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# Calibration of redox potential in sperm wash media and evaluation of oxidation–reduction potential values in various assisted reproductive technology culture media using MiOXSYS system

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## Summary

Oxidation–reduction potential describes the balance between the oxidants and antioxidants in fluids including semen. Various artificial culture media are used in andrology and IVF laboratories for sperm preparation and to support the development of fertilized oocytes under *in vitro* conditions. The composition and conditions of these media are vital for optimal functioning of the gametes. Currently, there are no data on the status of redox potential of sperm processing and assisted reproduction media. The purpose of this study was to compare the oxidation–reduction potential values of the different media and to calibrate the oxidation–reduction potential values of the sperm wash medium using oxidative stress inducer cumene hydroperoxide and antioxidant ascorbic acid. Redox potential was measured in 10 different media ranging from sperm wash media, freezing media and assisted reproductive technology one-step medium to sequential media. Oxidation–reduction potential values of the sequential culture medium and one-step culture medium were lower and significantly different ($p < 0.05$) from the sperm wash media. Calibration of the sperm wash media using the oxidant cumene hydroperoxide and antioxidant ascorbic acid demonstrated that oxidation–reduction potential and the concentration of oxidant or antioxidant are logarithmically dependent. This study highlights the importance of calibrating the oxidation–reduction potential levels of the sperm wash media in order to utilize it as a reference value to identify the physiological range of oxidation oxidation–reduction potential that does not have any adverse effect on normal physiological sperm function.

## Introduction

In the light of living in an aerobic environment, all cells in the human body, including spermatozoa, are exposed to different oxidation and reduction levels and have to cope with the relevant situation depending on the circumstances a specific cell is in (Naviaux, 2012; Agarwal *et al.*, 2016c,d). Oxidants also known as reactive oxygen species (ROS) are generated from either intrinsic or extrinsic sources (Saalu, 2010; Lavranos *et al.*, 2012; Agarwal *et al.*, 2014b). At low levels, ROS are required for physiological functions involved in the fertilization process, including capacitation, hyperactivation, acrosome reaction, and sperm– oolemma binding (de Lamirande & Cagnon, 1993; Agarwal *et al.*, 2006; de Lamirande & O'Flaherty, 2008; Kothari *et al.*, 2010; Guthrie & Welch, 2012),whereas at high levels, ROS are harmful to spermatozoa.

Oxidative stress (OS) is one of the major causes of male infertility (Aitken *et al.*, 1989; Sharma & Agarwal, 1996; Agarwal *et al.* 2006). Prolonged OS induces DNA damage (Agarwal *et al.*, 2003; Wang *et al.*, 2003; Aitken & Koppers, 2011; Dorostghoal *et al.*, 2017), and high levels of oxidants also negatively impact sperm motility, count, morphology, and viability (Agarwal *et al.*, 2016d; Agarwal & Wang, 2017). On the other hand, antioxidants act as a compensatory mechanism to reduce oxidative stress. However, supraphysiological levels of antioxidants result in reductive stress, which is defined as an excess of antioxidants. Reductive stress has the potential of being as harmful as oxidative stress (Castagne *et al.*, 1999). Despite the opposing extremes that spermatozoa have to face, there is no mention in the literature that specifies the impact of reductive stress on spermatozoa. Therefore, there is a need for maintaining a fine balance between oxidation and reduction for spermatozoa to function optimally. However, it is unclear at what level this balance is achieved and what the normal physiological range is. Ultimately, the accumulated damage in either direction to spermatozoa can lead to unsuccessful fertilization, pregnancy loss, and poor assisted reproductive technology (ART) outcomes (du Plessis *et al.*, 2008; Esteves *et al.*, 2016).

Higher concentrations of oxidants are seen in the semen of infertile men compared to fertile controls (Agarwal *et al.*, 2016c). This is generally accompanied by lower concentrations of antioxidants in their seminal plasma (Sharma *et al.*, 1999; Colagar & Marzony, 2009; Roychoudhury *et al.*, 2016). Oxidative stress is measured either directly by quantifying the ROS levels or indirectly by measuring the total antioxidant capacity (TAC) levels, and all these methods are either expensive or complex to carry out. Therefore, the oxidation–reduction potential (ORP), also known as redox potential, was introduced recently as a single measure of oxidative stress.

More recently, a novel method to measure ORP using male infertility oxidative (MiOXSYS) system was introduced and successfully used in a clinical validation study. This was designed to assess ORP levels and/or provide a snapshot of redox balance in semen sample. Patient samples with a significantly higher ORP value than that of the control samples confirmed the presence of OS as the redox balance shifted towards a pro-oxidant state (Agarwal *et al.*, 2016d). Subsequent studies using the MiOXSYS system with semen samples have been investigated in order to identify the impact and role of ORP levels in fertility. Higher levels of seminal ORP were exhibited by infertile males compared to the fertile controls (Agarwal *et al.*, 2016c,d). Studies have also identified a negative correlation between ORP levels and sperm count, concentration, motility, and morphology (Agarwal *et al.*, 2016c, d; Agarwal & Wang, 2017).

In assisted reproduction techniques (ART) procedures starting from sperm preparation to cryopreservation of spermatozoa and subsequent culturing of the embryos, different types of media are used. Hence, spermatozoa are continuously exposed to different culture media and incubating conditions. Even though the chemical composition and pH of these media are available from the manufacturers, there is no information about the redox

state of these media. Therefore, the aim of this study was to determine the ORP values for a number of commonly used media in sperm preparation and ART using the MiOXSYS system. Further, we calibrated ORP values using cumene hydroperoxide as an oxidizing agent (oxidative stress) and ascorbic acid as a reducing agent (reductive stress) to establish the ORP values in the various sperm wash and embryo culture media used in clinical andrology laboratory or in an ART laboratory setting. This study provides an initial platform for researchers to design and conduct the experiments for assessing the normal physiological range of ORP values for spermatozoa.

## Material and methods

### Selection of reagents and media

Ten different media used in andrology and IVF laboratories were tested. These included two sperm wash media: (i) ENHANCE WG (Vitrolife, San Diego, CA, USA); (ii) Quinn's™ Sperm Washing Medium (SAGE, In-Vitro Fertilization, Inc., Trumbull, CT, USA); and (iii) one sperm cryopreservation medium (Freezing Medium; Test Yolk buffer, Irvine Scientific, CA, USA). In addition, we tested seven ART culture media. These were (i) fertilization medium (G-IVF™ PLUS with HSA; Vitrolife, San Diego, CA, USA); (ii) Quinn's Advantage™ Fertilization (HTF) medium (SAGE, In-Vitro Fertilization, Inc., Trumbull, CT, USA); (iii) oocyte and embryo culture medium (G-MOPS™ PLUS with HSA); (Vitrolife, San Diego, CA, USA); (iv) G-TL™ with HSA (Vitrolife, San Diego, CA, USA); (v) Quinn's Advantage™ Cleavage Medium (SAGE, In-Vitro Fertilization, Inc., Trumbull, CT, USA); (vi) Quinn's Advantage™ Blastocyst Medium (SAGE, In-Vitro Fertilization, Inc., Trumbull, CT, USA); and (vii) SAGE 1-Step™ (SAGE, In-Vitro Fertilization, Inc., Trumbull, CT, USA). Considering that the redox potential is pH-dependent, we recorded the pH for all media at room temperature using pH paper (pH range 6.0–8.0). A drop of the media was added on the pH paper. The color was compared with the calibration strip to read the pH (WHO 2010).

### Measurement of ORP

ORP in the medium was measured at room temperature using the galvanostat-based MiOXSYS system that consists of the analyzer and the sensor (Aytu BioScience, Englewood, CO, USA). In brief, the disposable sensor has a built-in three electrode system that is placed onto the MiOXSYS analyzer holder. Thirty microliters of medium was loaded on the sensor. The test starts when the sample fills the reference cell and the electrochemical circuit is completed. After approximately 4 min, the ORP value in combine to read milliVolts (mV) is displayed on the screen (Agarwal *et al.*, 2016a). For each medium, ORP readings were taken on three separate days.

### Calibration of ORP in HTF-modified medium

All the reagents (cumene hydroperoxide and ascorbic acid) including different types of ART media and cryopreservation media were incubated at 37 °C for 20 min to attain the thermal homeostasis under *in vitro* conditions. ORP of the sperm wash media (ENHANCE WG) was measured. Working stock solutions of μM cumene hydroperoxide

(Sigma, St. Louis, MO, USA) and 1M ascorbic acid (Sigma) were prepared in the sperm wash media. These stock solutions were diluted further to attain 10 μM, 50 μM, 100 μM, 200 μM, 500 μM, and 1000 μM concentration of cumene hydroperoxide and 10 μM, 31.25 μM, 62.5 μM, 125 μM, 250 μM, 500 μM, and 1000 μM of ascorbic acid. ORP was measured at different dilutions of these solutions. The experiment was repeated thrice on three different days to check the consistency and reproducibility. Simultaneously, the pH was also recorded for each dilution separately.

**Statistical analysis**

Data were analyzed separately for each concentration of oxidant and reductant. Arithmetic mean and standard deviation (SD) for sperm wash media and different concentrations of solutions were calculated separately. The ORP value of the plain sperm wash media was subtracted, and the difference between ORP value of the plain sperm wash media and the ORP values of different concentrations of cumene hydroperoxide and ascorbic acid was then calculated. Using the difference in ORP values, the ORP–concentration response graph was plotted for the cumene hydroperoxide and ascorbic acid at different concentrations tested. The ORP values of the different media were compared using MedCalc Statistical Software (V. 17.8; (MedCalc Software bvba, Ostend, Belgium). An independent-sample *t*-test was carried out to check differences in the ORP values of the different ART media. The calibration curves were calculated with Excel 2013 (Microsoft, Redmond, WA, USA).

**Results**

The main focus of this experiment was to measure the ORP values of the sperm wash medium, cryopreservation medium, and different types of media used during ART procedure in andrology and IVF laboratories using the MiOXSYS system to measure ORP. Aside from this, calibration of ORP values was performed using cumene hydroperoxide as an oxidizing agent and ascorbic acid as a reducing agent to establish the ORP values in the HTF-modified sperm wash medium. Comparison of ORP values in the sperm wash media from two different manufacturers was also analyzed. The ORP values and pH levels for different media are shown in Table 1. The mean ORP values for ENHANCE WG (Vitrolife) and Quinn's Sperm Washing Medium were 267.27 mV and 269.50 mV, respectively, and no significant differences were seen between these two sperm wash media. The ORP and pH for the sperm cryopreservation medium were 233.13 mV and 6.8, respectively.

Different ART culture media exhibited significantly lower ORP values ($p < 0.05$) compared to the sperm wash medium except for the G-MOPS PLUS medium, which had a higher ORP of 273.83 mV (Fig. 1). Among all the media analyzed, the lowest ORP value was recorded for the SAGE-1-Step medium (208.63 mV).

The pH levels measured for all these media were the same (7.2), except for the SAGE-1-Step medium which had a pH of 7.6. The pH levels of all the ART culture media were in the normal physiological pH range (7.2–7.6).

The mean difference between the ORP value of the sperm wash medium alone and the ORP values with varying concentrations of cumene hydroperoxide and ascorbic acid is depicted in Table 2. Cumene hydroperoxide (an oxidizing agent) showed an increase and ascorbic acid (a reducing agent) a decrease in ORP values, respectively, when compared with the sperm wash medium. A gradual increase in the ORP values with differences from 6.4 mV to 104 mV was observed when 10 µM to 1000 µM of cumene hydroperoxide was added to the sperm wash medium (Fig. 2). On the contrary, when the medium was supplemented with 10 µM to 1000 µM of ascorbic acid, ORP values decreased with differences from -47.5 to -177 mV (Fig. 3).

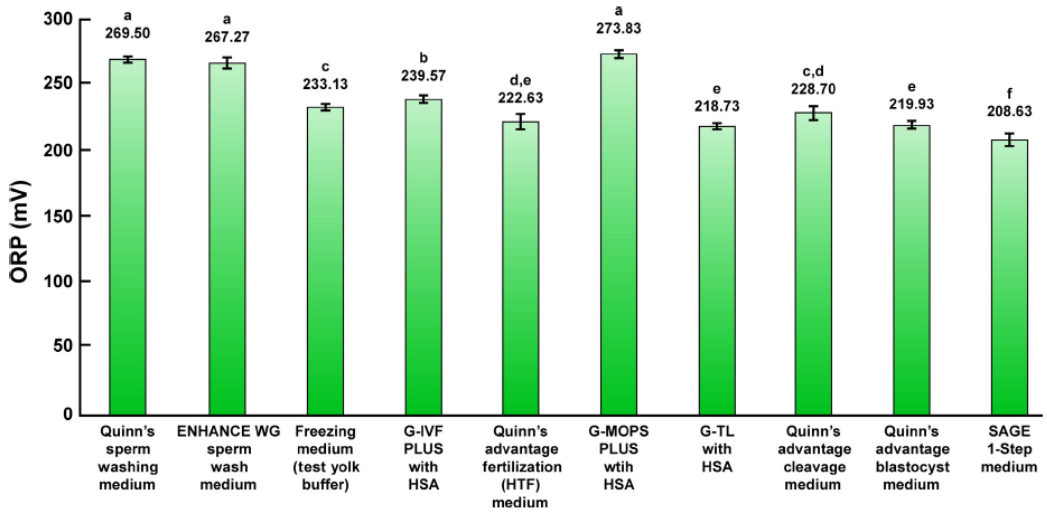
The pH in the media measured at different concentrations of cumene hydroperoxide and ascorbic acid did not change and remained stable at 7.2, whereas a slight drop in the pH to 7.0 was noticed in the media supplemented with 500 lM and 1000 lM of ascorbic acid (Table 2).

**Table 1** ORP and pH of some of the most commonly used media in the andrology and IVF laboratories

| No. | Medium | Usage | Mean ORP values (mV) | SD | Mean pH |
|---|---|---|---|---|---|
| 1 | Quinn's™ Sperm Washing Medium | Sperm preparation | 269.50[a] | 1.90 | 7.2 |
| 2 | ENHANCE WG (modified HTF with HSA) | | 267.27[a] | 3.61 | 7.2 |
| 3. | Freezing Medium (Test Yolk buffer) | Sperm Cryopreservation | 233.13[c] | 2.06 | 6.8 |
| 4 | G-IVF™ PLUS with HSA | IVF | 239.57[b] | 2.40 | 7.2 |
| 5 | Quinn's Advantage™ Fertilization (HTF) Medium | | 222.63[d,e] | 5.71 | 7.2 |
| 6 | G-MOPS™ PLUS with HSA | Oocyte and embryo culture | 273.83[a] | 3.01 | 7.2 |
| 7 | G-TL™ with HSA | | 218.73[e] | 2.01 | 7.2 |
| 8 | Quinn's Advantage™ Cleavage Medium | | 228.70[c,d] | 4.98 | 7.2 |
| 9 | Quinn's Advantage™ Blastocyst Medium | | 219.93[e] | 2.77 | 7.2 |
| 10 | SAGE 1-Step™ | | 208.63[f] | 4.77 | 7.6 |

ORP values of media labeled with a, b, c, d, e, and f in superscript indicate a significant ($p < 0.05$) difference based on the independent-sample *t*-test.

**Figure 1** ORP values for different types of routinely used sperm preparation and ART media measured using MiOXSYS. Media labeled with a, b, c, d, e, and f indicate a significant difference ($p < 0.05$) in ORP values.




To understand the pattern and changes in ORP in sperm wash media with different concentrations of oxidants (cumene hydroperoxide) and antioxidants (ascorbic acid), the oxidant/reductant concentration vs. the ORP values was plotted separately (Figs 2 and 3).

Analysis of the calculated ORP curve of the graph for cumene hydroperoxide followed the equation '$y = 22.192 \ln(x) - 52.54$' (Fig. 2). For ascorbic acid, the equation for the calculated curve was '$y = -28.27 \ln(x) + 15.864$' (Fig. 3). Further, S-shaped curve was derived by plotting the ORP values of sperm wash media with different concentrations of cumene hydroperoxide and ascorbic acid in same graph (Fig. 4).

## Discussion

Oxidative stress and its effect on the physiological function of spermatozoa still remain a topic of interest. Both researchers and clinicians are interested in accurately measuring oxidative stress in semen samples and further understanding its role for fertility treatment in general and male infertility in particular. The recently introduced novel galvanostatic technology MiOXSYS can determine the ORP in semen samples as a measure of oxidative stress (Agarwal *et al.*, 2016d). Higher levels of ORP were exhibited in infertile males compared to the fertile controls.

**Table 2** ORP values of the sperm wash (HTF-modified) medium alone calibrated with cumene hydroperoxide and ascorbic acid

| Concentration (μM) | | ORP (mV) | | Change in ORP (mV) | | pH |
|---|---|---|---|---|---|---|
| | | Mean | SD | Mean | SD | |
| Sperm wash media | | 267.3 | 3.6 | – | – | 7.2 |
| Cumene hydroperoxide | 10 | 273.7 | 2.2 | 6.4 | 4.2 | 7.2 |
| | 50 | 297.4 | 4.3 | 23.8 | 5.6 | 7.2 |
| | 100 | 313.7 | 2.4 | 46.4 | 4.4 | 7.2 |
| | 200 | 333.3 | 5.6 | 66.1 | 6.6 | 7.2 |
| | 500 | 354.3 | 3.2 | 87.0 | 4.8 | 7.2 |
| | 1000 | 371.2 | 7.0 | 104.0 | 7.8 | 7.2 |
| Ascorbic acid | 10 | 219.8 | 6.5 | −47.5 | 7.4 | 7.2 |
| | 31.25 | 189.6 | 2.4 | −77.6 | 4.3 | 7.2 |
| | 62.5 | 160.1 | 2.0 | −107.1 | 4.1 | 7.2 |
| | 125 | 144.9 | 4.0 | −122.3 | 5.4 | 7.2 |
| | 250 | 125.9 | 4.2 | −141.4 | 5.6 | 7.2 |
| | 500 | 108.3 | 2.4 | −159.0 | 4.3 | 7.0 |
| | 1000 | 90.3 | 2.3 | −177.0 | 4.3 | 7.0 |

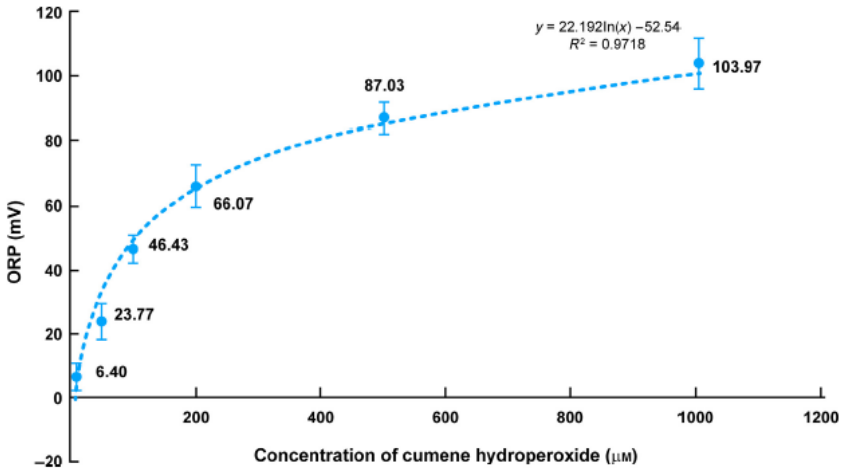



**Figure 2** Increase in the ORP values of sperm wash medium calibrated with cumene hydroperoxide.

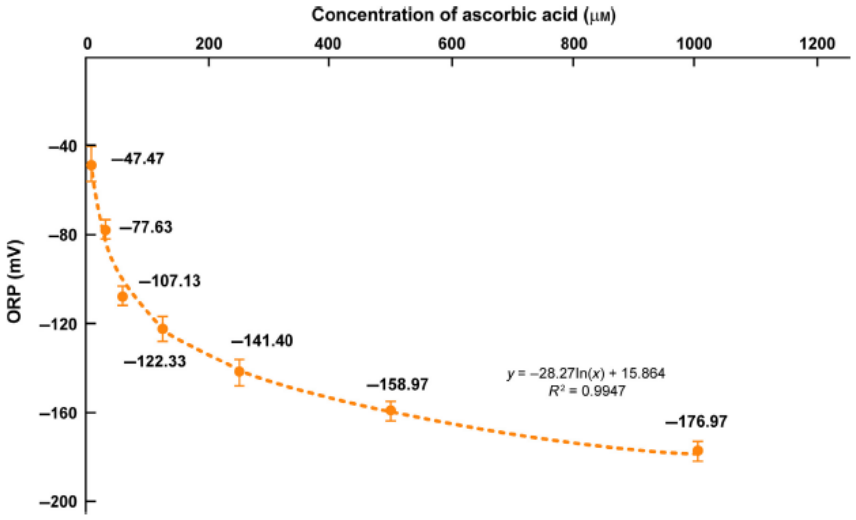
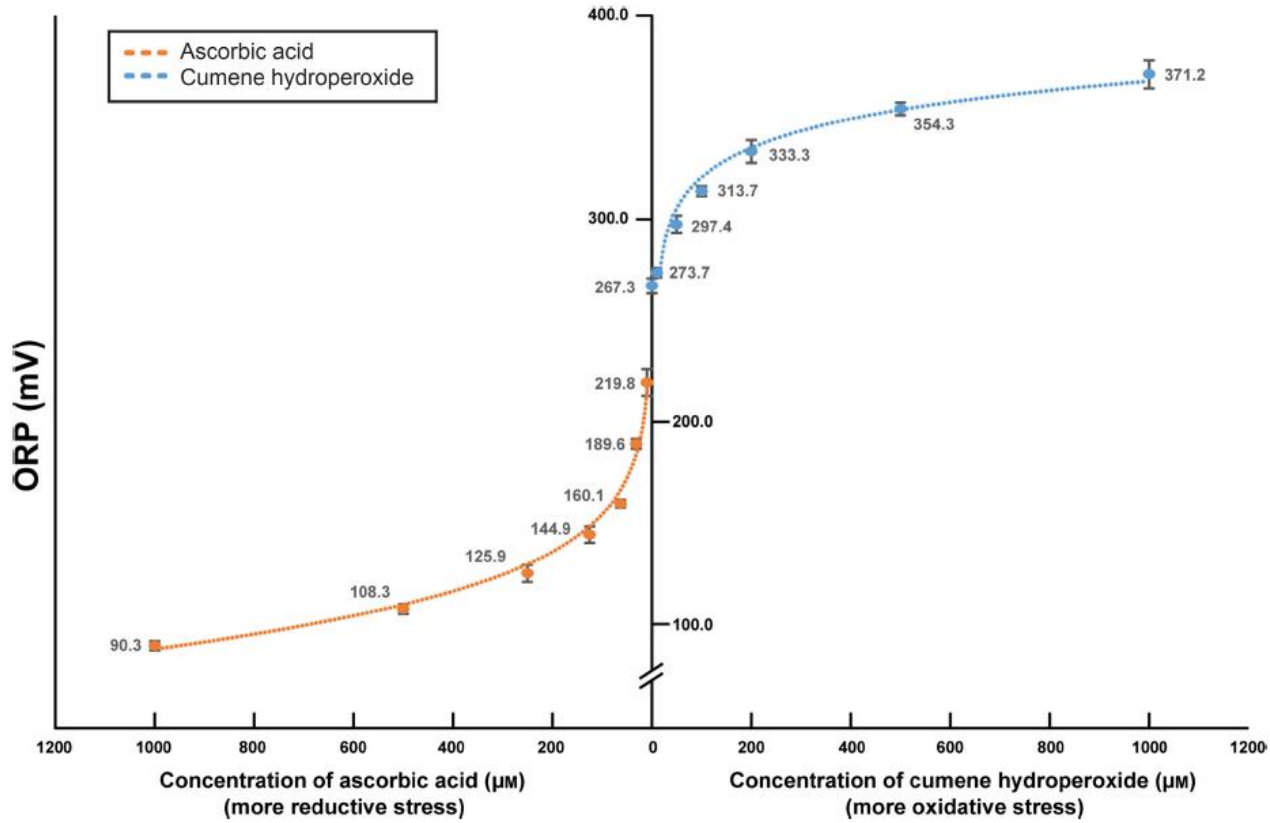



Figure 3 Decrease in the ORP values of sperm wash medium calibrated with ascorbic acid.

Figure 4 Calibration of ORP values in sperm wash medium using cumene hydroperoxide (more oxidative stress) and ascorbic acid (more reductive stress).




Previous studies have identified an ORP value of 1.36 mV/$10^6$ sperm/mL as the cutoff value to differentiate infertile men from fertile men (Agarwal *et al.*, 2016c). Similarly, a multicenter study has identified 1.42 mV/$10^6$/mL as the cutoff value to differentiate fertile from infertile men (Agarwal *et al.*, 2017). Studies also identified a negative correlation between ORP and sperm count, concentration, motility, and morphology, while

a positive correlation was observed with sperm nuclear DNA damage (Agarwal *et al.*, 2016c,d).

High seminal ORP values are indicative of poor semen quality, which are normally subjected to centrifugation during semen processing for sperm preparation in ART. The double density gradient centrifugation technique is routinely used to separate highly motile, morphologically normal spermatozoa from morphologically abnormal and immotile spermatozoa. Motile spermatozoa are then suspended in sperm wash medium before they are used for IUI (Agarwal *et al.*, 2016b). Even though composition and content of the sperm wash medium and other embryo culture media that are routinely used in andrology and IVF laboratories are available, information about the redox potential of these media, to the best of our knowledge, has not been investigated.

As poor quality spermatozoa from infertile men are used in ART, it is critical not only to optimize the chemical composition of media in order to increase the success rate in terms of fertilization and pregnancy, but also to control the redox conditions as ROS plays an essential role in the fertilization process. If the amount of ROS is too high, oxidative stress may occur leading to subsequent damages to the male germ cells that may have a negative impact on fertilization and subsequent embryo development. On the other hand, if the amount of antioxidants is too high, a condition termed 'reductive stress' can occur and prevent the fertilization process from happening by inhibiting capacitation and exerting a negative effect on the outcome of ART (Wendel, 1987; Henkel, 2011). Moreover, dysregulation of the fine redox balance can also lead to delayed embryo development (Ufer *et al.*, 2010) as this condition perturbs the blood–brain barrier with relevant neurological consequences (Mentor & Fisher, 2017). Reductive stress is also reported to be involved in the pathogenesis of other illnesses such as cardiac injuries (Brewer *et al.*, 2013) or neurological diseases such as Alzheimer's disease (Lloret *et al.*, 2016).

The goal of this pilot study was to assess the oxidation–reduction (redox) potential in sperm wash medium and to compare it with the other sequential and single-step media used for ART. The fertilization process is affected by high ROS production (Agarwal *et al.*, 2014c), especially during the centrifugation process of abnormal semen samples from infertile men resulting in an additional amount of ROS (Agarwal *et al.*, 2014a). Hence, the redox potential of the culture media is maintained on the lower side to neutralize and counteract ROS.

The results of this study indicate that the sequential culture medium has a significantly ($p < 0.05$) lower redox state compared to sperm wash medium, which is essential for spermatozoa to fertilize the oocyte. Further, the single-step medium showed a lower ORP level. This could be the reason for the increased blastocyst formation and delayed DNA methylation observed in the one-step media when compared with the sequential media for IVF (Sfontouris *et al.*, 2016), indicating that the redox potential of the medium might be involved in the regulation of the fertilization process. In fact, in the oviduct after

fertilization, the relative production of ROS is lowered for the embryos to implant (Ufer *et al.*, 2010).

Interestingly, the freezing medium used for cryopreservation of spermatozoa also showed low ORP values compared to the sperm wash medium due to the presence of the antioxidants such as carotenoids and vitamin E in the egg yolk used as major component of cryopreservation media (Nimalaratne & Wu, 2015). During cryopreservation and the thawing process, ROS production is increased and antioxidant levels are decreased (Said & Agarwal, 2012). We observed that the freezing medium exhibited a reduced redox potential which might be able to counteract the excess of ROS produced during the cryopreservation process.

In semen, the importance of a balanced redox system is increasingly being recognized and exhibited a strong correlation to sperm damage induced by oxidative stress. Antioxidants (enzymatic and non-enzymatic) act as counterparts of the oxidants and neutralize ROS (Aitken & Roman, 2008). To overcome the oxidative stress in infertile patients, antioxidant therapies are normally advised by physicians. However, different studies show conflicting results with regard to the effect of the antioxidant therapy on fertility. On the one hand, the use of antioxidants has been shown to increase sperm motility, concentration, and morphology and improve DNA integrity as well as increase pregnancy rates (Gupta & Kumar, 2002; Agarwal *et al.*, 2004; Durairajanayagam *et al.*, 2014; ElSheikh *et al.*, 2015; Ahmad *et al.*, 2017). On the other hand, however, no effects or even negative effects on various sperm functions were reported (Verma & Kanwar, 1998; Rolf *et al.*, 1999; Silver *et al.*, 2005; Menezo *et al.*, 2007; Giustarini *et al.*, 2008).

Like oxidative stress, reductive stress is detrimental to cells. Teratogenic changes during embryonic development are induced by reductive stress due to overdose of antioxidants (Ufer *et al.*, 2010). Reports suggest that reductive stress causes cardiac hypertrophy, cardiomyopathy, and heart failure (Brewer *et al.*, 2013). Moreover, reductive stress was shown to decrease the permeability of the blood–brain barrier (Zlokovic, 2011; Mentor & Fisher, 2017). The effect of reductive stress on spermatozoa is however neither described nor explained.

Spermatozoa depend on physiological levels of ROS for their functions including capacitation, hyperactivation, and acrosome reaction (Agarwal *et al.* 2006, Agarwal *et al.*, 2014c). Even more so, neither the amount of physiological ROS generated or required by the spermatozoa for normal function has been studied yet, nor the physiological range of the redox levels (oxidative and reductive stress) that can be tolerated by normal spermatozoa without affecting normal sperm functions is clearly defined. Under *in vitro* conditions, cumene hydroperoxide has been used to induce oxidative stress (Cho *et al.*, 2017) and ascorbic acid supplementation to reduce oxidative stress of spermatozoa (Ahmad *et al.*, 2017).

In this study, we have successfully demonstrated the increase in ORP levels in the sperm wash medium supplemented with cumene hydroperoxide and a decrease in ORP following use of ascorbic acid. This dose-dependent increase in the ORP values was achieved in the sperm wash medium by cumene hydroperoxide, and a decrease was obtained with ascorbic acid (Fig. 4) without affecting the pH of the medium. The results of this study could help in calibrating the ORP of sperm wash medium to obtain the desired redox potential state. This can be used for defining the normal redox potential values for the spermatozoa without affecting its functionality and also to compare the ORP values of patients with impaired sperm functions. Future *in vitro* experiments using live spermatozoa should be carried out to determine the normal physiological ORP range for healthy spermatozoa. One of the study limitations was that we did not examine the effect of alteration in ORP levels using different sperm wash media on sperm function such as acrosome reaction, alteration in mitochondrial membrane potential, and DNA fragmentation. Further in our current study, we did not evaluate the effect of changing ORP levels in the ART culture media on *in vitro* fertilization and subsequent steps of embryo development.

This is the first study to report the redox potential status in the different culture media used in andrology and IVF laboratories for sperm processing and embryo culture. An optimized redox potential is required for successful embryogenesis and to avoid teratogenesis (Ufer *et al.*, 2010). Oxidative stress affects the development of embryos (Dennery, 2007; Harvey *et al.,* 2002); hence, the differences in ORP levels of different culture media might be one of the factors regulating *in vitro* fertilization and development of embryos and therefore have to be taken into consideration. It further provides researchers the basis to design future experiments to establish the normal physiological range of the redox values to guide and improve sperm selection in assisted reproductive technology, allowing scientists to avoid the selection of spermatozoa affected by oxidative and reductive stress. This knowledge will improve not only the diagnosis, but also treatment of male infertility. In the end, the MiOXSYS system can potentially be used in a clinical setup to manipulate and optimize the ORP of media based on the ORP of patients' semen samples for better ART outcome.

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**Competing interests**
None of the authors declare competing financial interests.

**Authors' contributions**

AA and RH conceived and designed the study; MP was involved in carrying out the experiment. RH, RS, MKPS, and AA were involved in the analysis and interpretation of the data. All authors have read and approved the final version of the manuscript, and agreed with the order of presentation of the authors.

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