



Original article

The effect of oxidative and reductive stress on semen parameters and functions of physiologically normal human spermatozoa



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ABSTRACT

Both oxidative stress (OS) and reductive stress (RS) are the two extreme facets of redox imbalance that can have deleterious effects on sperm function. However, there is a lack of information on the physiological range of oxidation-reduction potential (ORP). The aim of this study was to investigate the effect of OS and RS on functions and associated molecular changes in normal spermatozoa in order to establish the physiological range of ORP. In the current study, total and progressive motility remained unchanged in spermatozoa exposed to ORP values 0.33 and 0.72 mV/10⁶ sperm/mL. However, a significant ($P < 0.05$) decline in total and progressive motility were observed at ORP values 1.48, 2.75, −11.24, −9.76 and −9.35 mV/10⁶ sperm/mL. Sperm vitality also decreased significantly ($P < 0.0001$) at 2.75, −11.24 and −9.76 mV/10⁶ sperm/mL. Spermatozoa exposed to ORP levels 2.75 and −11.24 mV/10⁶ sperm/mL showed a significant ($P < 0.01$) decrease in mitochondrial membrane potential. Intracellular reactive oxygen species (iROS) production increased ($P < 0.05$) in spermatozoa exposed to ORP levels of 1.48 and 2.75 mV/10⁶ sperm/mL, while iROS decreased ($P < 0.05$) at ORP levels −9.76 and −11.24 mV/10⁶ sperm/mL. No significant change in sperm DNA fragmentation was noted in sperm exposed to OS/RS and the values were below the reference range ($< 19.25\%$). Western blot analysis revealed decreased expression of CV-ATPase, CIII-UQCRC2 and CIV-MTCO1 proteins at 60 and 120 min ($P < 0.05$) in both OS and RS conditions. This is the first study to report physiological range of ORP (between −9.76 and 1.48 mV/10⁶ sperm/mL) and to elucidate the role of altered expression of oxidative phosphorylation (OXPHOS) complexes proteins in mediating detrimental effects of oxidative and reductive conditions on sperm functions. A decreased expression of OXPHOS proteins and associated mitochondrial dysfunction contributes to decreased sperm motility and vitality under oxidative and reductive stress.

1. Introduction

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are highly unstable molecules that are released during the cellular response to xenobiotics, cytokines, bacterial invasion and mitochondrial oxidative metabolism [1]. In spermatozoa, ROS are widely known for their role as second messenger in crucial cellular events related to the fertilization process, such as capacitation, acrosome reaction, hyperactivation and sperm-oocyte fusion [2]. Therefore, a physiological amount of ROS is

essential for normal male fertility potential [2]. White blood cells and abnormal and immature spermatozoa are responsible for excessive ROS production in the ejaculate [1,2]. Due to their molecular instability, excess ROS can damage cellular components resulting in lipid peroxidation of sperm membrane, loss of protein function and genomic and mitochondrial DNA fragmentation, thereby negatively affecting sperm functions and male reproductive potential [2].

Normally, the action of these highly reactive molecules is counterbalanced by several antioxidant systems that limit their harmful effects [3]. Consequently, a balance between ROS and antioxidant systems in

Abbreviations: CH, cumene hydroperoxide; AA, ascorbic acid; iROS, intracellular ROS; MMP, mitochondrial membrane potential; ORP, oxidation-reduction potential; OS, oxidative stress; ROS, reactive oxygen species; RS, reductive stress; SWM, sperm wash medium; TUNEL, terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling; DGC, density gradient centrifugation

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semen is necessary in order to attain the physiological redox homeostasis. Excessive ROS production or decreased concentration of available antioxidants result in a state of oxidative stress (OS). On the other hand, the reduced ROS production or shift in the redox balance towards a more reductive status results in reductive stress (RS), which is as harmful as OS [4]. Both, OS and RS conditions have detrimental effects on male fertility, resulting in alteration of semen parameters such as concentration, motility or normal sperm morphology [5–7]. In addition, OS leads to sperm DNA fragmentation (SDF) and consequently impairs normal fertilization, implantation, pregnancy and embryonic development [8–10]. Hence, excessive seminal ROS play a significant role in the etiology of male infertility [11].

Increased levels of ROS have been known to cause harmful effects at the molecular level of the spermatozoa. Cellular pathways such as energy metabolism and regulation, protein modification were compromised in men with high levels of seminal ROS [12]. Therefore, maintenance of mitochondrial activity is essential for the proper functioning of spermatozoa. Mitochondrial complex 1 protein, NADH: ubiquinone oxidoreductase core subunit S1 (NDUSF1) was downregulated in infertile men with OS [13]. Swain et al., demonstrated that chronic RS-induced OS can lead to sperm dysfunction [14] as a result of dysregulated oxidative phosphorylation (OXPHOS) complexes due to the up-regulation of antioxidant system [14]. Altered expression of sperm mitochondrial proteins such as NDFSU1, UQCRC2 and COX5B has been associated with OS and mitochondrial dysfunction [15].

Evaluation of ORP has been proposed to provide a comprehensive measure of the actual balance between seminal oxidants and antioxidants, instead of analyzing the individual marker i.e. ROS or antioxidants [16]. ORP reflects the seminal redox status which can be easily, quickly and cost-effectively determined in a clinical set up [17]. The analysis is based on the detection of the rate of electrons transferred from antioxidants to oxidants [18]. A cut-off value of 1.34 mV/ 10^6 sperm/mL has been identified to discriminate between normal and abnormal semen samples with high sensitivity [19]. However, the physiological ORP range in the seminal ejaculate has not been established yet. Therefore, the main objectives of this pilot study were to investigate the effects of OS and RS on a) total and progressive sperm motility, mitochondrial membrane potential (MMP), intracellular ROS (iROS) production and SDF, b) expression of proteins involved in OXPHOS pathway, and c) to establish a physiological ORP range.

2. Materials and methods

2.1. Study subjects

This pilot study was approved by the Institutional Review Board (IRB) of Cleveland Clinic, Cleveland, Ohio, United States (IRB #17–1027). All subjects provided written consent and were recruited from our existing pool of healthy donors between March and August 2019. A total of 66 semen samples were obtained from healthy donors after 2–7 days of ejaculatory abstinence. Samples of the same donor pool were used for evaluation of sperm motility, progressive motility, vitality assay, MMP testing, iROS assay and SDF testing. Sperm motility and progressive motility were evaluated in nine samples, whereas tests of sperm function were conducted on eight samples.

2.2. Inclusion and exclusion criteria

Normozoospermic samples were selected according to the WHO 5th edition guidelines (WHO, 2010). Semen samples with severe agglutination, and subjects with fever in the past 3 months or diagnosis of obstructive pathologies were excluded from the study. Samples with leukocytospermia and positive for the Endtz test have also been excluded.

2.3. Sperm parameters and processing of semen samples

After liquefaction, manual semen analysis was carried out using an aliquot of each sample (6 μ L) to analyze sperm parameters (sperm concentration, total sperm count and motility), using a Leja sperm counting chamber (Spectrum Technologies, Healdsburg, CA) with phase contrast optics set at x20 magnification. Semen samples were processed by double-density gradient centrifugation (DGC) to select a physiologically normal sperm fraction with motility > 80%. This was prepared with 2 mL of 40% and 80% gradient medium (PureCeption SAGE In-Vitro Fertilization, Inc., Trumbull, CT, USA) for the upper and lower phases, respectively. The liquefied semen sample was carefully overlaid on top of the prepared gradient column and centrifuged for 20 min at $300 \times g$. After gently removing the supernatant, the soft pellet was resuspended in 2 mL of sperm wash medium (SWM – ENHANCE WG, Vitrolife, San Diego, CA, USA) and centrifuged for 7 min at $300 \times g$. The supernatant was discarded, and the pellet was resuspended in SWM to a final volume of 0.5 mL.

2.4. Measurement and calibration of oxidation-reduction potential in sperm wash medium

The MiOXSYS (Aytu BioScience, Englewood, CO, USA) was used to measure the ORP levels in semen samples. Sensors were loaded with 30 μ L of sample and analyzed by the device. The results were expressed in milli Volt (mV) and normalized to the seminal sperm concentration (mV/ 10^6 sperm/mL) [20]. In our earlier study, we used different concentrations of cumene hydroperoxide (CH) and ascorbic acid (AA) to induce specific OS and RS conditions at defined ORP values [21]. The study provides a calibration curve of different concentrations for both molecules used [21]. The changes in the ORP levels had no effect on the pH of the medium after the addition of the oxidant (CH) and the reductant (AA). In the current study, we used the same approach for calibration of the SWM to obtain the desired ORP values. Working stock solutions of the OS and RS inducers 1 M CH and 1 M AA (Sigma, St. Louis, MO, USA) were prepared in SWM, respectively, and serially diluted to obtain defined ORP values. The ORP value of SWM was considered as baseline (Supplementary Table 1). Since sperm motility was not affected at ORP levels between -25 mV and -200 mV (data not shown), additional reductive stress conditions have been induced (Supplementary Table 1). Aliquots of 20×10^6 sperm/mL in SWM and a range of calibrated ORP levels were incubated at 37°C . Further, the normalized ORP values corresponded to 0.33, 0.72, 1.48 and 2.75 mV/ 10^6 sperm/mL for OS (Fig. 1, Supplementary Table 1) and -8.05 , -9.35 , -9.76 and -11.24 mV/ 10^6 sperm/mL for RS conditions (Fig. 2, Supplementary Table 1). SWM without the addition of CH and AA was used as control. Sperm motility was evaluated for all the above mentioned OS and RS conditions at 0, 30, 60, 90 and 120 min. Additional assays were conducted for the initial two redox conditions that significantly affected sperm motility at early stage of incubation (OS: 1.48 and 2.75 mV/ 10^6 sperm/mL; RS: -9.76 and -11.24 mV/ 10^6 sperm/mL) and considering only two time points of incubation (60 and 90 min). This was due to the long duration of the assays (MMP, iROS, TUNEL).

2.5. Semen analysis using LensHooke™ X1 PRO analyzer

Semen analysis was conducted on samples ($n = 9$) exposed to different OS and RS conditions. The semen samples from the same donor pool were used for both experimental conditions. Semen analysis was conducted according to the manufacturer's instructions for 0, 30, 60, 90 and 120 min by a LensHooke™ X1 PRO analyzer (Bonraybio Co., Ltd, Taichung, Taiwan) [22]. Forty microliters of semen samples were loaded on the LensHooke™ semen test cassette and analyzed.

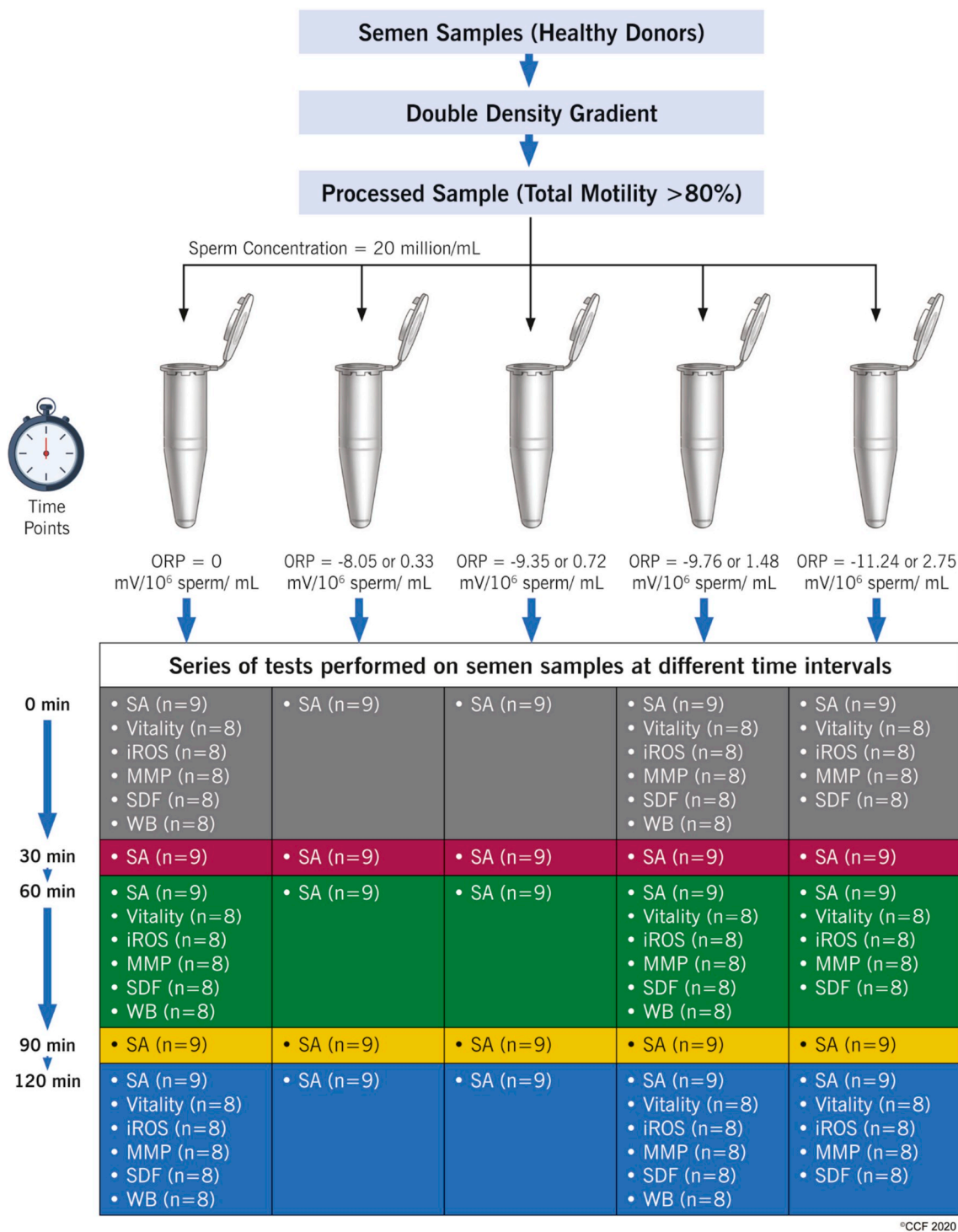


Fig. 1. Flowchart depicting the experimental design for oxidative and reductive stress conditions. SA: semen analysis, iROS: intracellular ROS, MMP: mitochondrial membrane potential, SDF: sperm DNA fragmentation, WB: Western blot for OXPHOS complex, ORP: oxidation-reduction potential. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

2.6. Staining for sperm vitality

Sperm vitality testing was conducted on samples (n = 8) exposed to different OS and RS conditions. Spermatozoa were exposed to ORP values (OS: 1.48 and 2.75 mV/10⁶ sperm/mL; RS: -9.76 and -11.24 mV/10⁶ sperm/mL) at time points of 0, 60 and 120 min. At these conditions, significant variations in sperm motility were observed. Eosin/Nigrosin staining was carried out to evaluate vitality. Semen was mixed with Eosin/Nigrosin in a ratio of 1:2. Ten microliters of stained

sample were smeared on frosted slides and evaluated under 100X oil-immersion in the bright field. The percentage of viable sperm was determined [23].

2.7. Mitochondrial membrane potential

MMP was measured in samples (n = 8) exposed to different OS (1.48 and 2.75 mV/10⁶ sperm/mL) and RS (-9.76 and -11.24 mV/10⁶ sperm/mL) conditions at 0, 60 and 120 min, using the BD™ MitoScreen

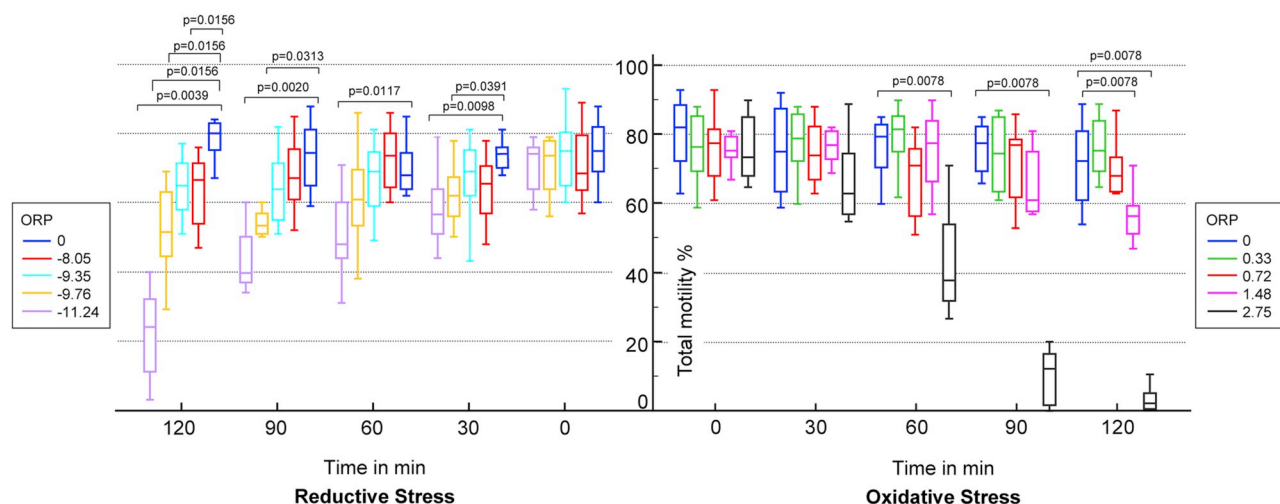


Fig. 2. Total motility of sperm at time points 0, 30, 60, 90 and 120 min after exposure to oxidative stress with ORP levels 0.33, 0.72, 1.48 and 2.75 mV/ 10^6 sperm/mL and reductive stress with ORP levels -8.05, -9.35, -9.76 and -11.24 mV/ 10^6 sperm/mL. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

kit (Becton and Dickinson, San Jose, CA, USA). Briefly, 1×10^6 sperm were washed with 1 mL 1X assay buffer by centrifuging at $300 \times g$ for 7 min. A positive control sample was prepared by incubating 1×10^6 sperm cells with 25 mM CCCP (carbonyl cyanide 3-chlorophenylhydrazone) at 37°C for 30 min. Sperm resuspended in 1 mL of 1X assay buffer were incubated with 0.5 mL JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazol carbocyanine iodide) at 37°C for 30 min. Further, 1 mL of 1X assay buffer was used to wash the sperm twice by centrifugation at $300 \times g$ for 7 min. MMP was assessed in duplicates using BD Accuri C6 bench top flow cytometer (Becton and Dickinson, San Jose, CA, USA). In sperm cells with intact MMP, JC-1 aggregates and emits intense red fluorescence, which is measured by the FL2 (590 nm) channel. JC-1 remains as a monomer and exhibits green fluorescence in sperm cells with low MMP, which is measured in the FL1 channel (527 nm). A total of 50,000 events were analyzed for each sample. The results were presented as percentage of cells having intact MMP.

2.8. Intracellular ROS assay

The iROS concentration was evaluated in samples ($n = 8$) exposed to different OS (1.48 and 2.75 mV/ 10^6 sperm/mL) and RS (-9.76 and -11.24 mV/ 10^6 sperm/mL) conditions at 0, 60 and 120 min. Briefly, 1×10^6 sperm were washed with 1 mL PBS to remove the CH and AA and then centrifuged for 7 min at $300 \times g$. After resuspending in 1 mL of PBS, sperm were incubated with 25 μM of 2',7'-dichlorofluorescein diacetate (DCFDA-Sigma, St. Louis, MO, USA) for 40 min at 37°C . DCFDA is a probe that is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) by hydroxyl and peroxyl ROS within the cell and emits green fluorescence. A positive control was incubated with 200 μM of *tert*-butyl hydroperoxide (TBHP - Sigma, St. Louis, MO, USA) 1X (30 min, 37°C). Further, 250 μL propidium iodide (PI-1.25 $\mu\text{g}/\text{mL}$, BD Pharmingen™, San Diego, CA, US) was added to each tube as a counterstain dye to exclude apoptotic spermatozoa, and incubated for 30 min at 37°C . DCF fluorescence was evaluated in the FL1 channel (529 nm), whereas the PI signal was detected in the FL2 channel (590 nm) using an Accuri C6 flow cytometer (Becton and Dickinson, San Jose, CA). A minimum of 10,000 events were examined for each sample. Results were expressed as DCF mean fluorescence intensity (MFI).

2.9. Sperm DNA fragmentation

SDF was evaluated with the TUNEL assay using the Apo-Direct kit (Becton and Dickinson, San Jose, CA) in samples ($n = 8$) exposed to

different OS (1.48 and 2.75 mV/ 10^6 sperm/mL) and RS (-9.76 and -11.24 mV/ 10^6 sperm/mL) conditions at 0, 60 and 120 min. Spermatozoa at a concentration of 2.5×10^6 cells were fixed in 3.7% paraformaldehyde for 30 min at 37°C , washed twice with 1X PBS, fixed in 1 mL of 70% ice-cold ethanol and stored at -20°C until tested for DNA damage. Ethanol was removed by centrifugation for 7 min at $300 \times g$, sperm were resuspended in 50 μL of freshly prepared staining solution and incubated for 60 min at 37°C . Rinse buffer was used to wash the samples in order to remove excess staining. Samples were resuspended in 0.5 mL of PI/RNase solution and incubated in the dark for 30 min at room temperature. Results were expressed as percent sperm showing DNA fragmentation, with samples showing less than 19.25% SDF being considered negative [24].

2.10. Western blot analysis of OXPHOS complex proteins

Expression of OXPHOS complex proteins (CV-ATPA, CIII-UQCRC2, CIV-MTCO1, CII-SDHB and CI-NDUFB8) was evaluated in samples ($n = 8$) exposed to OS (1.48 mV/ 10^6 sperm/mL) and RS (-9.76 mV/ 10^6 sperm/mL) conditions at 0, 60 and 120 min. Briefly, 20×10^6 sperm were washed twice with 1 mL PBS and centrifuged for 7 min at $300 \times g$ to remove the CH and AA. The sperm pellets were lysed by overnight incubation at 4°C in 20 μL radio-immunoprecipitation assay (RIPA; Sigma-Aldrich, St. Louis, MO, USA) buffer supplemented with Protease Inhibitor Cocktail (cOmplete™ ULTRA Tablets, EDTA-free; Roche, Mannheim, Germany). After centrifugation at $10,000 \times g$ for 30 min at 4°C , the supernatant was transferred into a clean tube and the protein concentration was estimated using the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Equal amounts of protein (20 μg) per sample were mixed with an equal volume of 2X loading buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% β -mercaptoethanol), incubated at 95°C for 10 min and then kept on ice for 5 min. Sperm proteins were separated by running on a 4%–15% gradient SDS-polyacrylamide gel electrophoresis for 2 h at 90 V, along with molecular weight markers (Sigma Chemical Co., St. Louis, MO, USA). The proteins were transferred from the gel to polyvinylidene difluoride (PVDF) membranes at 20 V for 30 min (transfer buffer: 25 mM Tris base, 192 mM glycine, and 20% methanol). Blocking of PVDF membranes was done with 5% bovine serum albumin (BSA) for 90 min at room temperature. Further, the membranes were briefly washed for 5 min and incubated with total OXPHOS Human WB Antibody Cocktail (mouse IgG; ab110411; Abcam, USA) at 4°C

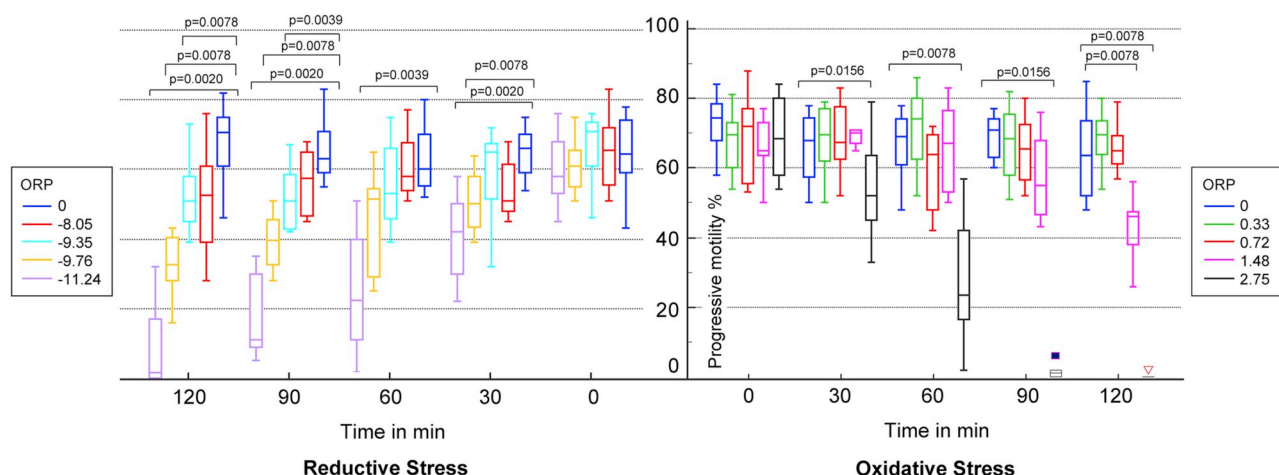


Fig. 3. Progressive motility of sperm at time points 0, 30, 60, 90 and 120 min after exposure to oxidative stress with ORP levels 0.33, 0.72, 1.48 and 2.75 mV/10⁶ sperm/mL and reductive stress with ORP levels −8.05, −9.35, −9.76 and −11.24 mV/10⁶ sperm/mL. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

overnight. The membranes were washed four times with 1X TBST (Tris-buffered saline, 0.1% Tween 20) for 10 min each by gentle shaking and were then incubated with horseradish peroxidase conjugated IgG (anti-mouse IgG; ab6728; Abcam, USA) in 1:10,000 dilution for 1 h at room temperature. Washed membranes were treated with enhanced chemiluminescence kit-Pierce™ ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) and chemiluminescence signals detected using Chemi-Doc™ MP Imaging System, Bio-Rad, Hercules, USA). The developed blots were subjected to densitometric analysis using Image Lab™ software (BioRad) normalizing with total proteins stained with colloidal gold (Bio-Rad, Hercules, USA).

2.11. Statistical analysis

Statistical analysis was conducted using MedCalc Statistical Software (version 19.0.5; MedCalc Software bvba, Ostend, Belgium). Normal distribution of the data was assessed by means of the Kolmogorov-Smirnov test. Friedman test and Kruskal-Wallis test with Jonckheere-Terpstra test for trend analysis were applied to highlight trends associated with ORP conditions and/or time points. Pairwise comparison analysis was carried out to detect significant differences. A $P < 0.05$ was considered statistically significant. For cases where a borderline significance was observed related to sperm motility, a retrospective sample size calculation was performed according to Machin et al. (2009) [25].

3. Results

3.1. Sperm parameters

Supplementary Table 2 provides the values for sperm concentration, motility and total motile sperm count (TMSC) before and after selection by DGC. Total sperm motility increased after DGC (from 63.7% to 89.1% for samples exposed to OS condition; from 69.7% to 91% for samples exposed to RS condition) (Supplementary Table 2), with an average sperm recovery rate of 53.3% and 65.5% for OS and RS tested samples, respectively.

3.2. Effect of oxidative and reductive stress on sperm motility

Supplementary Tables 3 and 4 provide the mean values of total sperm motility and progressive motility in samples exposed to different OS and RS conditions. Total and progressive motility remained unchanged for sperm incubated in SWM (control) and exposed to ORP

values of 0.33 and 0.72 mV/10⁶ sperm/mL up to 120 min. However, significant decline in both total and progressive motility were observed in sperm exposed to an ORP value of 1.48 mV/10⁶ sperm/mL at 120 min ($P = 0.0078$). At ORP of 2.75 mV/10⁶ sperm/mL, significant decrease in total motility was noted at 60 min ($P = 0.0078$) and progressive motility at 30 min ($P = 0.0156$). Under RS conditions, a significant decline in total motility was observed in sperm exposed to ORP values of −9.76 and −11.24 mV/10⁶ sperm/mL for 30 min ($P = 0.0391$ and $P = 0.0098$, respectively), −9.35 mV/10⁶ sperm/mL for 90 min ($P = 0.0313$) and −8.05 mV/10⁶ sperm/mL for 120 min ($P = 0.0156$) (Fig. 2). A dramatic decline in progressive motility was observed at ORP levels of −9.76 and −11.24 mV/10⁶ sperm/mL after 30 min ($P = 0.0078$ and $P = 0.0020$, respectively) and −9.35 mV/10⁶ sperm/mL after 90 min exposure ($P = 0.0039$) (Fig. 3).

3.3. Effect of oxidative and reductive stress on sperm vitality

Supplementary Table 5 provides the mean values of sperm vitality in samples exposed to different OS and RS conditions. A decrease in vitality was observed at 60 min when sperm were exposed to ORP values of −9.76 ($P = 0.0049$), −11.24 mV/10⁶ sperm/mL ($P = 0.0010$) and 2.75 mV/10⁶ sperm/mL ($P = 0.0007$) as well as at 120 min after incubation at 1.48 mV/10⁶ sperm/mL ($P = 0.0484$) (Fig. 4).

3.4. Effect of oxidative and reductive stress on mitochondrial membrane potential

Supplementary Table 6 provides the mean values of MMP in samples exposed to different OS and RS conditions. MMP remained unchanged in sperm incubated in SWM and after exposure to ORP values of 1.48 and −9.76 mV/10⁶ sperm/mL for up to 120 min. A decrease in MMP was observed in sperm exposed to ORP value of 2.75 mV/10⁶ sperm/mL at 120 min ($P = 0.0313$). Similarly, a significant decline in MMP was observed at −11.24 mV/10⁶ sperm/mL up to 60 min ($P = 0.0078$) (Fig. 5).

3.5. Intracellular ROS production in oxidative and reductive stress conditions

The iROS concentration was measured and expressed either only in the live sperm fraction or in the global (live and dead sperm) fraction. The live sperm population showed no significant variation in iROS under both OS and RS conditions (Fig. 6). Including the dead sperm

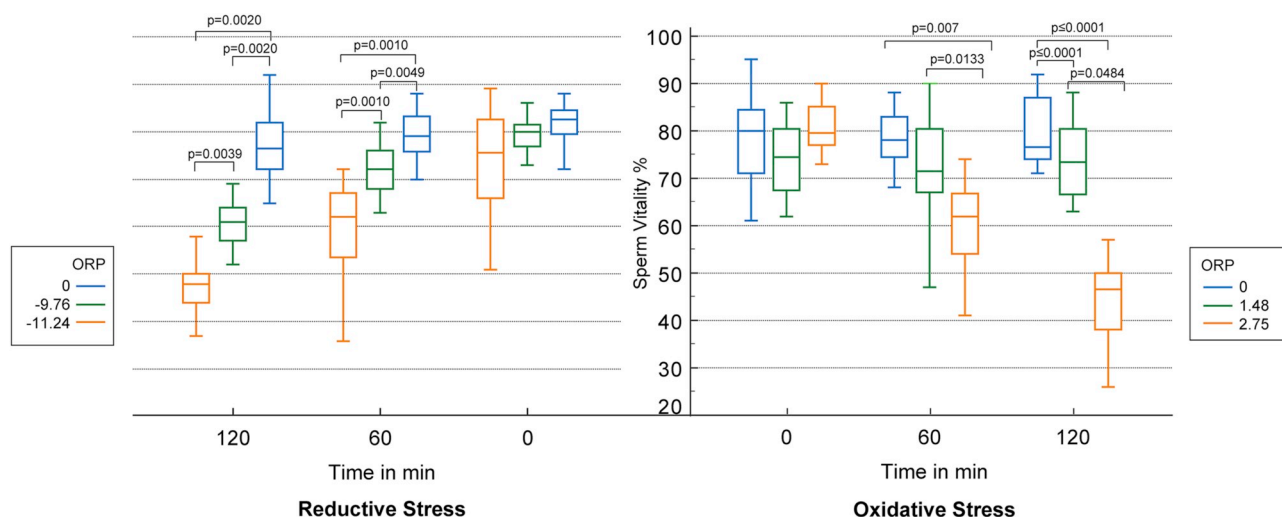


Fig. 4. Vitality of sperm at time points 0, 60 and 120 min after exposure to oxidative stress with ORP levels 1.48 and 2.75 mV/10⁶ sperm/mL and reductive stress with ORP levels -9.76 and -11.24 mV/10⁶ sperm/mL. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

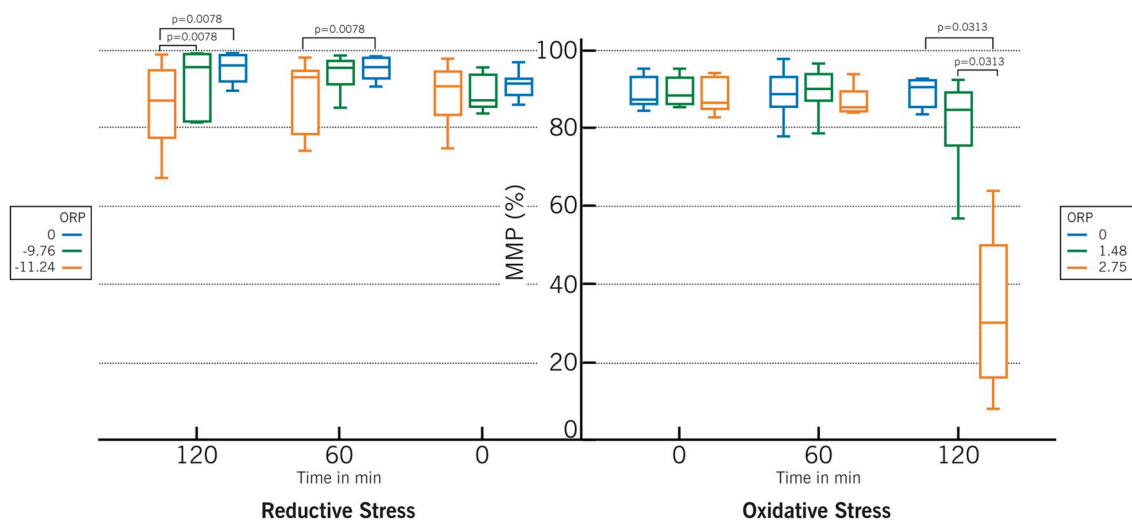


Fig. 5. Mitochondrial membrane potential of sperm at time points 0, 60 and 120 min exposed to oxidative stress with ORP levels 1.48 and 2.75 mV/10⁶ sperm/mL and reductive stress with ORP levels -9.76 and -11.24 mV/10⁶ sperm/mL. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

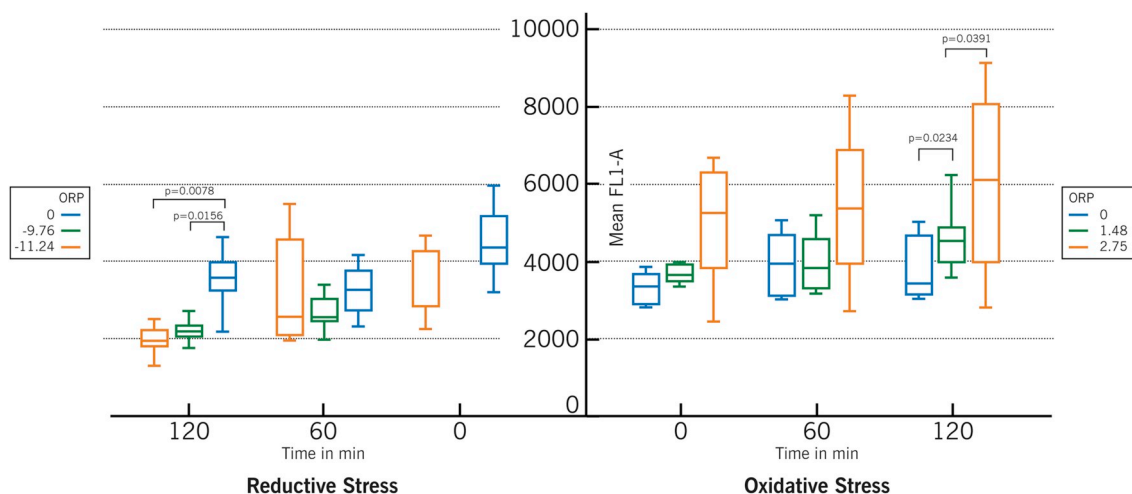


Fig. 6. Intracellular ROS production at time points 0, 60 and 120 min from live cells exposed to oxidative stress with ORP levels 1.48 and 2.75 mV/10⁶ sperm/mL and reductive stress with ORP levels -9.76 and -11.24 mV/10⁶ sperm/mL. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

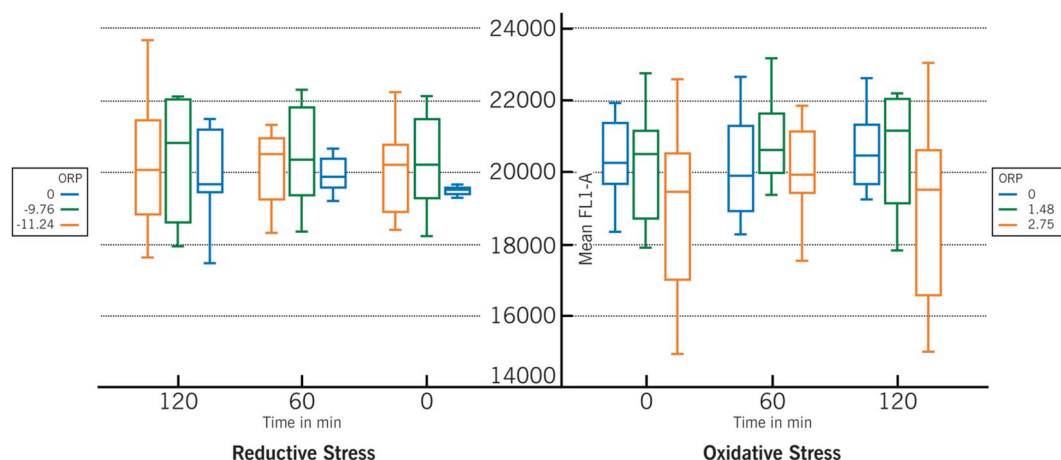


Fig. 7. Intracellular ROS production at time points 0, 60 and 120 min from all cells exposed to oxidative stress with ORP levels 1.48 and 2.75 mV/10⁶ sperm/mL and reductive stress with ORP levels −9.76 and −11.24 mV/10⁶ sperm/mL. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

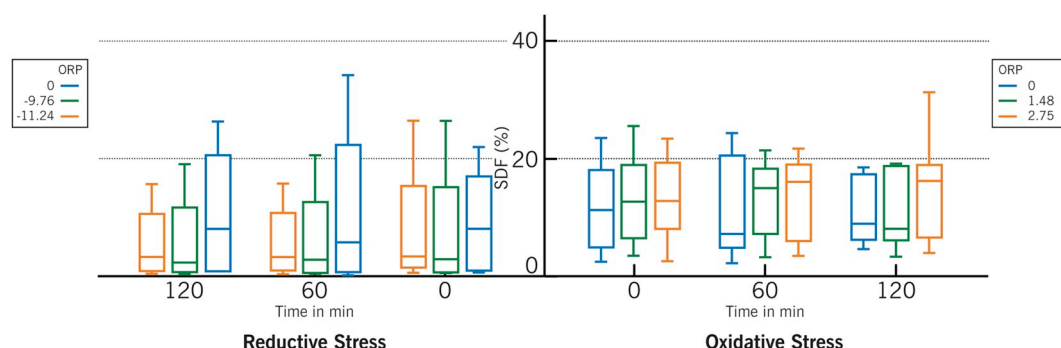


Fig. 8. Sperm DNA fragmentation at time points 0, 60 and 120 min exposed to oxidative stress with ORP levels 1.48 and 2.75 mV/10⁶ sperm/mL and reductive stress with ORP levels −9.76 and −11.24 mV/10⁶ sperm/mL. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

fraction in the calculations, a significant increase in iROS was observed at ORP 1.48 mV/10⁶ sperm/mL ($P = 0.0234$). However, sperm exposed to RS conditions corresponding to −9.76 ($P = 0.0156$) and −11.24 ($P = 0.0078$) mV/10⁶ sperm/mL showed a significant decrease in iROS production (Fig. 7). Supplementary Table 7 provides the mean values of iROS production in global fraction exposed to different OS and RS conditions.

3.6. Effect of oxidative and reductive stress on sperm DNA integrity

Supplementary Table 8 provides the mean values of SDF in samples exposed to different OS and RS conditions. No significant change in SDF was observed after exposure to OS and RS conditions (Fig. 8). Moreover, the average SDF values were found to be negative ($< 19.25\%$) at different time points.

3.7. Expression profile of OXPHOS complex proteins

Since oxidative and reductive conditions resulted in significant changes in sperm motility and MMP, we determined the effect of OS and RS on the expression profile of OXPHOS complex proteins (CV-ATPA, CIII-UQCRC2, CIV-MTCO1, CII-SDHB and CI-NDUFB8). Supplementary Table 9 provides the mean relative fold change of OXPHOS complex proteins in samples exposed to different OS and RS conditions. Expression of CV-ATPA, CIII-UQCRC2 and CIV-MTCO1 proteins decreased at 60 and 120 min ($P < 0.05$) (Fig. 9), whereas the expression of CII-SDHB and CI-NDUFB8 proteins did not show any change.

4. Discussion

A plethora of evidence emphasizes the pivotal role of ROS in mediating the crucial functions of spermatozoa associated with fertilization [26,27]. On the other hand, elevated levels of ROS are known to have adverse consequences on the structural and functional integrity of sperm via inducing peroxidative damage to lipids, proteins and DNA of spermatozoa [28–30]. Therefore, maintaining a crucial balance between the levels of ROS and antioxidants is the key element in establishing a physiological redox homeostasis. A shift in the redox balance towards oxidative or reductive condition results in OS/RS, which in turn has negative implications on spermatozoa [19,31]. Though several studies have acknowledged the significance of the redox balance with respect to sperm functions, the physiological ORP range that determines the functional ability of spermatozoa is still unknown.

Until recently, scientific studies only focused on OS as a major factor contributing to male infertility, but lacked clinical awareness about RS [32]. Ménéz et al. reported the negative effects of commonly recommended antioxidants such as vitamin C and vitamin E [33]. Incorrect usage of antioxidants may scavenge the physiologically necessary ROS resulting in sperm not being able to undergo capacitation and acrosome reaction [34,35]. It is possible that a patient can be overdosed with antioxidants which could possibly lead to reductive stress and infertility [31]. Therefore, it is essential to not only understand this redox balance, but also know the physiological ORP range in which sperm are able to fulfill their normal physiological functions.

Our results from the present study suggest that the physiological ORP range between −9.76 and 1.48 mV/10⁶ sperm/mL did not have

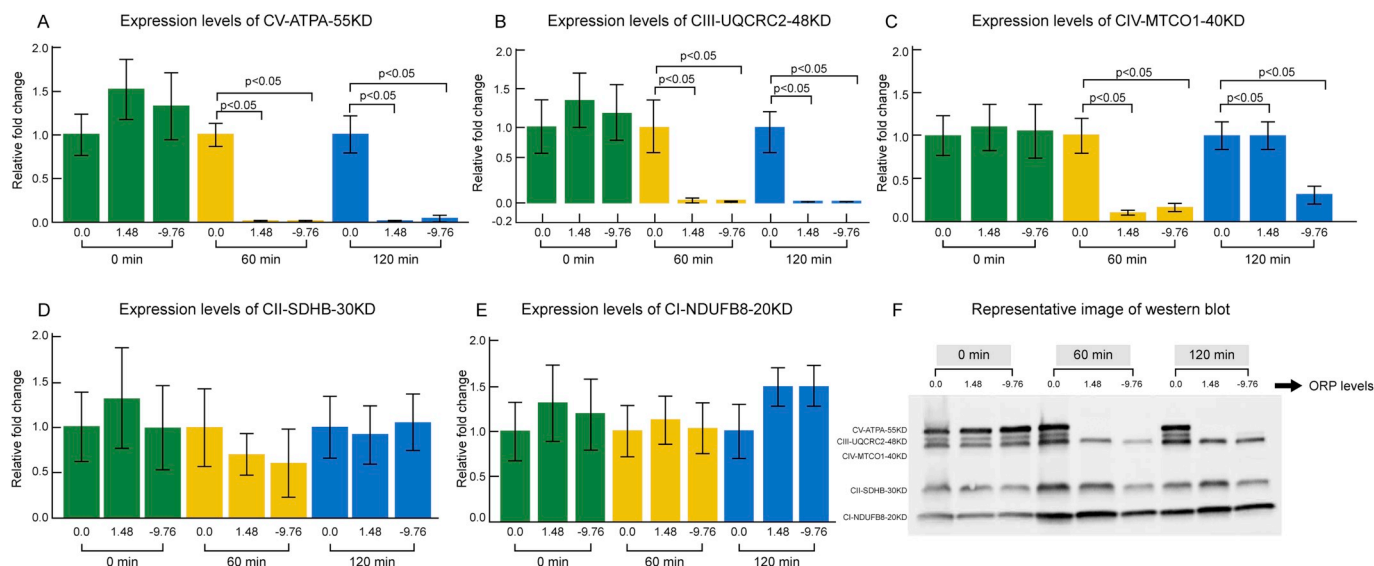


Fig. 9. Expression profile of OXPHOS complex proteins at time points 0, 60 and 120 min after sperm exposure to oxidative stress with ORP level 1.48 mV/10⁶ sperm/mL and reductive stress with ORP level -9.76 mV/10⁶ sperm/mL. (A) CV-ATPA (B) CIII-UQCRC2 (C) CIV-MTCO1 (D) CII-SDHB (E) CI-NDUFB8 and (F) Representative image of Western blot. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography© 2020. All Rights Reserved.

any deleterious effect on the human sperm with respect to total and progressive motility, MMP and SDF. Seminal ORP values ≤ -9.76 mV/10⁶ sperm/mL resulted in RS, while values ≥ 1.48 mV/10⁶ sperm/mL resulted in OS. The upper limit of this range is in close agreement with the previously reported ORP cut-off value of 1.34 mV/10⁶ sperm/mL [36]. Our study demonstrates an impairment of total and progressive motility as well as sperm vitality at both higher (≥ 1.48 mV/10⁶ sperm/mL) and lower (≤ -9.76 mV/10⁶ sperm/mL) ORP levels. A negative correlation between high levels of ROS and decreased total and progressive motility has been reported in asthenozoospermic patients [37,38]. In fact, high ROS concentrations impair the physiological phosphorylation pathway of axonemal proteins, a process important for the generation of motility [39]. In addition, the inhibition of glucose-6-phosphate dehydrogenase results in the reduced bioavailability of antioxidant cofactor, NADPH, and consequently, reduced antioxidant activity [40].

On the other hand, the impact of RS on seminal parameters is still unclear. Several studies have tried to investigate the impact of antioxidant supplementation on semen quality [31]. Verma and Kanwar reported a detrimental effect of AA supplementation on sperm motility and vitality of normozoospermic samples [41]. Paradoxically, they observed a higher rate of lipid peroxidation, evidenced by malondialdehyde synthesis. The excess antioxidants can act as pro-oxidants, which was described by Halliwell (2000) as 'antioxidant paradox' [42]. In the study by Verma and Kanwar, the impairment was observed at AA concentrations higher than 1 mM. In contrast, we did not observe any alteration at concentration < 12 mM AA. This could be due to the differences in study designs. In our study, we removed the round cells and leukocytes by density gradient centrifugation and the pure sperm fraction was exposed to AA, whereas Verma and Kanwar did not remove the round cells and leukocytes [41]. Therefore, the role of leukocytes and round cells in increasing the sperm damage under RS conditions cannot be excluded.

To understand the molecular changes in the sperm exposed to OS and RS conditions, we analyzed parameters (such as MMP, iROS and SDF) that are directly correlated with the fertilization potential of spermatozoa. Mitochondria are the site where reduction of O₂ to H₂O and the synthesis of ATP takes place in an electron transport linked oxidative phosphorylation. The latter is directly related to the formation of an electrochemical gradient across the inner mitochondrial membrane and provides the energy required for sperm motility

[43–45]. Therefore, an alteration of the MMP has a negative impact on ATP synthesis and sperm motility [46]. In our study, a decline in MMP was observed after induction of OS, suggesting a direct negative impact on mitochondrial function, resulting in decreased sperm motility. Furthermore, we observed an increase in ROS production in the total cell fraction. Though the iROS concentration remained essentially unaltered in the live fraction, the dramatic increase of iROS detected in the combined fraction of live and dead sperm strongly supports the idea of a direct ROS-mediated cellular damage. Similar negative associations of iROS with sperm quality, particularly sperm motility and vitality as well as mitochondrial dysfunction have been reported [2,47]. Although OS is a well-described factor affecting sperm DNA integrity [48], a variation of SDF percentage was not observed in our experimental conditions, where the average SDF levels were below the reference value (19.25%) for established infertile men [24]. Sperm DNA is strictly compacted by protamines, protecting it from oxidative assaults [49]. Therefore, it is possible that longer exposure to OS is required to compromise sperm chromatin structure and DNA integrity.

In the current study, we observed a decrease in MMP under reductive conditions. Interestingly, the observed decline was not as dramatic as noticed in OS conditions. Several *in vitro* studies reported a negative impact of AA on cellular vitality, due to its rapid mitochondrial uptake [37,50,51]. Being hydrophilic, AA uses specific membrane transporters, such as sodium-AA co-transporters (SVCT), to cross the cellular membrane, while its oxidized form dehydroascorbic acid (DHA) can cross the mitochondrial membrane by means of glucose transporters (GLUTs) [52]. Kramarenko et al. demonstrated that, although AA usually acts as an antioxidant, it can react with oxygen to synthesize DHA and H₂O₂ [53]. In mitochondria, DHA is quickly reduced to ascorbic acid by NADPH and NADH dependent reductases that increase the concentration of AA *in situ* [52,54] and reduce the availability of NADPH and NADH cofactors. Several antioxidants, such as catalase and thioredoxin reductase (TrxR), use these cofactors to catalyze antioxidant chemical reactions [51,55]. Therefore, intra-mitochondrial accumulation of high concentrations would reflect in a reduced cellular antioxidant capacity [37,56]. As in OS, we did not observe a change in SDF in RS conditions, probably due to the limited exposure of sperm to the OS or RS. The findings of our study clearly demonstrate that the establishment of a reductive environment due to high antioxidant concentration has a deleterious impact on sperm motility, vitality and mitochondrial activity.

High levels of AA can induce cellular death by enhancing the synthesis of ROS from Complex III of mitochondrial electron transport chain [57,58]. Guidarelli et al. suggested a role of AA in driving the conversion of iron Fe^{3+} in Fe^{2+} , the latter being used by Complex III for ROS generation [58]. In addition, Fe^{2+} is also involved in lipid peroxidation, being able to trigger a chain reaction at the membrane level and enhance the cellular damage [2,59]. Assuming the same mechanism in sperm, uptake of AA in mitochondria would result in the reduction of MMP observed after sperm exposed to RS environment, increasing the mitochondrial ROS generation. However, flow cytometry analysis did not reveal a significant difference of iROS production at different points in the live sperm fraction, while a decrease in iROS production was observed in the combined (live and dead) cellular fraction. Therefore, in our experimental design, molecular mechanisms different from a local increase of mitochondrial ROS seem to affect sperm functions critically. Additional studies are warranted to clarify these interesting observations. In this context, the idea that RS could have triggered the mitohormesis phenomenon with reduced iROS production has been reported in myoblasts [60]. This observation is tempting, but needs further investigations.

For the first time, our results show molecular sources for the observed effect of OS and RS on human sperm exposed to low ($-9.76 \text{ mV}/10^6 \text{ sperm/mL}$) and high ($1.48 \text{ mV}/10^6 \text{ sperm/mL}$) ORP levels. The expression of proteins associated with mitochondrial OXPHOS complexes was significantly altered under both OS and RS conditions. While the expression of mitochondrial ATP synthase α -subunit (ATPA; protein of Complex V), Ubiquinol-Cytochrome C Reductase Core Protein 2 (UQCRC2; protein of Complex III) and Cytochrome c oxidase subunit I (MTCO1; protein of Complex IV) significantly decreased immediately under RS and OS conditions, mitochondrial Succinate dehydrogenase (ubiquinone) iron-sulfur subunit (SDHB; protein of Complex II) was not affected. On the other hand, NADH:Ubiquinone Oxidoreductase Subunit B8 (NDUFB8; protein of Complex I) reached its highest expression (though not significant) after 2 h of exposure to RS and OS conditions. Mitochondrial Complexes I to IV are involved in electron transfers from NADH to oxygen. While Complexes I, III and IV are proton pumping complexes that create a transmembrane electrochemical gradient, Complex II produces reduced ubiquinone via the oxidation of FADH_2 to FAD and thus contributes 2 electrons to the electron transfer chain. Complex V, at the end of the oxidative phosphorylation process, is involved in chemiosmosis and produces ATP [32,61]. Our WB results clearly indicate that the onset of changes at the molecular level (OXPHOS complexes) preceded MMP changes in spermatozoa. Ferramosca et al. (2013) showed that patients with OS had reduced sperm motility and postulated that this is due to reduced mitochondrial functionality [62]. Our result shows that the expression of UQCRC2 (Complex III) and MTCO1 (Complex IV) is reduced. Under-expression of these proteins leads to a reduced electron transfer and associated decline in the electrochemical MMP, which was also observed in sperm exposed to RS and OS. UQCRC2 is negatively associated with sperm fertility and motility [63,64]. Samanta et al. (2018) showed that mitochondrial dysfunction in terms of very low expression of UQCRC2 is linked with poor sperm motility in patients with varicocele [15] and concluded that mitochondrial dysfunction may lead to sperm OS, reduced ATP synthesis and sperm dysfunction [15]. Shukla et al. (2013) reported that decreased expression of UQCRC2 is correlated with lower sperm kinematics, ATP production and sperm capacitation [65]. In addition, reduced expression of the mitochondrial ATP synthase α -subunit (ATPA; Complex V) results directly in a reduced production of ATP, which is important for sperm motility [66–68]. Based on our results, we conclude that the mitochondrial proteins associated with the functional aspects of the sperm are altered under OS and RS conditions.

4.1. Limitations of the study

The samples used in this pilot study were pooled after DGC to obtain

desired concentration of sperm to conduct experiments with different ORP levels at multiple time points. Furthermore, the sperm function tests such as MMP, iROS production and SDF testing were conducted only at two different time points due to long assay protocols. Hence, it was not feasible to analyze time points earlier than 60 min.

5. Conclusion

In this study, we identified the physiological ORP range for human sperm to maintain normal semen parameters such as total motility, progressive motility and vitality, as well as mitochondrial functionality of spermatozoa between -9.76 and $1.48 \text{ mV}/10^6 \text{ sperm/mL}$. This knowledge can help to identify men with reductive stress or male oxidative stress infertility (MOSI). Semen samples with ORP values $\leq -9.76 \text{ mV}/10^6 \text{ sperm/mL}$ and $\geq 1.48 \text{ mV}/10^6 \text{ sperm/mL}$ can be characterized as pathological RS and OS, respectively. Although the effect on semen parameters are comparable in both oxidative and reductive stress groups, the management for both groups are at opposite extremes. Hence, it is extremely important to identify the category they fit, i.e. OS or RS. This study paves way for research on treatment options for men of both groups and the management thereof. In addition, the availability of an easy and reliable method to assess the seminal redox status during the treatment course allows customization of the treatment. Furthermore, the proposed physiological range indicates that when physiologically normal sperm are exposed to severe OS and RS conditions during their transit through the female reproductive tract, this can alter the expression of vital proteins associated with sperm functions, resulting in fertilization failure. Additionally, the knowledge of this physiological range of the redox equilibrium could be important for improving sperm separation and embryo culture conditions in all assisted reproductive methods.

Author contributions

Conceptualization: Ashok Agarwal, Ralf Henkel. Data curation: Manesh Kumar Panner Selvam, Saradha Baskaran, Ralf Henkel. Formal analysis: all authors. Funding acquisition: NA. Investigation: NA. Methodology: Manesh Kumar Panner Selvam, Renata Finelli, Concetta Iovine, Kathy Robert. Project administration: Ashok Agarwal. Resources: American Center for Reproductive Medicine. Software: Manesh Kumar Panner Selvam, Ralf Henkel. Supervision: Ashok Agarwal. Validation: Manesh Kumar Panner Selvam. Visualization: NA. Writing – original draft: Manesh Kumar Panner Selvam, Renata Finelli, Kathy Robert, Concetta Iovine, Saradha Baskaran, Ralf Henkel, Ashok Agarwal. Writing – review & editing: Saradha Baskaran, Ralf Henkel, Manesh Kumar Panner Selvam, Ashok Agarwal. Approval of the final manuscript: all authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.freeradbiomed.2020.03.008>.

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