 2002; Mahfouz et al., 2010).

The MiOXSYS system is a new technology that determines seminal OS (Agarwal, Gupta, & Sharma, 2016; Agarwal, Roychoudhury, Bjugstad, & Cho, 2016; Agarwal, Sharma, Roychoudhury, Plessis, & Sabanegh, 2016). This novel technology determines the balance between all oxidants and antioxidants by measuring the oxidation–reduction potential (ORP) in semen or seminal plasma samples (Agarwal, Gupta, et al., 2016; Agarwal, Roychoudhury, et al., 2016; Agarwal, Sharma, et al., 2016). The measurement of ORP has been identified as a marker for male infertility (Agarwal, Henkel, Sharma, Tadros, & Sabanegh, 2018) since higher levels are associated with a decrease in sperm concentration and motility (Agarwal, Gupta, et al., 2016; Agarwal, Roychoudhury, et al., 2016; Agarwal, Sharma, et al., 2016). A cut-off value of 1.34 mV/10^6 spermatozoa/ml has been established to discriminate between fertile and infertile men (Agarwal, Panner Selvam, et al., 2019). However, the contribution of leucocytes to the seminal equilibrium between oxidation and reduction is still unclear.

Therefore, this study aims to investigate the contribution of leucocytes to the seminal ORP and sperm DNA fragmentation (SDF) as an OS-related marker.

2 | MATERIAL AND METHODS

2.1 | Study design

This study was conducted on data collected from 1,068 men attending the Male Fertility Unit of Hamad Medical Center, Doha, Qatar, a tertiary medical centre, over a period of 6 months (January 2018–June 2018). Only patients tested for both SDF and ORP were included in the study. Patients with azoospermia, testicular malignancy, receiving chemotherapy or radiotherapy as well as under treatment with antioxidants, antibiotics or hormonal therapy were excluded.

This study was approved by the Institutional Review Board of the Hamad Medical Center.

2.2 | Semen analysis

All samples were tested for conventional semen analysis, presence of leucocytospermia (≥1×10^6 WBC/ml), seminal ORP and SDF. Semen samples were collected in the centre or at home by masturbation following 2–4 days of ejaculatory abstinence. Seminal analysis was performed within 1 hour of collection according to WHO criteria post-liquefaction at 37°C (WHO, 2010).

2.3 | Quantification of seminal leucocytes

Of the 1,068 samples included in this study, 59 presented at least 1×10^6 round cells/ml of semen. The Endtz test was used to discriminate peroxidase-positive leucocytes from other round cells and quantify them as recommended by the WHO (2010). Brown-stained peroxidase-positive leucocytes as well as unstained peroxidase-negative cells were counted and expressed as 10^6 cells/ml. A leucocyte concentration of ≥1×10^6 cells/ml is classified as leucocytospermia (Politch, Wolff, Hill, & Anderson, 1993).

2.4 | Measurement of oxidation–reduction potential (ORP)

Oxidation–reduction potential was measured using the galvanostat-based MiOXSYS (Aytu BioScience; Agarwal, Gupta, et al., 2016; Agarwal, Roychoudhury, et al., 2016; Agarwal, Sharma, et al., 2016). In brief, an aliquot of 30 µl of the unprocessed liquefied semen was placed on a disposable sensor and inserted into the analyser holder. The test results, provided after about 4 min and expressed in millivolt (mV), are a 'snapshot' of the current balance between oxidants and reductants. These unadjusted ORP values were then normalised to sperm concentration (sORP) and the data presented as mV/10^6 spermatozoa/ml. A sORP value of 1.34 mV/10^6 spermatozoa/ml was considered as cut-off, with values above or equal to this cut-off being regarded as high sORP and those less than the cut-off as low sORP (Agarwal, Panner Selvam, et al., 2019; Agarwal, Parekh, et al., 2019). Additionally, unadjusted ORP has also been normalised for the total motile sperm concentration (motORP) and the total motile sperm count (TMSC-ORP), expressed as mV/10^6 motile spermatozoa/ml and mV/10^6 motile spermatozoa, respectively.

2.5 | Determination of sperm DNA fragmentation (SDF)

Sperm DNA fragmentation was evaluated by means of the sperm chromatin dispersion (SCD) assay (Halosperm G2 test kit, Halotech

DNA SL) as per manufacturer's instructions. A mixture of equal volumes of the semen sample and 6.5% agarose gel was treated with an acid solution to denature fragmented DNA, for 7 min at room temperature. Subsequently, the samples were treated with a lysis solution for 20 min and stained in eosin and thiazine-based solutions (7 min each). The evaluation was performed using bright-field microscopy. Intact DNA loops around the agarose-embedded sperm nucleus form a characteristic 'halo' appearance. Spermatozoa with fragmented DNA show very small halos or do not exhibit any halos. A threshold of 30% was used to distinguish between patients with high and normal SDF (Majzoub, Agarwal, Cho, & Esteves, 2017).

2.6 | Statistical analysis

Statistical analysis was performed using MedCalc Statistical Software version v19.0.3 (MedCalc Software Ltd.). After testing the data for normal distribution using the chi-squared test, group comparisons were performed with respect to quantitative and categorical variables using the Mann–Whitney or Fisher's exact test. Spearman's rank correlation was used to analyse the relationship between the leucocyte concentration, ORP values and SDF. Receiver operator characteristic (ROC) analyses were used to determine if ORP values could predict the rate of leucocytospermia. The diagnostic value of the presence of leucocytospermia for identifying high or low sORP and SDF was also assessed. ROC curves were described by the following parameters: area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). A p value < .05 was considered significant.

3 | RESULTS

3.1 | Correlations between ORP values, seminal leucocytes concentration and SDF

Summary statistics of all parameters investigated in this study are depicted in Table 1. Table 2 shows the correlations between the seminal leucocyte concentration, ORP values and SDF. The seminal leucocyte concentration did not correlate with unadjusted ORP, sORP, motORP, TMSC-ORP and SDF. On the other hand, weak but significant positive correlations were found between SDF and ORP values. Unadjusted ORP, sORP, motORP and TMSC-ORP showed a highly significant positive correlation with each other.

Grouping of patients into leucocytospermic/non-leucocytospermic and high/low sORP and subsequent analysis with Fisher's exact test did not result in the identification of any significant subgroups ($p = .58$). However, the grouping between leucocytospermic/nonleucocytospermic and high/low SDF was with rather borderline significance ($p = .0386$). On the other hand, Fisher's exact test for high/low sORP and high/low SDF was highly significant ($p < .0001$; Tables S1–S3).

3.2 | ROC curve analysis

Receiver operator characteristic curve analyses to determine if leucocytospermia can predict the presence of high sORP values (>1.34 mV/10^6 spermatozoa/ml) or if sORP can predict leucocytospermia were negative, indicating that none of these predictions ($p = .872$ and $p = .286$, respectively) are possible (Figures 1a and 2). On the other hand, a leucocyte concentration lower than 1.2×10^6/

TABLE 1 Summary statistics of the main parameters analysed in this study

Variables	All samples (n = 1,068)		None leucocytospermia (n = 33)		Leucocytospermia (n = 26)	
	Mean (SD)	Median (IQ range)	Mean (SD)	Median (IQ range)	Mean (SD)	Median (IQ range)
Age (years)	35.98 (7.78)	35.00 (31.00–40.00)	38.36 (9.93)	36.00 (32.00–43.50)	35.07 (7.86)	35.00 (28.00–40.00)
Volume (ml)	3.24 (3.05)	3.00 (2.00–4.00)	2.47 (1.31)	2.00 (1.50–3.00)	3.06 (1.88)	2.50 (2.00–4.00)
Sperm conc. (count/ml)	33.53 (24.07)	28.00 (15.00–45.00)	24.03 (15.77)	22.00 (11.75–32.00)	32.61 (19.18)	28.00 (17.00–50.00)
Total motility (%)	50.92 (18.39)	55.00 (40.00–62.00)	45.33 (26.97)	40.00 (23.75–60.00)	47.04 (12.32)	46.50 (40.00–55.00)
Progressive motility (%)	11.46 (11.53)	10.00 (0.00–20.00)	10.15 (13.89)	0.00 (0.00–17.50)	8.81 (9.81)	5.00 (0.00–20.00)
Normal morphology (%)	5.182 (8.09)	4.00 (2.00–5.00)	2.91 (3.05)	3.00 (0.00–4.00)	4.77 (5.14)	3.00 (2.00–5.00)
SDF (%)	28.49 (18.36)	24.00 (15.00–35.00)	41.27 (24.90)	37.00 (15.00–65.25)	24.69 (12.65)	21.00 (17.00–31.00)
Unadjusted ORP (mV)	54.87 (32.03)	51.40 (37.55–66.90)	63.30 (39.03)	54.80 (39.35–73.32)	52.54 (21.98)	48.20 (38.40–61.30)
sORP (mV/10^6 spermatozoa/ml)	2.94 (3.90)	1.76 (1.01–3.47)	4.50 (4.63)	2.82 (1.20–5.77)	2.50 (2.01)	1.72 (0.89–4.09)
motORP (mV/10^6 motile spermatozoa/ml)	11.11 (41.05)	3.48 (1.86–7.84)	14.85 (18.21)	7.45 (3.26–21.05)	6.11 (6.37)	3.45 (1.86–7.73)
TMSC-ORP (mV/10^6 motile spermatozoa)	4.81 (20.20)	1.29 (0.60–3.12)	7.93 (10.29)	3.28 (1.60–10.38)	2.52 (2.55)	1.92 (0.60–2.75)
Leucocytes (×10^6/ml)			0.39 (0.24)	0.40 (0.20–0.60)	2.51 (1.46)	2.00 (1.50–3.00)

TABLE 2 Summary correlation table of leucocyte count (*n* = 59), unadjusted ORP (*n* = 1,068), sORP (*n* = 1,068), motORP (*n* = 1,068), TMSC-ORP (*n* = 1,068) and SDF (*n* = 1,068)

Parameters	Leucocyte count (×10⁶/ml)	Unadjusted ORP (mV)	sORP (mV/10⁶ spermatozoa/ml)	motORP (mV/motile spermatozoa/ml)	TMSC-ORP (mV/10⁶ motile spermatozoa)	SDF (%)
Leucocytes (×10⁶/ml)		−0.090 0.4955	−0.160 0.2264	−0.234 0.0750	−0.215 0.1020	−0.250 0.0566
Unadjusted ORP (mV)	−0.090 0.4955		0.456 <0.0001	0.435 <0.0001	0.372 0.0001	0.181 <0.0001
sORP (mV/10⁶ spermatozoa/ml)	−0.160 0.2264	0.456 <0.0001		0.917 <0.0001	0.791 <0.0001	0.218 <0.0001
motORP (mV/motile spermatozoa/ml)	−0.234 0.0750	0.435 <0.0001	0.917 <0.0001		0.884 <0.0001	0.387 <0.0001
TMSC-ORP (mV/10⁶ motile spermatozoa)	−0.215 0.1020	0.372 <0.0001	0.791 <0.0001	0.884 <0.0001		0.334 <0.0001
SDF (%)	−0.250 0.0566	0.181 <0.0001	0.218 <0.0001	0.387 <0.0001	0.334 <0.0001	

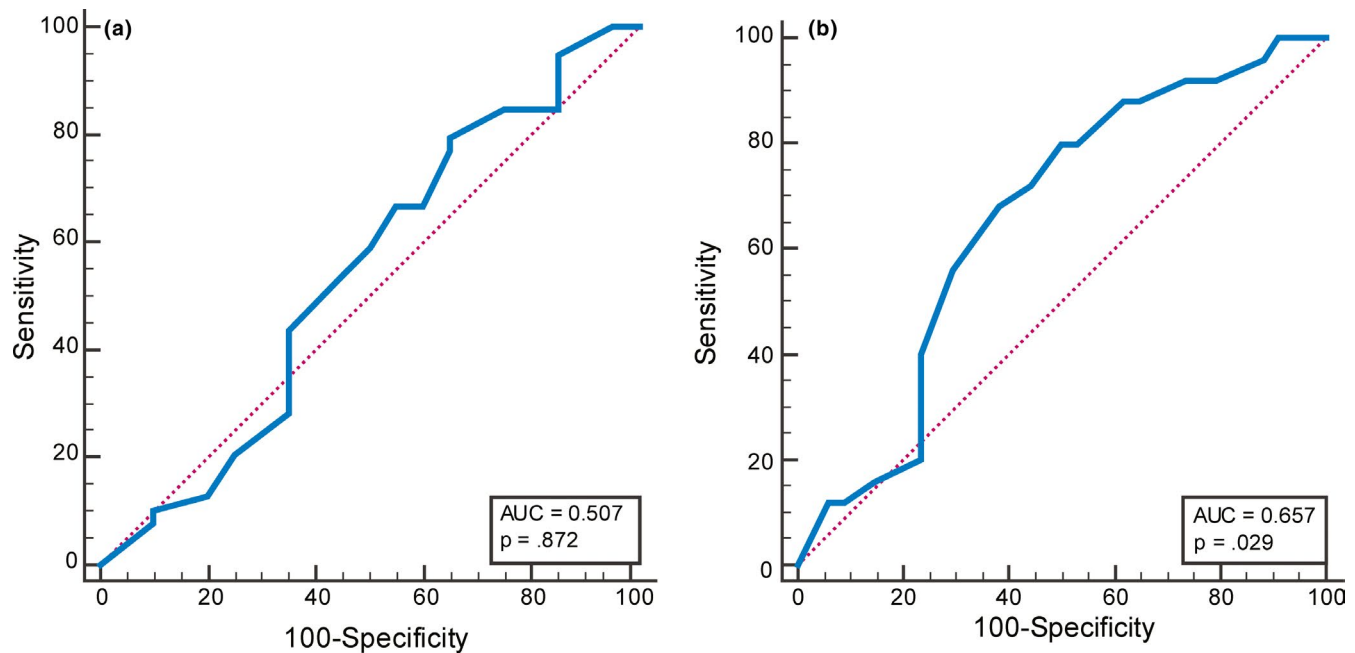*Note:* Data are reported as correlation coefficient (significance *p* value).

FIGURE 1 ROC curve analysis. Leucocytospermic condition was used to predict high values of (a) sORP (>1.34 mV/10⁶ spermatozoa/ml) and (b) SDF (>30%)

ml predicts a low SDF rate (<30%) with an AUC of 0.657, a sensitivity of 80.0%, specificity of 50.0%, PPV of 54.1% and NPV of 77.3% (*p* = .0298; Figure 1b). Vice versa, the ROC analysis shows that SDF ≥45% can significantly predict leucocytospermia with the following parameters: AUC: 0.679; specificity: 45.5%; sensitivity: 92.3%; positive likelihood ratio: 1.69; negative likelihood ratio: 0.17; *p* = .011 (Figure 2).

Receiver operator characteristic curves were generated for motORP and TMSC-ORP to predict the rate of leucocytospermia (Figure 2). In the first case, leucocytospermia can be predicted at a cut-off value of 11.46 mV/10⁶ motile spermatozoa/ml (AUC: 0.666; *p* = .0195) with a specificity of 42.4%, a sensitivity of 88.5%, a PPV of 54.8% and a NPV of 82.4%. For TMSC-ORP, a value equal to 2.97 mV/10⁶ motile spermatozoa can significantly (*p* = .0085) predict leucocytospermia (AUC = 0.683) with a specificity of 54.5%, sensitivity of 80.8%, PPV of 58.3% and NPV of 78.3%. The two ROC curves did not differ statistically (*p* = .6810).

4 | DISCUSSION

Many authors suggest that leucocytospermia negatively impacts semen quality as this condition causes and exponentially increases
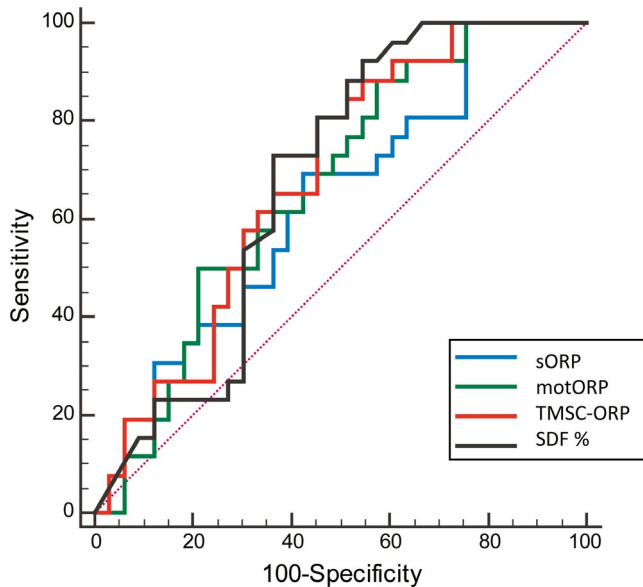
FIGURE 2 Comparison between ROC curves. The sORP (in blue), motORP (in green), TMSC-ORP (in red) and SDF% (in black) variables were used to predict the condition of leucocytospermia

the amount of seminal ROS, leading to OS (Aziz et al., 2004; Henkel et al., 2005). Recent studies demonstrate sORP as a novel marker of semen quality (Agarwal et al., 2018; Agarwal & Wang, 2017). However, the impact of leucocytes on the seminal redox status and therefore their relationship with the seminal ORP is still unexplored.

In this study, no association between the seminal leucocyte concentration and ORP values (unadjusted ORP, sORP, motORP and TMSC-ORP) was observed. This result was unexpected as leucocytes are thought to be the main source of ROS production in semen and would therefore not only contribute significantly to seminal OS, but have also a significant detrimental effect on sperm function (Plante et al., 1994). However, it has to be considered that ORP provides a global picture of the redox balance in semen, that is the sum of all oxidants and antioxidants, unlike normal luminometric ROS measurements, which assess only one end of the spectrum, the oxidants. ORP considers the interplay of all escalating and diminishing factors of OS. Hence, correlating a single parameter such as leucocyte concentration, which is responsible for increasing the amount of oxidants, with the ORP is not reasonable. Moreover, the actual leucocyte number that can cause harm to spermatozoa may be underestimated by determining the leucocyte concentration with the Endtz test, as it only identifies peroxidase-positive leucocytes by staining the peroxisomes (Shekarriz, Sharma, et al., 1995; Shekarriz, Thomas, et al., 1995). In semen, numerous types of leucocytes exist, such as polymorphonuclear (PMN) leucocytes and macrophages, representing 50%–60% and 20%–30% of all seminal leucocytes, respectively (Plante et al., 1994; Thomas et al., 1997; Wolff, 1995). In fact, several authors have shown peroxidase-positive leucocytes to be the major source of seminal ROS production (Aitken, West, & Buckingham, 1994; Plante et al., 1994; Wolff, 1995). However, the Endtz test cannot identify inactivated cells or those which have

already released their peroxisomes, resulting in an underestimation of the final leucocytes count. Therefore, other infection/inflammatory markers should be used to investigate their association with ORP.

Although the leucocyte concentration did not significantly correlate with the ORP values, the parameters motORP and TMSC-ORP were able to predict leucocytospermia. Both ORP values are calculated considering sperm motility, which is the main parameter to be affected by OS (Mahfouz et al., 2010). Therefore, it is not surprising that they were able to predict leucocytospermic condition. However, a limitation of this study is that extracellular and intracellular ROS could not be discriminated, with the latter being mainly responsible for the impairment of sperm motility rather than DNA damage (Henkel et al., 2005). In fact, OS affects motility mainly through the disturbance of the mitochondrial membrane potential and the membrane-associated electron transport chain, responsible for ATP production (Agnihotri et al., 2016; Wang et al., 2003). Therefore, a role of spermatozoa-generated ROS cannot be excluded, and it may explain the relatively moderate predictive values observed for motORP and TMSC-ORP.

Leucocytes produce several types of ROS (hydrogen peroxide—H_2O_2, superoxide anion—O_2^- or the hydroxyl radical—OH). Of these, only H_2O_2 is persistent, can penetrate the sperm plasma membrane and hence contribute to an increased intracellular ROS concentration. The other ROS, O_2^- and $\cdot$OH, cannot penetrate the lipid membranes although they contribute to phospholipids peroxidation, thereby affecting the sperm functions and morphology. According to Henkel et al. (2005) the localisation of ROS modulates their impact on DNA integrity, wherein extracellular ROS has a comparatively much lesser effect. Moreover, an association between the leucocyte count and the amount of intracellular ROS in semen was reported (Henkel et al., 2005). Hence, not only the seminal leucocyte count, but also the different ROS generated may play a role in affecting sperm functions as well as DNA integrity. With OS being a major cause of SDF, it was not surprising to observe a positive association between ORP values and SDF. However, several factors other than OS can affect sperm DNA integrity (Morris, 2002; Muratori et al., 2015; Sakkas, Seli, Bizzaro, Tarozzi, & Manicardi, 2003). This may explain why we did not observe any significant association between leucocytospermia and SDF, although a leucocyte concentration lower than 1.2×10^6/ml was able to predict a low SDF rate of <30%.

In conclusion, our data confirm that all ORP values do not correlate with leucocytospermia. The sORP is not able to predict leucocytospermia, but motORP and TMSC-ORP, as parameters based on sperm motility, may be better predictive factors of such condition. Although all of these parameters are indicative of seminal OS, however, ORP values, SDF and leucocytospermia should be considered independent parameters for the evaluation of seminal redox status, as they investigate distinct seminal features. In order to clarify the impact of leucocytes on sperm functions, different methods of identifying leucocytes than the Endtz test need to be employed.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

AUTHORS CONTRIBUTION

Mohamed Arafa, Ashok Agarwal and Ralf Henkel conceived the study and analysed the data. Ahmad Majzoub and Haitham ElBardisi were involved in collection of the samples. Kathy Robert and Renata Finelli drafted the article. All authors reviewed and approved the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.