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MiOXSYS® and OxiSperm® II assays appear to provide no clinical utility for determining oxidative stress in human sperm—results from repeated semen collections

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Abstract

Background: Oxidative stress in semen contributes up to 80% of all infertility diagnosis. Diagnostics to measure oxidative stress in semen was recently added to the 6th edition WHO methods manual, although diagnostic predictive values need to be interpreted with caution as there are still several research questions yet to be answered.

Objectives: To determine the natural fluctuations in semen redox indicators (MiOXSYS® and OxiSperm® II) within and between men and their association with markers of sperm oxidative stress.

Materials and methods: Total, 118 repeat semen samples from 31 generally healthy men aged 18–45 years, over 6 months. Standard semen analysis as per 5th WHO manual. Semen redox levels measured via MiOXSYS® and OxiSperm® II. Additional attributes of sperm quality; HBA® binding assay and sperm hyperactivation and oxidative stress; DNA fragmentation (Halo® Sperm) and lipid peroxidation (BODIPY™ 581/591 C11) were assessed.

Results: Samples with high redox-potential (MiOXSYS® ≥1.47 sORP/10^6 sperm/ml) had lower sperm, motility, morphology and higher DNA fragmentation ($P < 0.05$). Upon further analysis, these associations were driven solely by the adjustment of sperm concentration (10^6/ml) in normalised redox-potential. No significant associations between NBT-reactivity (OxiSperm® II) and measures of the sperm function or oxidative stress were observed ($P > 0.05$). Fluctuations in semen redox levels varied greater between men than within men over the study period.

Discussion: Neither MiOXSYS® nor OxiSperm® II assays were predictive of sperm function or sperm oxidative stress. This was likely due at least in part to limited understanding of their biochemistry and clinical application. As a result, these assays seem to provide no additional clinical utility beyond that of a standard semen analysis,

highlighting the imperative for the development of new robust point-of-care devices for accurately determining sperm oxidative stress.

Conclusion: These findings suggest that MiOXSYS® and OxiSperm® II systems for the measurement of sperm oxidative stress may have limited diagnostic potential.

KEYWORDS
fertility, infertility, ROS, seminal plasma, spermatazoa

1 | INTRODUCTION

Sperm counts are declining globally, with infertility affecting one in 20 men, and a male factor contributing to 30%–50% of all couples seeking assisted reproductive technology (ART) treatment.[1] Currently, the most widely used tool for the diagnosis of male infertility is the semen analysis.[39] However, while sperm count, motility, and morphology reveal useful information for providing a starting point for the evaluation of male infertility, none of these parameters is themselves a direct measure of fertility potential.[25]

Increased semen and sperm reactive oxygen species (ROS) and associated oxidative stress are shown to contribute to up to 80% of all male infertility diagnoses.[37] More recently, diagnostics to measure semen oxidative stress have been added to the recently released 6th edition of the WHO laboratory manual for the examination and processing of human semen, under advanced examinations.[39] Importantly, the 6th edition highlights that their diagnostic predicative values need to be interpreted with caution,[39] as there is still a number of research questions left to be answered before their potential clinical utility may be realised. These include: the fundamental knowledge of their natural fluctuations in men and their ability to correctly determine sperm oxidative stress.[16]

The MiOXSYS® system is a point-of-care device requiring only 30 μl of neat semen in order to assess oxidative stress based on galvanostatic measure of electron availability in semen.[6] It is relatively cheap and takes between 2 and 5 min to run.[16] To date, MiOXSYS® remains the most extensively studied system, reported as being highly sensitive and predictive of male infertility when assessing abnormal semen parameters[2] and sperm DNA fragmentation.[19] However, most of the published literature supporting its clinical effectiveness has been generated by a single research group.[16] To date, only one published study has evaluated whether semen redox-potential as measured by MiOXSYS® fluctuates within individuals over time, reporting in two repeat semen samples that levels varied based on baseline semen analysis results; concentration and motility.[7] Additionally, no studies have assessed the system against measurements of sperm oxidative stress such as sperm lipid peroxidation and/or oxidised DNA damage.

OxiSperm® II (manufactured by Halotech®) measures redox activity through the nitro blue tetrazolium (NBT) assay via a membrane-based gel. In this assay, the yellow NBT molecules are reduced to the insoluble blue crystal (formazan), in the presence of oxidoreductases [i.e., superoxide anion, P450-and cytochrome b5- reductases, nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate].[9] Briefly, at the end of the reaction, the membrane displays a varying intensity of blue colour depending on the level of NBT-reactivity which can then be used to qualitatively categorise a sample as having low, medium, or high redox activity based on the colour scale provided. A further benefit of this assay is the ability to measure redox activity in neat semen, seminal plasma and in sperm cells independently. As such, this second generation assay helps to remove some of the intrinsic biases associated with the original OxiSperm® assay, where high levels of reductase present in seminal plasma could lead to false positive results.[9] While the original OxiSperm® test has previously been shown to be an indicator of male infertility (medium to high reactivity),[17,22,23,33] the clinical utility of the newer OxiSperm® II assay is yet to be confirmed by validation studies.

The aim of this current study was first to evaluate whether oxidative stress, when measured using commercially available methods purported for measuring levels in human semen, fluctuates within and between individual men over time. Understanding the extent of this variation will inform the establishment of decision limits/reference ranges/thresholds to be employed clinically when evaluating redox activity. Second, we aimed to determine whether a relationship exists between measures of the sperm function (hyperactivation and binding to hyaluronan) and oxidative stress (sperm DNA fragmentation and lipid peroxidation) when redox levels are measured using the MiOXSYS® and OxiSperm® II assays.

2 | MATERIALS AND METHODS

2.1 | Ethics/participant recruitment

This project was approved by Human Research Ethics Committee at the University of Adelaide (H-2020-163), and all protocols followed The National Statement on Ethical Conduct in Human Research (2007)—Updated 2018. All participants provided informed consent and were reimbursed for their time. In total, 31 English speaking men between the age of 18 and 45 years were recruited from the general population (2020–2021), providing four semen samples over a 6-month period, at least 4 weeks apart (The ROSS cohort). Twenty-nine participants provided all four semen samples, while two participants provided only a single semen sample, equating to a total of 118 samples. All samples were analysed at the Adelaide Health and Medical

Sciences Building at the University of Adelaide. Exclusion criteria were men with a history of vasectomy or vasectomy reversal, men with undescended testicles or genetic conditions affecting their fertility (i.e., Prader–Willi, Klinefelter's Syndrome etc.), and men with known infectious status, such as HIV/AIDS.

2.2 | Participant characteristics

All men completed a general health questionnaire, providing information such as age, chronic health conditions (i.e., diabetes), current medication use, smoking status, alcohol consumption (frequency/per/week and units consumed) and previously fathered pregnancies at the first semen collection. The questionnaire also included the SF-36 short-form health status survey which provides scores from 0 to 100 about general physical and mental health with a high score indicating better overall health.[15] For subsequent collections men filled in a shortened questionnaire, which included the SF-36 and indications for any changes in chronic conditions, medication use, smoking status, alcohol consumption or fathering of a pregnancy since the previous semen collection. Participant height was measured with a stadiometer (cm) and weight (kg) was measured with electronic scales. Body mass index (BMI) was calculated using the formula weight/height^2 and categorised based as being underweight (< 18.5 kg/m^2), normal weight (18.5–24.9 kg/m^2), overweight (25.0–29.9 kg/m^2) and obesity (> 30.0 kg/m^2). Waist circumference was measured using a standard measuring tape set around the waist at a level midway between the lower rib and the iliac crest. Participants were instructed to fully exhale, with the abdomen relaxed.

2.3 | Semen anaysis

Participants were asked to abstain from ejaculation for 2–7 days prior to semen collection. Semen samples were produced in one of the private, clinical rooms in the University of Adelaide's Clinical Research Facility and collected in a sterile container or produced at home and brought in within 45 min of ejaculation. Only approved lubricant (Ovoil, Vitrolife, Goteborg, Sweden) was used in the study at participant request. After liquefaction at room temperature, a standard semen analysis was performed within 1 h of ejaculation as per WHO V guidelines for the assessment of human semen.[38] Semen volume was measured using a 10 ml serological pipette and semen pH measured using pH strip indicators ranging from pH 4.5 to 10 (Merck, Darmstadt, Germany). Sperm concentration and motility were measured on the CASA® semi-automatic semen analyser (Microptic, Spain, Barcelona). Low- and high-quality control beads (Microptic) were run prior to each sample analysis. A pre-set human count/motility program was used to calculate sperm concentration (10^6/ml), total count (10^6/ejaculate) and proportion of progressive [straightness (STR) > 80% (STR = straight linear velocity/average path velocity×100)], non-progressive (STR < 80%) and immotile sperm. Total motility was calculated by the proportion of progressive and non-progressive sperm. Sperm morphology was assessed after 10 μl sperm smears were made on glass slides and fixed in 100% methanol for 10 min. Diff-Quik® (RAL Diagnostic, Martillac, France) stain was then applied and sperm morphology assessed under 60× objective with 200 sperm classified as either abnormal or normal morphology according to the Kruger strict criteria.[38] The proportion (expressed as %) of sperm with normal morphology was then calculated.

2.4 | Redox-potential: MiOXSYS® system

The MiOXSYS® system (Aytu Bioscience, CO, USA) was used to detect total static oxidative–reductive potential (sORP), or redox-potential, within a semen sample as per manufacture's instructions.[5] The sORP (mV) value for samples was automatically generated on the MiOXSYS® reader and divided by the samples sperm concentration to calculate normalised sORP/10^6 sperm/ml. High and low controls supplied separately by the manufacturer were run monthly, ensuring consistency in machine detection, with the lowest detectable limit being 0.001 sORP (mV). Samples having values greater than or equal to 1.47 sORP/10^6 sperm/ml were classified as having high redox potential.[29]

2.5 | NBT-reactivity: OXISPERM II® test

OxiSperm® II (Halotech DNA, Madrid, Spain) was used as per manufacturer's instructions. The protocol allows for the detection of the redox activity in neat semen, seminal plasma and sperm separately. Seminal plasma was prepared, by spinning the neat semen at 6,000×*g* for 10 min and transferring the seminal plasma fraction to a clean tube. The remaining sperm pellet was resuspended in 50 μl of PBS and re-spun at 6,000×*g* for 10 min a further two times. Following the last spin, the sperm pellet was re-suspended in 7.5 μl of the provided sperm reactivity induction solution and left at room temperature for 5 min. Five microlitres of each sample (neat, seminal plasma and spermatazoa) was added to membrane base and allowed to sit at room temperature for 15 min. Colour reactivity of the membrane gel for each semen component was compared with the colour of the scheme and classified as NBT-LOW (1+), NBT-MEDIUM (2+) or NBT-HIGH (3+). Negative controls were run at each analysis by placing the same volume of PBS onto the control membrane.

2.6 | Sperm hyperactivation

Sperm hyperactivation was assessed on the CASA® semi-automatic system (Microptic) based on the vigorous motility and flagella movements of spermatazoa. Washed motile sperm was allowed to capacitate in G-IVF PLUS (Vitrolife) at 37°C at 5% O_2 and 6% CO_2 for 45 min during the swim up preparation. The percentage of sperm hyperactivation was calculated via the CASA® system based on the proportion of sperm with sperm motility kinetics amplitude of lateral head displacement > 3.5 μM, curvilinear velocity > 80 μM/s and linearity < 20% (straight line velocity/curvilinear velocity ×100).

2.7 | Sperm binding: HBA® hyaluronan binding assay

Sperm binding was assessed using the HBA® hyaluronan binding assay (CooperSurgical Fertility Solutions, Knardrupvej, Denmark) according to Jakab et al.[24] Briefly, 10 μl of semen was loaded into the assay chamber and a Cell-Vu® gridded cover-slip was installed. The sample was incubated for 15 min at 20–30°C. A total of 200 motile sperm was then counted and classified as either bound (head attached and tails moving) or unbound (freely moving). Scores of 80% or higher were classified as normal binding and those below this threshold were classified as displaying reduced binding.[24]

2.8 | Sperm DNA fragmentation: halosperm G2®

Sperm DNA fragmentation was measured using HaloSperm G2® (Halotech DNA, Madrid, Spain) as previously described by Fernandez et al.,[20] and visualised and assessed using the CASA® semi-automatic semen analyser (Microptics) under the pre-set human sperm DNA fragmentation program. Spermatozoa was classified as having small (smaller than 1/3 diameter of the nuclei core), medium (150−170 μm^2), or large (250–280 μm^2) halos, as well as degraded or absent halos. Sperm DNA fragmentation index (DFI) percentage was calculated with the following equation: (fragmented + degraded /200) × 100; in which fragmented DNA included small and absent halos. A pooled control comprised of 16 randomly selected participant samples was stained and analysed at the time of each test to ensure consistency within the assay and reagents; a maximum standard error of 3% was considered an acceptable range.

2.9 | Lipid peroxidation: BODIPY™ 581/591 C11

Motile sperm (1×10^6/ml, > 95% progressive motility) collected following a swim up in G-IVF PLUS (Vitrolife) were incubated in 5 μM of BODIPY 581/591 fluorescent probe for 30 min at 37°C, as previously described by Aitken et al.[14] This reagent localises to membranes throughout live cells and upon oxidation by lipid hydroperoxides, displays a shift in peak fluorescence emission from ~590 nm (red) to ~510 nm (green). Sperm were then centrifuged at 400×*g* for 5 min, the supernatant removed and sperm resuspended in pre-equilibrated (37°C, 5% O_2 and 6% CO_2) G-IVF minus albumin (Vitrolife, Sweden). Lipid peroxidation was assessed on a BD FACSCanto™ II Flow Cytometer (BD Bioscience, NSW, Australia), which had CS&T research beads run daily to ensure fluorescence was kept consistent on measurement days. Then, 10,000 cells per sample were examined and non-specific events gated out. Positive controls of 3,000 μM of hydrogen peroxide spiked preparation were run monthly. Negative controls consisted of spermatozoa incubated in G-IVF medium alone. Lipid peroxidation was expressed as both the ratio shift in red to green fluorescence (a lower red to green ratio indicative of higher lipid peroxidation[30]) and the proportion of sperm that had high lipid peroxidation.[14] Average shift in sperm red to green ratio due to hydrogen peroxide exposure was −1.72 from untreated or a 4.67-fold increase in lipid peroxidation compared with unexposed sperm, indicating that the dye was able to differentiate sperm lipid peroxidation induced from oxidative stress. Details of our gating strategy are presented in Figure S1.

2.10 | Statistical analysis

GraphPad Prism v9.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and IBM SPSS Statistics for Windows, version 26 (IBM Corp., New York, USA) were used for all statistical analyses. In determining whether parameters varied within and between participants, and which contributed greater to the overall variation observed, a repeated measure one-way ANOVA test was performed. Corresponding *F*-values were used, with values closer to 1 indicating less variation.

To determine the relationship between clinical cut-offs for MiOXSYS® (1.47 sORP/10^6 sperm/ml and 38.2 sORP mV) and OxiSperm® II NBT-HIGH (3+) with measure of the sperm function (count, motility, morphology, hyperactivation, binding) and oxidative stress (DNA fragmentation and lipid peroxidation) a general linear model (GLM) was performed. High/low concentrations of ROS for each assay were added as a fixed factor, participant ID as a random factor and the measures of sperm function/oxidative stress as the dependent variable.

To further understand relationships between MiOXSYS® and OxiSperm® II and sperm function and oxidative stress, partial correlations controlling for participant ID initially, and then sperm concentration and normalised sORP/10^6 sperm/ml were used to analyse quantitative data relationships between MiOXSYS®, sperm concentration, sperm function and oxidative stress, while point biserial correlations were performed on associations of OxiSperm® II (count data) with sperm function and oxidative stress.

Except for total sperm motility, NBT-reactivity (OxiSperm® II) and lipid peroxidation ratio, all GLMs and correlation analysis were run on data transformed by natural logarithm which normalised the data. In all cases, statistical significance was inferred when $P < 0.05$.

3 | RESULTS

3.1 | Participant characteristics and baseline semen results

Men recruited into the study were of prime reproductive age, averaging 27 years (ranging 19–37 years), non-smokers (only 2/31 participants) who consumed approximately 1–3 standard alcoholic drinks per week (Table 1). Men in the study were also relatively healthy, with a median general health score of 75/100, median emotional wellbeing score of 72/100, an average BMI of 24.6 kg/m^2 (range 19.0–33.5 kg/m^2) and 26% (8/31) taking prescription medication (predominantly antidepressants) (Table 1). It was also noted

TABLE 1 Participant characteristics

	%	Mean	Median	Range
Anthropological measures				
Age (years)		27	26	19–37
Height (m)		1.79	1.79	1.67–2.02
Weight (kg)		79.7	77.0	56.7–115.4
BMI (kg/m^2)		24.6	24.4	19.0–33.5
Waist circumference (cm)		85.2	83.0	66.0–114.6
Smokes tobacco (yes/no)	6.5 (2/31)			
Alcohol consumption (days/week)		0.9	1	0–3.5
Alcohol consumption (units/per/session)		3.1	1.5	1.5–9
Prescription medication (yes/no)	25.8 (8/31)			
General health score		71.4	75.0	(25–100)
Emotional wellbeing score		68.1	72.0	(20–92)
Semen analysis				
Abstinence (days)		3.5	3.0	0.5–10
pH		8.5	8.5	7.5–9.0
Volume (ml)		3.1	2.9	0.5–11.1
Sperm concentration (10^6/ml)		47.5	39.0	3.0–188.6
Total sperm count (10^6/ejaculate)		145.5	106.5	4.9–603.5
Progressive motility (%)		42.9	39.8	5.0–82.8
Total motility (%)		58.9	57.4	14.7–97.0
Normal morphology (%)		8.1	7.0	0.0–25.0
Reactive oxygen species				
MiOXYSYS® (sORP mV)		39.6	38.2	0.1–200.0
MiOXYSYS® (sORP/10^6 sperm/ml)		1.58	0.87	0.0–14.4
OxiSperm® II (semen)		2.3	2.0	1.0–3.0
OxiSperm® II (seminal plasma)		2.3	2.0	1.0–3.0
OxiSperm® II (sperm)		1.2	1.0	1.0–3.0
Sperm function				
Hyperactivation (%)		15.3	13.0	0.0–42.2
Sperm binding (%)		75.2	79.0	29.5–95.0
Oxidative stress				
DNA fragmentation (%)		13.6	12.5	3.0–29.5
Lipid peroxidation (ratio)		2.75	2.86	0.45–7.01
Lipid peroxidation (%)		25.6	16.4	0.2–99.6

Note: Data are representative of $N = 118$ samples from 31 men. BMI, Body mass index.

that 23% (7/31) of participating men had recorded conceiving a pregnancy.

Of the samples collected, 56.7% (67/118) reported at least one abnormal semen characteristic (Figure S2A), with decreased progressive motility (< 32%) being most prevalent (31%) (Figure S2B). When assessing samples produced from the same man, 58% of men were found to have at least one abnormal semen parameter over their repeat collections (Figure S2B).

When assessing clinical measures of oxidative stress, 67% (21/31) of participants recorded at least one semen sample with high redox potential (≥1.47 sORP/10^6 sperm/ml), while 33% (39/118) of all collected samples were above the suggested cut-off (Figure S2A,B), as measured by MiOXSYS®. The range in baseline sORP mV readings was high across participants (0.1–200) with a median value 38.2 (Table 1). For OxiSperm® II, 74% of men recorded at least one sample with high levels of NBT reactivity (3+) in their neat semen (31% of all semen

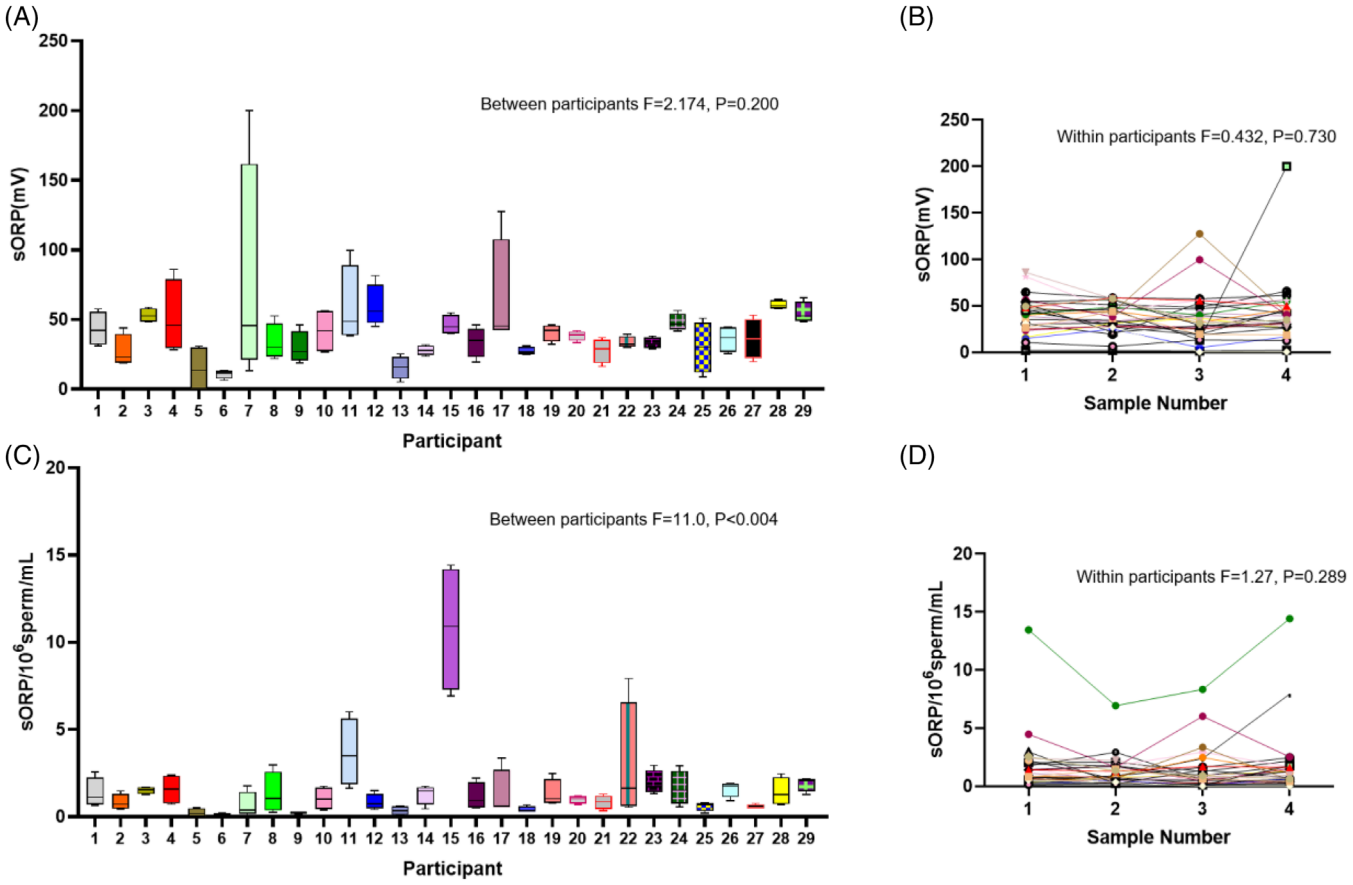

FIGURE 1 Variations in semen redox potential between and within individual men as measured using the MiOXSYS® system. Semen oxidative redox potential sORP (mV) as (A) box and whisker plots for each individual (the box depicts the 25th, 50th and 75th percentiles and the whiskers minimum and maximum values) or (B) line plots for each participant over the collection period. Normalised sORP/10^6 sperm/ml [sORP(mV) divided by sperm concentration 10^6/ml] as (C) box and whisker plots for each individual (the box depicts the 25th, 50th and 75th percentiles and the whiskers minimum and maximum values) or (D) line plots for each participant over the four collections. Data are representative of $N = 116$ samples from 29 men and analysed by a repeated measures one-way ANOVA with corresponding *F*-values closer to one indicating less variation.

samples tested) and this was contributed by high levels of reactivity in their seminal plasma (96%) and not their sperm (4%) (Figure S2A,B). Table 1 also shows the means, medians and ranges of all semen parameters, sperm function tests and oxidative stress indicators measured.

3.2 | Within and between participant variation in clinical measures of oxidative stress MiOXSYS® and OxiSperm® II

We first assessed the variations between and within participants for both MiOXSYS and OxiSperm II by assessing *F*-values. Semen redox potential absolute values sORP (mV) showed low variation both within and between participants ($F = 2.17$ and 0.43 respectively, $P > 0.05$) (Figure 1). Normalised semen redox potential (sORP/10^6 sperm/ml) also showed low variation within participants ($F = 1.27$, $P > 0.05$) (Figure 1), however, between participant variation was found to be much higher ($F = 11.0$, $P < 0.01$) (Figure 1). Interestingly, NBT-reactivity as determined by OxiSperm® showed the opposite, with much higher within participant variation (neat semen: $F = 6.89$, seminal plasma: $F = 6.74$ and spermatazoa: $F = 4.28$, $P < 0.05$) than between participant variation (neat semen: $F = 2.11$, seminal plasma: $F = 2.48$ and spermatazoa: $F = 2.79$, $P > 0.05$).

Between participant variation contributed more greatly to the variation in semen parameters (abstinence: $F = 8.46$, pH: $F = 4.01$, volume: $F = 7.55$, concentration: $F = 7.38$, total count: $F = 5.68$, progressive motility: $F = 6.82$, total motility: $F = 7.13$, and morphology: $F = 7.24$, $P < 0.05$) (Table S1), sperm function (hyperactivation: $F = 7.10$ and sperm binding: $F = 4.44$, $P < 0.05$) (Table S1) and oxidative stress indicator of DNA fragmentation ($F = 8.35$, $P < 0.05$) (Table S1), compared to within participant variation (Table S1). Interestingly, within participant variation contributed greater to the distribution of lipid peroxidation ratio ($F = 3.33$, $P < 0.05$) than between participant variation (Table S1).

3.3 | Relationship between high MiOXSYS and Oxisperm II with sperm function and oxidative stress

Using the clinical cut-offs of ≥1.47 sORP/10^6 sperm/ml for MiOXSYS® and 2+ and 3+ (medium-high NBT-reactivity) for OxiSperm® II, we

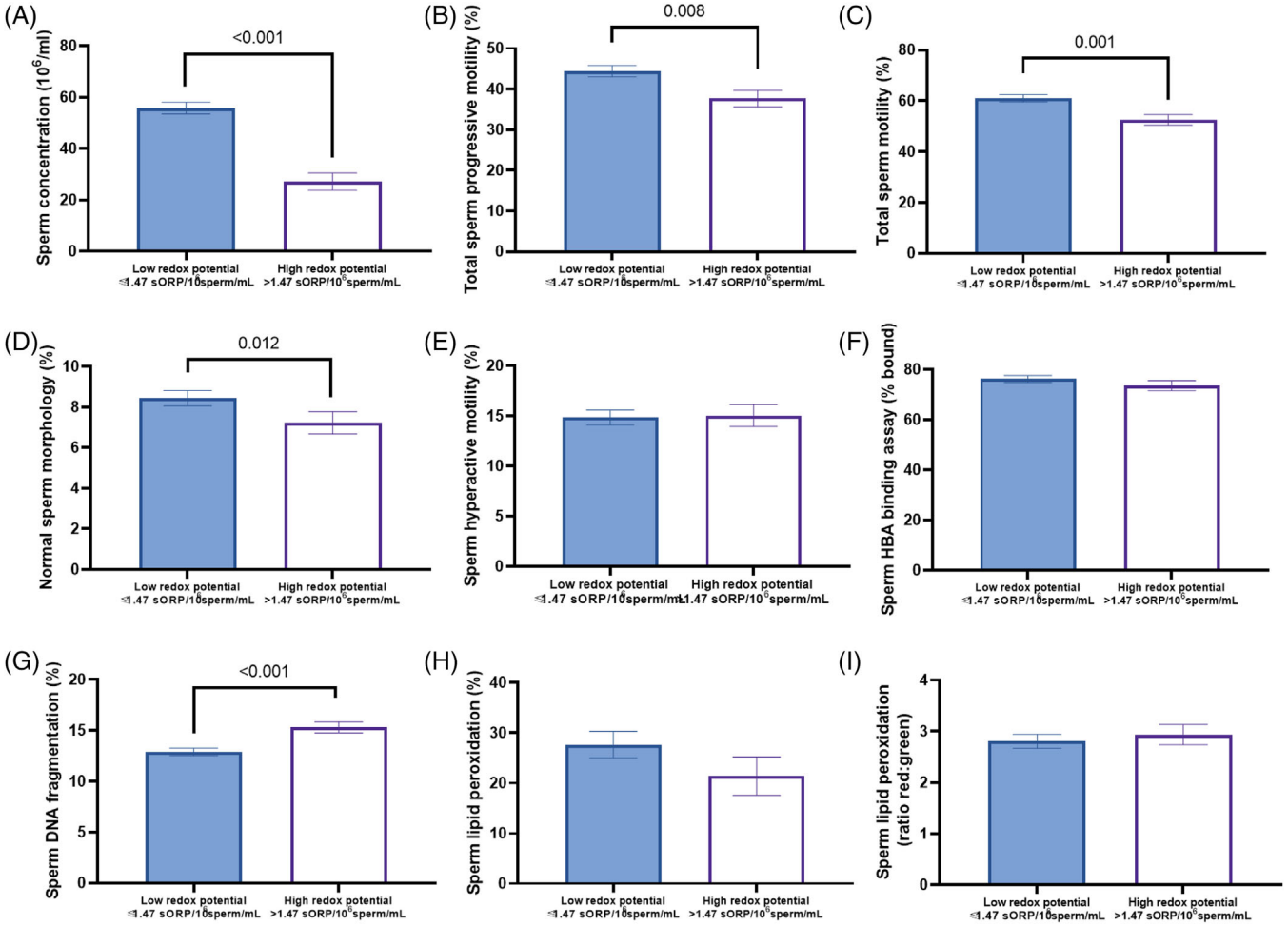

FIGURE 2 The relationship between high semen redox potential (MiOXSYS®) with measures of sperm function and oxidative stress when assessing sORP/10^6 sperm/ml. Normalised high redox potential (> 1.47 sORP/10^6 sperm/ml) or low redox potential (< 1.47 sORP/10^6 sperm/ml) on measures of sperm function (A) concentration 10^6/ml, (B) progressive motility, (C) total motility, (D) normal morphology, (E) hyperactive motility, (F) sperm binding and sperm oxidative stress, (G) DNA fragmentation, (H) proportion of lipid peroxidation and (J) lipid peroxidation ratio of red:green fluorescence. Data are representative of $N = 118$ samples from 31 men and analysed by a general linear model with participant added as a random factor.

next assessed their potential relationships with sperm function and oxidative stress. Samples with normalised sORP/10^6 sperm/ml readings above 1.47 were found to have lower sperm concentration ($P < 0.001$), progressive motility ($P = 0.008$), total sperm motility ($P = 0.001$) and normal sperm morphology ($P = 0.012$) and higher sperm DNA fragmentation ($P < 0.001$) (Figure 2). No differences were seen for sperm hyperactive motility, sperm binding or sperm lipid peroxidation ($P > 0.05$) (Figure 2). However, as normalised sORP is adjusted by sperm concentration we also sought to determine whether any relationship existed between high semen redox potential as measured by baseline sORP mV reading. Using the median sORP mV value from our population (38.2), we reassessed outcomes following classification as either high > 38.2 or low < 38.2 sORP mV. There was no effect of high semen redox potential, expressed as sORP mV, with any measure of sperm function or oxidative stress ($P > 0.05$) (Figure 3).

There were no differences between low (1), medium (2) or high (3) levels of NBT-reactivity in neat semen (OxiSperm® II) on sperm function (concentration, progressive motility, total motility, normal morphology, hyperactive motility, sperm binding) or oxidative stress (DNA fragmentation and lipid peroxidation) ($P > 0.05$) (Figure 4). No differences were also found for most measures of sperm function and oxidative stress using NBT-reactivity in seminal plasma ($P > 0.05$) (Figure S3) and sperm ($P > 0.05$) (Figure S4). However, sperm DNA fragmentation was found to be reduced in those samples with seminal plasma NBT-reactivity of medium (2+) or high (3+) compared with those that scored low (1+, $P < 0.05$) (Figure S3) and normal sperm morphology was reduced in those samples with medium/high (2+) NBT-reactivity in spermatazoa compared with those that scored low (+1, $P < 0.05$) (Figure S4). Further, lipid peroxidation ratios were increased in those samples with medium/high (2+) NBT-reactivity in sperm compared with those that scored low (+1, $P < 0.05$, Figure S4).

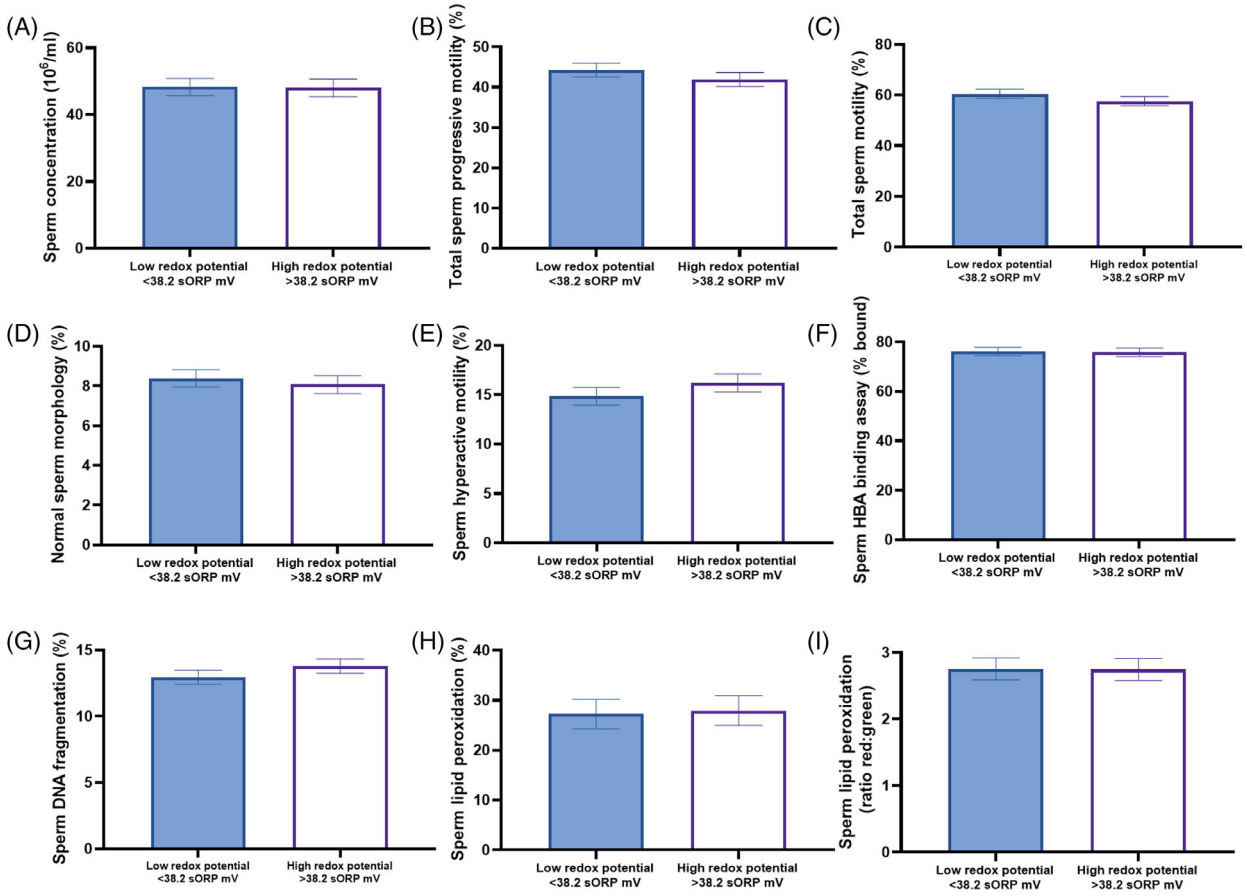

FIGURE 3 The relationship between high semen redox potential (MiOXSYS®) with measures of sperm function and oxidative stress when assessing sORP (mV). High redox potential (> 38.2 sORP mV) or low redox potential (< 38.2 sORP mV) on measures of sperm function (A) concentration 10^6/ml, (B) progressive motility, (C) total motility, (D) normal morphology, (E) hyperactive motility, (F) sperm binding and sperm oxidative stress, (G) DNA fragmentation, (H) proportion of lipid peroxidation and (J) lipid peroxidation ratio of red:green fluorescence. Data are representative of $N = 118$ samples from 31 men and analysed by a general linear model with participant added as a random factor.

3.4 | Correlations between clinical measures of ROS (MiOXSYS and OxiSperm II) to sperm function and oxidative stress

Next, we assessed whether MiOXSYS® and OxiSperm® II values correlated with measures of sperm function and oxidative stress, irrespective of their suggested clinical cut-offs. The only relationship observed between semen absolute sORP (mV), as measured by MiOXSYS®, and any sperm function or oxidative stress indicator, was a weak positive association with the proportion of sperm positive for lipid peroxidation (0.167, $P < 0.05$) (Table 2). Normalised sORP/10^6 sperm/ml was strongly negatively associated with sperm concentration (−0.749, $P < 0.001$) and moderately negatively associated with total sperm count (−0.584, $P < 0.001$, Table 2). This is not surprising given sperm concentration is the denominator used to determine normalised sORP. Further, normalised sORP/10^6 sperm/ml was moderately negatively associated with total sperm motility (−0.385, $P < 0.001$) and weakly negatively associated with progressive sperm motility (−0.224, $P < 0.01$), sperm normal morphology (−0.282, $P < 0.01$), sperm binding (−0.176, $P < 0.05$) and sperm DNA fragmentation (−0.277, $P < 0.01$) (Table 2).

Next, since normalised sORP/10^6 sperm/ml is calculated by dividing sORP (mV) by sperm concentration, we wanted to examine whether these associations persisted after adjusting for sperm concentration. Following adjustment for sperm concentration, the negative associations observed previously between normalised sORP/10^6 sperm/ml and sperm function and oxidative stress were no longer present ($P > 0.05$) (Table 3). However, new correlations were revealed, with normalised sORP/10^6 sperm/ml weakly positively associated with abstinence (0.203, respectively, $P < 0.05$) (Table 3) and proportion of sperm positive for lipid peroxidation (0.224, $P < 0.05$) (Table 3). To further investigate whether the adjustment for sperm concentration in the calculation of normalised sORP/10^6 sperm/ml was the primary driver of the association of MiOXSYS® with measures of the sperm function and oxidative stress, we again performed partial correlations of the sperm concentration adjusting for normalised sORP/10^6 sperm/ml. In these analyses, the sperm concentration was strongly positively associated with total sperm count (0.643, $P < 0.001$),

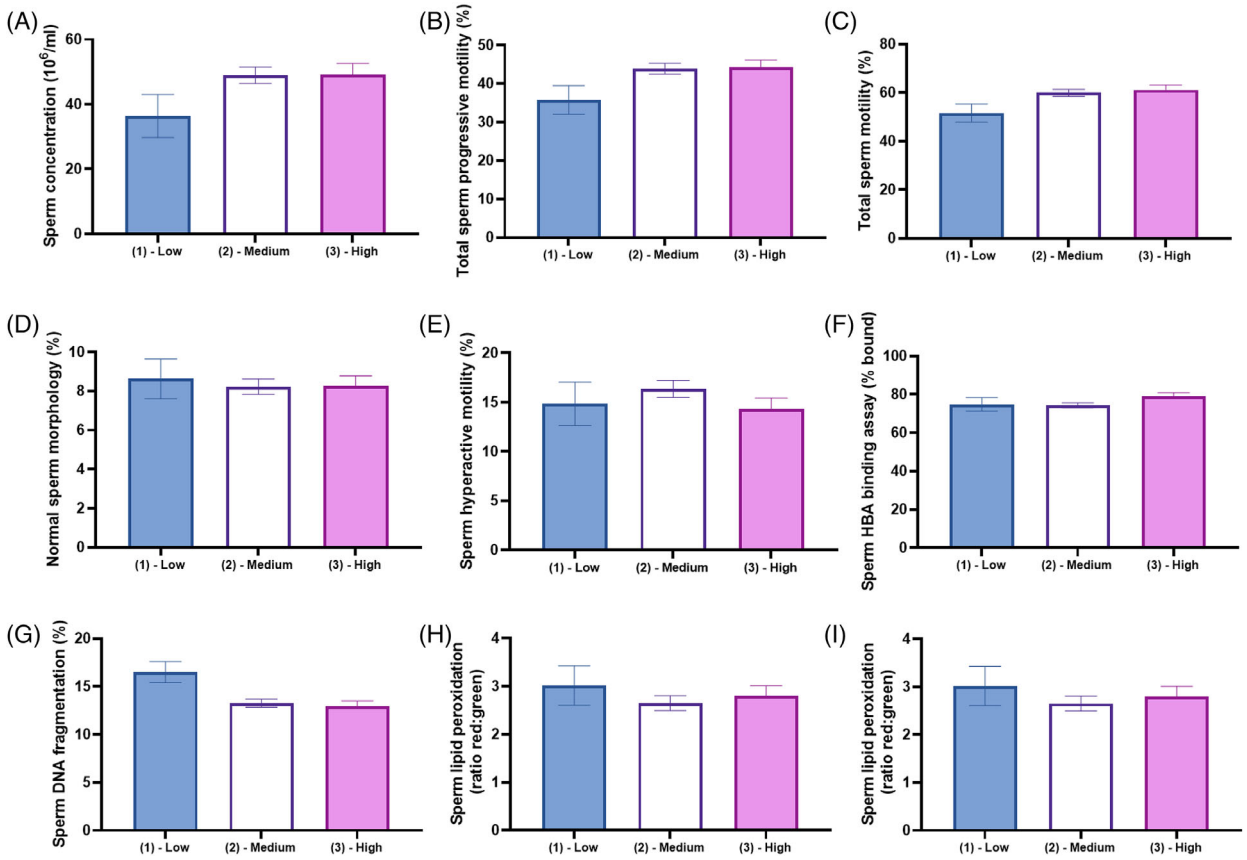

FIGURE 4 The relationship between high neat semen redox activity (OxiSperm® II) with measures of sperm function and oxidative stress. Effect of differing levels of NBT-reactivity (1—low, 2—medium and 3—high) on measures of sperm function (A) concentration 10^6/ml, (B) progressive motility, (C) total motility, (D) normal morphology, (E) hyperactive motility, (F) sperm binding and sperm oxidative stress, (G) DNA fragmentation, (H) proportion of lipid peroxidation and (J) lipid peroxidation ratio of red:green fluorescence. Data are representative of $N = 118$ samples from 31 men and analysed by a general linear model with participant added as a random factor.

moderately positively associated with total motility (0.356, $P < 0.001$) and normal morphology (0.400, $P < 0.01$), moderately negatively associated with DNA fragmentation (−0.310, $P = 0.001$) and weakly positively associated with progressive motility (0.230, $P = 0.008$) (Table S2).

Very few associations were observed between NBT-reactivity, as measured by OxiSperm® II, and either sperm function or oxidative stress (Table 2). High levels of NBT reactivity in both semen and seminal plasma were weakly positively associated with sperm binding (0.223 and 0.262, respectively, $P < 0.05$), and weakly negatively associated with proportion of sperm positive for high lipid peroxidation (−0.170 and −0.195, respectively, $P < 0.05$), while high NBT reactivity in spermatozoa was weakly negatively associated with normal sperm morphology (−0.225, $P < 0.01$) and positively with lipid peroxidation ratio (0.257, $P < 0.01$) (Table 2).

There were also no correlations found between NBT reactivity in semen, seminal plasma or spermatozoa with sORP (mV) (−0.081, −0.108 and 0.07, respectively, $P > 0.05$) or normalised sORP/10^6 sperm/ml as measured by MiOXSYS® (−0.145, −0.131 and 0.085, respectively, $P > 0.05$).

4 | DISCUSSION

High concentrations of ROS in sperm are considered one of, if not, the main contributor to male idiopathic infertility.[3,37] Despite this, there remains no widespread, point-of-care assay that can be employed in clinics to give a rapid assessment of sperm oxidative stress. A potential explanation for this is because fundamental research questions remain, with their ability to accurately measure sperm oxidative stress and whether natural fluctuations in oxidative stress occur within and between men over time are not fully understood.[16] Utilising two commercially available tests marketed as clinical diagnostics for the redox activity in semen (MiOXSYS® and OxiSperm® II), we aimed to examine whether natural fluctuations in semen redox levels occur over time as well as understand their associations with measures of sperm function and oxidative stress over a 6-month period. We found that while normalised redox potential (≥1.47 sORP/10^6 sperm/ml), as measured by MiOXSYS®, was associated with lower sperm concentration, motility, morphology and higher DNA fragmentation, these associations were solely driven by sperm concentration (10^6/ml). Further supporting sperm concentration as independently driving these

TABLE 2 Correlations between MiOXSYS® and OxiSperm® II to sperm function and oxidative stress

	MiOXSYS (sORP mV)	MiOXSYS (sORP/10^6sperm/ml)	OxiSperm II (semen)	OxiSperm II (seminal plasma)	OxiSperm II (sperm)
Abstinence (days)					
R^2	0.149	−0.048	−0.036	−0.033	−0.084
P-Value	0.063	0.312	0.355	0.367	0.189
Concentration (10^6/ml)					
R^2	−0.88	**−0.749**	0.100	0.044	−0.059
P-Value	0.182	**<0.001**	0.148	0.324	0.269
Total count (10^6/ejaculate)					
R^2	−0.072	**−0.584**	0.243	0.013	−0.071
P-Value	0.230	**<0.001**	0.112	0.445	0.227
Progressive motility (%)					
R^2	−0.008	**−0.244**	0.119	0.073	0.020
P-Value	0.468	**0.006**	0.105	0.222	0.416
Total motility (%)					
R^2	−0.031	**−0.385**	0.064	0.006	-0.026
P-Value	0.374	**<0.001**	0.253	0.474	0.393
Morphology (%)					
R^2	0.017	**−0.282**	−0.073	−0.094	**−0.225**
P-Value	0.429	**0.001**	0.223	0.162	**0.009**
Sperm binding (%)					
R^2	−0.033	**−0.176**	**0.233**	**0.262**	0.032
P-Value	0.368	**0.034**	**0.007**	**0.003**	0.369
Hyperactivation (%)					
R^2	−0.075	−0.150	0.025	0.035	0.000
P-Value	0.219	0.059	0.399	0.361	0.499
DNA fragmentation (%)					
R^2	−0.001	**0.277**	−0.135	−0.097	−0.029
P-Value	0.494	**0.002**	0.079	0.155	0.382
Lipid peroxidation (ratio)					
R^2	0.003	0.117	0.081	0.069	**0.257**
P-Value	0.486	0.114	0.198	0.236	**0.003**
Lipid peroxidation (%)					
R^2	**0.167**	−0.100	**−0.170**	**−0.195**	−0.045
P-Value	**0.037**	0.144	**0.037**	**0.020**	0.320

Note: Data are representative of $N = 118$ samples from 31 men and analysed by partial correlations adjusting for participant ID for MiOXSYS® and point biserial for NBT-reactivity (OxiSperm® II). Bold values indicate the statistical significance.

correlations and not redox-potential, was the observation that baseline sORP mV values had little to no relationship with sperm function or oxidative stress. Further, low (1+), medium (2+) or high (3+) NBT-reactivity (OxiSperm® II) and measures of sperm function or oxidative stress (DNA fragmentation and lipid peroxidation) were only minimally associated, suggesting these assays may have little to no diagnostic potential for accurately measuring sperm oxidative stress in humans.

This study initially set out to use two commercial assays to measure oxidative stress in human semen in order to understand the normal variation that occurs within and between individuals from the general population to help determine their clinical decision limits. Unfortunately, in this study we found MiOXSYS® and OxiSperm® II to have significant limitations. First, it appears that the clinical utility of the MiOXSYS® system may be exaggerated by adjusting to the sperm concentration. Using the sperm concentration as a denominator when expressing the sORP results was originally introduced as it was believed that sperm would be the major source of electrons in the ejaculate.[4] However, a recent publication by Joao et al.[26] instead shows that the presence of sperm in seminal plasma is unrelated to the baseline sORP (mV) readings, and as a result, using the ratio

TABLE 3 Correlation between MiOXSYS®, sperm function and oxidative stress after adjusting for sperm concentration

	MiOXSYS® (sORP/10^6 sperm/ml)
Abstinence (days)	
R^2	**0.203**
P-Value	**0.018**
Total count (10^6/ejaculate)	
R^2	0.002
P-Value	0.493
Progressive motility (%)	
R^2	0.005
P-Value	0.481
Total motility (%)	
R^2	0.481
P-Value	0.105
Morphology (%)	
R^2	0.118
P-Value	0.112
Sperm binding (%)	
R^2	−0.025
P-Value	0.401
Hyperactivation (%)	
R^2	−0.026
P-Value	0.396
DNA fragmentation (%)	
R^2	−0.034
P-Value	0.363
Lipid peroxidation (ratio)	
R^2	−0.085
P-Value	0.191
Lipid peroxidation (%)	
R^2	**0.224**
P-Value	**0.008**

Note: Data are representative of $N = 118$ samples from 31 men and analysed by partial correlations adjusting for participant ID and sperm concentration (10^6/ml). Bold values indicate the statistical significance.

correction method to normalise data overestimates the sORP/10^6 sperm/ml value in patients with low sperm concentration. This is because normalisation to sperm concentration does not take into account the observation that sperm concentration itself has independent associations with measures of sperm function and oxidative stress.[11] In this study, we found that all the associations of normalised redox potential (sORP/10^6 sperm/ml) with sperm function and oxidative stress were being driven by its adjustment with sperm concentration, and the independent negative associations low sperm concentration had with count, motility, morphology and hyperactive motility and positive associations with DNA fragmentation. As such, for MiOXSYS® to be clinically meaningful, baseline sORP (mV) readings need to be the measure used for determining its ability to predict both male infertility and sperm oxidative stress. In our study, sORP (mV) was found to be largely unrelated to measures of sperm function and oxidative stress, although we do acknowledge a weak positive association with sperm lipid peroxidation.

A key reason why MiOXSYS® may not be able to accurately predict sperm function and oxidative stress may come down to its underlying biochemistry and biological application. Oxidation–reduction measures the potential for electrons to move from one chemical species to another thereby being reduced or oxidised, respectively, with oxidisers having a positive ORP value and reducers having a negative ORP value.[36] Therefore, ORP is dependent on the total concentrations of reductants and oxidants in a particular system with positive ORP suggestive of a larger oxidant concentration.[36] ROS molecules including superoxide anion, hydroxyl radicals, hydrogen peroxide, nitric oxide, peroxynitrites all contribute to the oxidant level, while molecules including: thiols, vitamin C, tocopherol, β-carotene, lycopene, uric acid, bilirubin and flavonoids contribute as reductants.[27] Importantly, ROS are not the only molecules that contribute to the oxidant levels of biological fluids.[31] It has since been established that semen sORP (mV) readings are not influenced by sperm,[26] which leads to the question of 'how is the redox status of a fluid that is created instantaneously at ejaculation by sex accessory glands (seminal plasma) going to be predictive of the oxidative stress status of sperm?'. This is because the majority of oxidative damage to sperm occurs between spermiogenesis and epididymal maturation prior to mixing with seminal plasma at ejaculation.[8,10,12] Additionally, seminal plasma is a potent source of some of the most highly specialised antioxidants and scavenging enzymes of any biological fluid [i.e., glutathione peroxidase (GPx5), extracellular superoxide dismutase (SOD), uric acid, vitamin C, tyrosine and polyphenols] which act to protect against oxidative damage to sperm.[12] As such, we should consider whether there is any benefit at all to focus on developing point-of-care devices that measure redox status of seminal plasma if what we really need to understand is the status of sperm?

Another important consideration when developing new methods for measuring ROS/oxidative stress in semen for evaluating male infertility is which component of the ejaculate is most informative? One might argue that the oxidative stress status of the motile sperm fraction would be the most important, given these sperm are those most likely to go on to fertilise the egg and contribute to fetal development. However, one may also contend that given recent advances in better understanding the complex role seminal plasma plays in promoting fertility and fecundity through its beneficial effects on the female immune system,[35] that a measure of oxidative stress of the entire ejaculate might be more informative.

There are also many factors that limit oxidation reduction potential interpretation, including; temperature, pH, irreversible reactions, slow electrode kinetics, non-equilibrium, small exchange currents and presence of multiple redox couples.[28] Recently, a study by Garcia-Segura et al.[21] reported that despite correcting for semen pH, semen redox-potential remained unrelated to measures of sperm DNA

fragmentation as assessed by the single-cell gel electrophoresis assay. Therefore, in this study it appears that MiOXSYS® provides no additional diagnostic information for the assessment of male infertility, or sperm oxidative stress, beyond that of a standard semen analysis.

Similar to the MiOXSYS® system, the inability of the OxiSperm® II assay to determine sperm oxidative stress is also likely related to the biochemistry of the assay. While it is correct that the NBT will undergo reduction in the presence of superoxide, any molecule capable of giving up an electron is equally able to reduce NBT, including NADH and phenazine methosulphate.[32] Further, in vitro studies using human sperm have shown that exposure to the sperm mitochondrial ROS generators arachidonic acid or 4-hydroxynonenal were unable to modify NBT reactivity.[13] However, only the potent redox cycler menadione was able to modify NBT reactivity in human sperm, although high background readings were also present.[13] As such, it cannot be stated with certainty that the high levels (3+) of NBT reactivity are the result of high superoxide concentrations in the ejaculate. The lack of association between OxiSperm® II and sperm function and oxidative stress observed in this study is consistent with previous studies using the original OxiSperm® assay. In these, it was reported that NBT-reactivity did not correlate with male infertility, sperm parameters, fertility rates, sperm DNA damage nor pregnancy and live birth rates, with only 31%–76% of infertile men displayed medium to high redox activity (2+ and 3+).[17,22,23,33] Further, superoxide production in sperm, as measured using the MitoSOX red probe, has been previously reported as being predictive of human sperm DNA damage as measured by Halosperm® and the Comet assay,[34] suggesting the ability of OxiSperm® II to correctly identify superoxide concentrations is limited.

Although, the newer OxiSperm® II assay offers the ability to assess the redox activity in individual components of the ejaculate (neat semen, seminal plasma, and spermatozoa), the qualitative nature of the assay creates considerable risk of bias, thereby limiting the utility of the assay. This bias is likely to take the form of subjective differences in colour perception between operators, with the potential to result in different interpretations as to what is classified as low (1+), medium (2+) or high (3+).[16] Even though ~58% of participants in our study had at least one abnormal semen analysis over the collection period, the majority of the high (3+) OxiSperm® II observations came from the seminal plasma fraction. This is consistent with evidence showing that high levels of reductase present in seminal fluid reduce the NBT to formazan, creating false positive results.[9] As a result, it appears that the newer OxiSperm® II assay may also be unable to correctly determine sperm oxidative stress which acts to limit its clinical application.

Studies examining between- and within-individual variation in semen redox status are also limited. Using chemiluminescence, Zorn et al.[40] reported ROS levels as being relatively consistent in 25 infertile men who had repeat measures taken over a 6-month period. In contrast, a longitudinal study (21-month period) conducted in healthy, fertile men found ROS concentrations to be quite variable, reporting these fluctuations as appearing independent to sperm count, motility and morphology.[18] The investigators concluded that the variation was likely attributable to changes in ejaculation frequency and seasonal/lifestyle variation. Interestingly, in our study, frequency of ejaculation and BMI were stable within individuals over time, as was good general health, which likely contributed to the low level of variation we observed in semen redox-potential and activity over time.

An important limitation of our study was only accessing relatively healthy men from the general population, thereby limiting the number of infertile men in our cohort. However, we did not want to bias our findings by only evaluating men seeking infertility treatment. Despite this limitation, approximately 30% of all samples analysed in our study were found to have an abnormal characteristic in their basic sperm parameters. Further, it must be noted that majority of high (3+) redox activity readings in neat semen in our study were due to increased seminal plasma redox activity and not sperm redox activity, raising the possibility that our samples were biased towards those with low-sperm redox activity. Finally, further studies are required to determine whether similar observations to those described in our study are also observed in sub fertile populations.

5 | CONCLUSION

Appropriately designed and suitably powered studies are required to determine the clinical utility of both MiOXSYS® and OxiSperm® II assays, as the balance of evidence suggests that these tests may have been prematurely introduced clinically, before a rigorous understanding of their underlying biochemistry was known. As such, it appears from this study that these assays provide no additional clinical utility beyond that of a standard semen analysis. The development of new robust point-of-care devices for accurately determining oxidative stress in spermatozoa is therefore warranted.

AUTHOR CONTRIBUTIONS

Acquisition, data analysis, drafting of manuscript: Patience Castleton. *Acquisition, data analysis*: Prabin Gyawali. *Conception, design, acquisition*: Nicola Mathews. *Acquisition, data analysis*: Shadrack Mulinge Mutuku. *Design, data analysis, interpretation, editing of manuscript*: David James Sharkey. *Conception, design, acquisition, analysis, interpretation, drafting and editing of manuscript*: Nicole Olivia McPherson.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Agarwal A, Baskaran S, Parekh N, et al. Male infertility. *The Lancet*. 2021;397:319-333.
2. Agarwal A, Panner Selvam MK, Arafa M, et al. Multi-center evaluation of oxidation-reduction potential by the mioxsys in males with abnormal semen. *Asian J Androl*. 2019;21:565-569.
3. Agarwal A, Parekh N, Panner Selvam MK, et al. Male oxidative stress infertility (mosi): proposed terminology and clinical practice guidelines for management of idiopathic male infertility. *World J Mens Health*. 2019;37:296-312.

4. Agarwal A, Roychoudhury S, Bjugstad KB, Cho CL. Oxidation–reduction potential of semen: what is its role in the treatment of male infertility? *Ther Adv Urol*. 2016;8:302-318.
5. Agarwal A, Sharma R, Roychoudhury S, Du Plessis S, Sabanegh E. Mioxsys: a novel method of measuring oxidation reduction potential in semen and seminal plasma. *Fertil Steril*. 2016;106:566-573.e510.
6. Agarwal A, Sharma R, Roychoudhury S, Du Plessis S, Sabanegh E. Mioxsys: a novel method of measuring oxidation reduction potential in semen and seminal plasma. *Fertil Steril*. 2016;106:566-573 e510.
7. Agarwal A, Wang SM. Clinical relevance of oxidation-reduction potential in the evaluation of male infertility. *Urology*. 2017;104:84-89.
8. Aitken J, Fisher H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays*. 1994;16:259-267.
9. Aitken RJ. Nitroblue tetrazolium (nbt) assay. *Reprod Biomed Online*. 2018;36:90-91.
10. Aitken RJ, Baker MA. Reactive oxygen species generation by human spermatozoa: a continuing enigma. *Int J Androl*. 2002;25:191-194.
11. Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, Wu FC. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl*. 1989;10:214-220.
12. Aitken RJ, Drevet JR. The importance of oxidative stress in determining the functionality of mammalian spermatozoa: a two-edged sword. *Antioxidants (Basel)*. 2020;9(2):111. https://doi.org/10.3390/antiox9020111
13. Aitken RJ, Smith TB, Lord T, et al. On methods for the detection of reactive oxygen species generation by human spermatozoa: analysis of the cellular responses to catechol oestrogen, lipid aldehyde, menadione and arachidonic acid. *Andrology*. 2013;1:192-205.
14. Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. Analysis of lipid peroxidation in human spermatozoa using bodipy c11. *Mol Hum Reprod*. 2007;13:203-211.
15. Brazier JE, Harper R, Jones NM, et al. Validating the sf-36 health survey questionnaire: new outcome measure for primary care. *BMJ*. 1992;305:160-164.
16. Castleton PE, Deluao JC, Sharkey DJ, McPherson NO. Measuring reactive oxygen species in semen for male preconception care: a scientist perspective. *Antioxidants (Basel)*. 2022;11(2):264. https://doi.org/10.3390/antiox11020264
17. Degirmenci Y, Demirdag E, Guler I, Yildiz S, Erdem M, Erdem A. Impact of the sexual abstinence period on the production of seminal reactive oxygen species in patients undergoing intrauterine insemination: a randomized trial. *J Obstet Gynaecol Res*. 2020;46:1133-1139.
18. Desai NR, Mahfouz R, Sharma R, Gupta S, Agarwal A. Reactive oxygen species levels are independent of sperm concentration, motility, and abstinence in a normal, healthy, proven fertile man: a longitudinal study. *Fertil Steril*. 2010;94:1541-1543.
19. Elbardisi H, Finelli R, Agarwal A, Majzoub A, Henkel R, Arafa M. Predictive value of oxidative stress testing in semen for sperm DNA fragmentation assessed by sperm chromatin dispersion test. *Andrology*. 2020;8:610-617.
20. Fernández JL, Muriel L, Goyanes V, et al. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertil Steril*. 2005;84:833-842.
21. Garcia-Segura S, Ribas-Maynou J, Lara-Cerrillo S, et al. Relationship of seminal oxidation–reduction potential with sperm DNA integrity and pH in idiopathic infertile patients. *Biology (Basel)*. 2020;9(9):262. https://doi.org/10.3390/biology9090262
22. Gosalvez J, Coppola L, Fernandez JL, et al. Multi-centre assessment of nitroblue tetrazolium reactivity in human semen as a potential marker of oxidative stress. *Reprod Biomed Online*. 2017;34:513-521.
23. Iommiello VM, Albani E, Di Rosa A, et al. Ejaculate oxidative stress is related with sperm DNA fragmentation and round cells. *Int J Endocrinol*. 2015;2015:321901.
24. Jakab A, Sakkas D, Delpiano E, et al. Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. *Fertil Steril*. 2005;84:1665-1673.
25. Jequier AM. Semen analysis: a new manual and its application to the understanding of semen and its pathology. *Asian J Androl*. 2010;12:11-13.
26. Joao F, Duval C, Belanger MC et al. Reassessing the interpretation of oxidation–reduction potential in male infertility. *Reprod Fertil*. 2022;3:67-76.
27. Kohen R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol Pathol*. 2002;30:620-650.
28. Moore GR, Pettigrew GW, Rogers NK. Factors influencing redox potentials of electron transfer proteins. *Proc Natl Acad Sci USA*. 1986;83:4998-4999.
29. Selvam P, K M, Agarwal A, et al. The effect of oxidative and reductive stress on semen parameters and functions of physiologically normal human spermatozoa. *Free Radic Biol Med*. 2020;152:375-385.
30. Pap EH, Drummen GP, Winter VJ, et al. Ratio-fluorescence microscopy of lipid oxidation in living cells using c11-bodipy(581/591). *FEBS Lett*. 1999;453:278-282.
31. Papi A, Chicca M, Pandit A, Caramori G. Oxidants and antioxidants | antioxidants, nonenzymatic. In: Laurent GJ, Shapiro SD, eds. *Encyclopedia of Respiratory Medicine*. Academic Press; 2006:266-271.
32. Ponti V, Dianzani MU, Cheeseman K, Slater TF. Studies on the reduction of nitroblue tetrazolium chloride mediated through the action of NADH and phenazine methosulphate. *Chem Biol Interact*. 1978;23:281-291.
33. Pujol A, Obradors A, Esteo E, et al. Oxidative stress level in fresh ejaculate is not related to semen parameters or to pregnancy rates in cycles with donor oocytes. *J Assist Reprod Genet*. 2016;33:529-534.
34. Riley L, Ammar O, Mello T, Giovannelli L, Vignozzi L, Muratori M. Novel methods to detect ROS in viable spermatozoa of native semen samples. *Reprod Toxicol*. 2021;106:51-60.
35. Robertson SA, Sharkey DJ. Seminal fluid and fertility in women. *Fertil Steril*. 2016;106:511-519.
36. Shapiro HM. Redox balance in the body: an approach to quantitation. *J Surg Res*. 1972;13:138-152.
37. Tremellen K. Oxidative stress and male infertility—a clinical perspective. *Hum Reprod Update*. 2008;14:243-258.
38. WHO. In: Wilson JS, ed. *WHO Laboratory Manual for the Examination and Processing of Human Semen* 5th ed. Cambridge University Press; 2010.
39. WHO. *WHO Laboratory Manual for the Examination and Processing of Human Semen*. 6th ed. Cambridge University Press; 2021.
40. Zorn B, Osredkar J, Meden-Vrtovec H, Majdic G. Leptin levels in infertile male patients are correlated with inhibin b, testosterone and SHBG but not with sperm characteristics. *Int J Androl*. 2007;30:439-444.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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