Ascorbic acid reduces redox potential in human spermatozoa subjected to heat-induced oxidative stress

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Summary
Oxidation–reduction potential (ORP) is a new measure of oxidative stress. It is a balance between the total available oxidants and reductants. This study measures the efficiency of ascorbic acid (AA) against oxidative stress induced by either heat alone or heat and hydrogen peroxide in sperm suspensions using the MiOXSYS System. Two concentrations of ascorbic acid (400 and 600 μmol/L) were tested against heat- and heat plus hydrogen peroxide-induced oxidative stress in sperm suspensions after 2 and 4 hr of incubation. Sperm motility and static oxidation reduction potential (sORP) were measured at 2 and 4 hr of incubation at three different temperatures. A significant decrease in sORP was observed as a function of AA concentration. The 600 μmol/L AA had more pronounced reduction in sORP compared to 400 μmol/L AA (p = .001). Significant decreases in sperm motility ranging from 4.89% to 14.02% were observed both as a function of incubation time and addition of H₂O₂ (p < .001). Ascorbic acid is efficacious to reduce heat-induced oxidative stress in sperm preparations in vitro. The supplementation of ascorbic acid may be advantageous for semen preparations in IUI, IVF and ICSI.

KEYWORDS
ascorbic acid, heat, hydrogen peroxide, oxidation–reduction potential, spermatozoa

1 | INTRODUCTION

Reactive oxygen species (ROS) are oxygen- or nitrogen-derived array of molecules endowed with unpaired electrons. These are the by-products generated during normal cell metabolism and several cellular functional processes (Chen, Allam, Duan, & Haidl, 2013). Physiological levels of ROS are prerequisite to normal sperm function such as capacitation, hyperactivation and sperm–oocyte fusion (Du Plessis, Agarwal, Halabi, & Tvrda, 2015). Naturally, a balance exists between the ROS and antioxidant levels in the semen. When ROS overwhelm the seminal antioxidant capacity, oxidative stress (OS) occurs. Oxidative stress is a contributory factor to male infertility in 30%–80% of the affected men (Agarwal, Nallella, Allamaneni, & Said, 2004). It is associated with many aetiology conditions and risk factors such as varicocele, cryptorchidism, subclinical infections, advanced paternal age, cancer, radiation, smoking, obesity, use of gonadotoxins and occupational exposure to high temperature (Aitken & Koppers, 2011; Alvarez et al., 2002; Chen et al., 2013). In the semen, leucocytes as well as immature and defective spermatozoa produce pathological levels of ROS, which are detrimental to normal sperm function. Excessive ROS levels have been associated to sperm membranes’ lipid peroxidation and DNA damage (Aitken & Koppers, 2011; Alvarez et al., 2002; Chen et al., 2013).

Among the several aforementioned factors associated with oxidative stress, heat stress attains special attention because of the temperature-sensitive testicular physiology. The human testes are located in the scrotum where spermatogenesis is optimal at a temperature 2.5°C lower than the core body temperature (Rao et al., 2015).
The pampiniform plexus and testicular artery ensure maintenance of temperature at lower level through the counter-current heat exchange mechanism (Mieusset et al., 1987). Elevation of scrotal temperature has been associated with semen quality deterioration in men exposed to occupational (e.g., firefighters, bakery workers, farmers, construction workers, miners, boiler room workers and factory workers) and recreational (e.g., sauna and hot tubs) heat stress, as well as patients with varicocele and cryptorchidism (Garolla et al., 2013; Hjollund, Bonde, Jensen, & Olsen, 2000; Liu & Li, 2010; Shiraishi, Takihara, & Matsuyama, 2010; Thonneau, Ducot, Bujan, Mieusset, & Spira, 1997). Heat stress increases the production of mitochondrial superoxide anion ($\cdot$O$_2^-$), which is the first by-product of the one-electron reduction of oxygen. This reaction occurs at specific sites of the electron transport chain (ETC), which is an important source of oxidising agent and that after catalysis is converted to highly reactive anion ($\cdot$O$_2^-$).

Recreational (e.g., sauna and hot tubs) heat stress, as well as patients with varicocele and cryptorchidism (Garolla et al., 2013; Hjollund, Bonde, Jensen, & Olsen, 2000; Liu & Li, 2010; Shiraishi, Takihara, & Matsuyama, 2010; Thonneau, Ducot, Bujan, Mieusset, & Spira, 1997). Heat stress increases the production of mitochondrial superoxide anion ($\cdot$O$_2^-$), which is the first by-product of the one-electron reduction of oxygen. This reaction occurs at specific sites of the electron transport chain (ETC), which is an important source of oxidising agent and that after catalysis is converted to highly reactive anion ($\cdot$O$_2^-$).

Heat stress has also shown to decrease superoxide dismutase 1 (SOD-1) mRNA levels, cytoplasmic SOD protein and enzyme activity, leading to increased ROS generation (Turrens, 2003). Specifically, spermatozoa B, pachytene spermatocytes and spermatids are highly vulnerable to heat stress (Hadziselimovic & Herzog, 2001).

Antioxidants, including ascorbic acid (AA), have a balancing role in controlling oxidative stress, and do this by their characteristic nature of having extra electrons that mitigate the unpaired valence electrons of oxidative molecules. These include enzymatic high-molecular-weight antioxidants such as superoxide dismutase, catalase, glutathione peroxidase/reductase, as well as nonenzymatic antioxidants such as low-molecular-weight taurine, hypotaurine, vitamin E, ascorbate, carnitine, glutathione, α-tocopherol, β-carotene, lycopene, albumin, ubiquinol epinephrine, lactate and pyruvate (Agarwal et al., 2004). Despite the potential advantages of antioxidant therapy, a definitive conclusion of its indication could not be drawn yet. Most published studies have been poorly designed and lack controls, and the few good-quality studies achieved conflicting results, which prompts a need for additional trials (Agarwal et al., 2004; Donnelly, McClure, & Lewis, 1999; Hamada, Esteves, & Agarwal, 2013; Menezo et al., 2007; Tremellen, 2008).

The aim of the study was to investigate whether ascorbic acid can alleviate heat-induced oxidative stress in human sperm in vitro. The experiments were designed to create a condition of heat stress. For this, we exposed spermatozoa to excessive heat (39.5°C) and compared with temperatures reflecting the normal human testicular temperature (34.5°C) and normal body temperature (37°C; Ahmad, Moinard, Esquerre-Lamare, Mieusset, & Bujan, 2012). Moreover, we examined the effectiveness of AA to alleviate heat-induced oxidative stress plus by addition of hydrogen peroxide ($H_2O_2$), which is a strong oxidising agent and that after catalysis is converted to highly reactive hydroxyl radicals.

## MATERIAL AND METHODS

### 2.1 Subjects, semen collection and analysis

Following approval by our Institutional Review Board (IRB), semen samples were obtained from healthy male volunteers ($n = 26$). All subjects provided written consent, and samples were collected and analysed according to the 5th edition of World Health Organization (WHO) manual for the examination of human semen (WHO, 2010). Semen samples were collected after 2-3 days of ejaculatory abstinence. After liquefaction, an aliquot was removed for standard semen analysis.

Analysis of sperm concentration, motility and round cells was performed manually on wet preparation using a MicroCell counting chamber (Vitrolife, San Diego, CA) with phase optics set at 20× magnification. Samples with a high concentration of round cells (>5 per high-power field) were examined for the presence of polymorphonuclear leukocytes (PML) using the peroxidase (Endtz) test. For this, a 20-μl well-mixed aliquot of the semen sample was mixed with one volume of PBS and 2 volumes of working Endtz solution in an amber coloured Eppendorf tube. After 5 min, a drop of the aliquot was placed on a Makler chamber and examined for the presence of dark brown cells under a 10× bright field objective. Leukocytespermia was defined as the presence of >1 × 10<sup>6</sup> PML/ml according to the WHO guidelines (World Health Organization, 2010). Samples with the presence of round cells >1 × 10<sup>6</sup> or positive to the Endtz test were excluded.

### 2.2 Sperm preparation by double density gradient centrifugation technique

All selected semen samples underwent double density gradient centrifugation to further ensure the separation of any leukocytes in the ejaculates. Gradients were prepared by layering 2 ml of the 80% gradient medium as a lower phase and 2 ml of the 40% gradient medium as an upper phase (PureCeption SAGE In-Vitro Fertilization, Inc., Trumbull, CT, USA). Liquefied semen was then loaded on top of the gradient set and centrifuged for 20 min at 300 x g, after which the supernatant was removed and the pellet was resuspended in 2 ml of washing media (Quinn’s Advantage medium with HEPES, Origio, Malov, Denmark). Centrifugation was repeated for 7 min at 300 x g, the supernatant was discarded and the pellet was resuspended in washing media. The final sperm concentration was adjusted to 10-15 × 10<sup>6</sup> spermatozoa/ml and used for exposure to heat-induced OS.

### 2.3 Exposure to heat stress

Two experiments were conducted in duplicate run. In the first experiment, OS was induced by heat using three different temperatures: 34.5°C (i.e., physiological temperature of testes), 37°C (i.e., core body temperature) and 39.5°C (encountered during fever, hot baths or when exposed to high occupational temperatures such as chefs, factory workers, welders and long-distance drivers; Bujan, Daudin, Charlet, Thonneau, & Mieusset, 2000). In the second experiment, in addition to heat as in the first experiment, OS was induced by hydrogen peroxide ($H_2O_2$, 200 μmol/L). Three incubators were designated for these experiments, each one set with the temperature under investigation, namely 34.5, 37 and 39.5°C at 6% CO<sub>2</sub>. Specimens were analysed after 2 and 4 hr of incubation.
The goal was to determine the levels of oxidative stress generated under experimental conditions.

2.4 | Ascorbic acid supplementation

Two concentrations of ascorbic acid, namely 400 and 600 μmol/L (L-ascorbic acid A4403, Sigma Aldrich, St. Louis, MO, USA), were used after induction of heat stress in each set of experiments described above. Stock solution of one molar (1 mol/L) ascorbic acid was prepared in phosphate buffer saline (PBS) and diluted with human tubal fluid (HTF, Irvine Scientific, Irvine, CA, USA) to achieve the final working concentrations.

In the first set of experiments (heat only), an aliquot containing sperm was resuspended in HTF only and served as control. A second aliquot contained sperm suspension + 400 μmol/L ascorbic acid whereas the third aliquot contained sperm suspension + 600 μmol/L ascorbic acid. The samples were incubated at 34.5, 37 and 39.5°C and analysed after 2 and 4 hr of incubation.

In the second set of experiments (heat + H$_2$O$_2$), aliquots containing sperm resuspended in HTF only, 400 μmol/L ascorbic acid and 600 μmol/L ascorbic acid were mixed with 200 μmol/L H$_2$O$_2$ and incubated at the three aforementioned temperatures as described above.

The same experimental conditions were used throughout experiments. A detailed illustration of sample incubation and reagents is given in Figure 1.

2.5 | Measurement of oxidation–reduction (redox) potential

Oxidation–reduction was measured using a novel galvanostat-based technology (MiOXSYS™ System; Aytu Bioscience, Englewood, CO, USA; Figure 2). Briefly, a 30 μl sample suspension was exposed to the MiOXSYS sensor. The MiOXSYS system provides an estimation of the static oxidation–reduction potential (sORP), measured in mV. It represents the integrated measure of the existing balance between total oxidants and reductants in a biological system (Agarwal, Sharma, Roychoudhury, Du Plessis, & Sabanegh, 2016). Two readings were measured from each aliquot after 2 and 4 hr of incubation for sperm count and motility and sORP, and the average values were recorded. Data were then normalised to sperm concentration to control for differences in cell numbers, and sORP was presented as mV/10$^6$ sperm/ml.

2.6 | Statistical analysis

Quantitative variables, including the changes between heat alone and heat + H$_2$O$_2$, are presented as mean ± standard error of the mean (SEM). Repeated measures models were used to assess the effects of ascorbic acid concentrations on motility and sORP while aggregating over other factors. The effect of ascorbic acid on motility and sORP are presented as covariate-adjusted differences by means and 95% CI.
3 | RESULTS

Descriptive data on sperm motility and sORP in heat only and heat and H$_2$O$_2$ groups as a function of ascorbic acid supplementation are shown in Table 1.

Heat stress alone had no significant impact on sperm motility (Table 2). When H$_2$O$_2$ was added to heat, a significant decrease in motility ranging from 6.7% to 14.02% was observed ($p < .001$). The degree of change in motility with the inclusion of H$_2$O$_2$ was dependent on the incubation temperature, and likewise, the change in motility with added heat was dependent on the inclusion of H$_2$O$_2$ ($p < .001$) for the interaction between H$_2$O$_2$ and temperature. Increasing the temperature from 34.5 to 37.0°C resulted in a significant decrease in motility (~4.77%) after inclusion of H$_2$O$_2$ ($p = .004$). Elevating the temperature to 39.5°C did not decrease motility further. A significant decrease in motility (~4.89%) was also observed as a function of incubation time. A significant decrease in sORP was observed as a function of AA concentration (Table 3). The mean reduction in sORP for 400 μmol/L AA compared to control was 6.70 mV/10$^6$ sperm/ml ($p < .001$), and 7.31 mV/10$^6$ sperm/ml for 600 μmol/L AA compared to control was ($p < .001$; Table 3). A reduction of 0.61 mV/10$^6$ sperm/ml was observed when 600 μmol/L AA was compared to 400 μmol/L AA ($p = .001$). However, none of the other factors (incubation time, temperature and H$_2$O$_2$) were significantly associated with mean sORP and no significant interactions were found among the factors in the repeated measures model (Table 3). Therefore, unlike the model for motility, the effects of each variable on sORP as shown in Table 3 were described without subsetting to specific values of the remaining variables.

**FIGURE 2** Measurement of oxidation-reduction potential by the MiOXSYS System. (a) MiOXSYS Analyzer showing the sensor socket and the sensor module and (b) sensor showing the reference cell and the sample port where the sample is loaded.
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4 | DISCUSSION

The goal of our pilot study was to examine the combined effect of heat and ROS on sperm motility using an in vitro model and examine the potential benefit of ascorbic acid in reducing the increased effect of ROS and/or heat on sperm prepared by density gradient. We found that ascorbic acid supplementation provided a dose-dependent sperm protection against oxidative stress, as assessed by the static oxidation-reduction (redox) potential (sORP). Both ascorbic acid concentrations used lead to a marked decline in sORP compared to controls, but results favoured the higher dosage of 600 μmol/L. Heat stress caused a reduction in sperm motility after addition of H$_2$O$_2$, but ascorbic acid in the concentrations used was unable to sustain motility in specimens exposed to a combination of heat stress and H$_2$O$_2$. In the initial experiments performed by us during standardisation of the MiOXSYS System, we examined the use of H$_2$O$_2$ as a positive inducer of ROS and measuring the ORP by the MiOXSYS System. We tested H$_2$O$_2$ concentration from 100 to 8000 μmol/L along with semen sample, but the change in ORP ranged from only 168 to 208.4 mV. Furthermore, we found that the increase in ORP was not linear even after 2–3 hr of incubation. This may be because of the interference of H$_2$O$_2$ with the source of the sensor electrode. This could be one of the reasons for the lack of a dramatic increase in sORP when the samples were incubated with 200 μmol/L of H$_2$O$_2$.

**TABLE 2** Repeated measures model for sperm motility (%) in heat-induced oxidative stress

<table>
<thead>
<tr>
<th>Variable</th>
<th>Covariate-adjusted Difference in Mean (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>63.58 (53.81, 73.35)</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid (μmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 AA (vs Control)</td>
<td>-0.83 (-3.12, 1.45)</td>
<td>.47</td>
</tr>
<tr>
<td>600 AA (vs Control)</td>
<td>-2.24 (-4.53, 0.04)</td>
<td>.055</td>
</tr>
<tr>
<td>H$_2$O$_2$ (if Temp = 34.5°C)</td>
<td>-6.70 (-9.94, -3.46)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>H$_2$O$_2$ (if Temp = 37.0°C)</td>
<td>-14.02 (-17.25, -10.78)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>H$_2$O$_2$ (if Temp = 39.5°C)</td>
<td>-11.00 (-14.24, -7.77)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Time 4 hr (vs 2 hr)</td>
<td>-4.89 (-6.52, -2.79)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Temperature (if heat only)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37.0°C (vs 34.5°C)</td>
<td>2.54 (-0.69, 5.78)</td>
<td>.12</td>
</tr>
<tr>
<td>39.5°C (vs 34.5°C)</td>
<td>1.14 (-2.09, 4.38)</td>
<td>.49</td>
</tr>
<tr>
<td>39.5°C (vs 37.0°C)</td>
<td>1.40 (-4.64, 1.84)</td>
<td>.40</td>
</tr>
<tr>
<td>Temperature (if heat + H$_2$O$_2$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37.0°C (vs 34.5°C)</td>
<td>-4.77 (-8.01, -1.54)</td>
<td>.004</td>
</tr>
<tr>
<td>39.5°C (vs 34.5°C)</td>
<td>-3.16 (-6.40, 0.07)</td>
<td>.055</td>
</tr>
<tr>
<td>39.5°C (vs 37.0°C)</td>
<td>1.61 (-1.62, 4.85)</td>
<td>.33</td>
</tr>
</tbody>
</table>

**TABLE 3** Repeated measures model for semen sORP (mV/10^6 sperm/ml) in heat-induced oxidative stress

<table>
<thead>
<tr>
<th>Variable</th>
<th>Covariate-adjusted Difference in Mean (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>11.39 (9.68, 13.10)</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 AA vs Control</td>
<td>-6.70 (-7.07, -6.34)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>600 AA vs Control</td>
<td>-7.31 (-7.68, -6.94)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>600 AA vs 400 AA</td>
<td>-0.61 (-0.97, -0.24)</td>
<td>.001</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>0.159 (-0.138, 0.457)</td>
<td>.29</td>
</tr>
<tr>
<td>Time 4 hr vs 2 hr</td>
<td>0.074 (-0.024, 0.371)</td>
<td>.63</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37.0°C vs 34.5°C</td>
<td>0.110 (-0.256, 0.476)</td>
<td>.55</td>
</tr>
<tr>
<td>39.5°C vs 34.5°C</td>
<td>0.229 (-0.134, 0.592)</td>
<td>.22</td>
</tr>
<tr>
<td>39.5°C vs 37.0°C</td>
<td>0.119 (-0.246, 0.484)</td>
<td>.52</td>
</tr>
</tbody>
</table>
Vitamin C (ascorbic acid) is a water-soluble vitamin found in dietary sources. It is considered a synthetic antioxidant because it cannot be produced by the human body enzymatically. Vitamin C is found at higher concentrations in the seminal fluid of fertile men than in infertile men and is therefore assumed to play an important role as a seminal antioxidant (Colagar & Marzony, 2009). Strategies to alleviate OS using vitamin C have been attempted in both in vitro and in vivo studies, with mixed results. Some have reported an increase in semen parameters, including motility, concentration, morphology and DNA integrity, and also higher pregnancy rates after antioxidant therapy (Cyrus, Kabir, Goodarzi, & Moghimi, 2015; Dawson, Harris, Teter, & Powell, 1992; EISheikh et al., 2015; Greco, Iacobelli et al., 2005; Greco, Romano et al., 2005; Kodama, Yamaguchi, Fukuda, Kasai, & Tanaka, 1997; Menezo et al., 2007; Piomboni et al., 2008; Showell et al., 2014; Suleiman, Ali, Zaki, El-Malik, & Nasr, 1996). On the contrary, others have found either no improvement on the aforementioned parameters or a detrimental effect of vitamin C supplementation, such as increased rates of sperm DNA decondensation (EISheikh et al., 2015; Menezo et al., 2007; Moilanen & Hovatta, 1995; Piomboni et al., 2008; Silver et al., 2005). Along the same lines, the empiric prescription of oral antioxidants is commonplace among clinicians providing care to infertile couples. It is based on the premise that seminal oxidative stress is due, in part, to a deficiency in seminal antioxidants (Fanaei et al., 2014). Moreover, oral antioxidants lack major side effects and are relatively inexpensive.

Our results are in accordance with previous in vitro studies, which showed reduction in OS levels in human sperm exposed to ascorbic acid supplementation (Donnelly et al., 1999; Fanaei et al., 2014). Nevertheless, our technique to measure OS differs than the aforementioned studies as we employed a novel galvanostat-based technology that provides an estimation of the static oxidation-reduction potential (sORP) based on the balance between total oxidants and reductants in the semen.

Physiological levels of ROS are necessary for important functions such as capacitation, hyperactivation and acrosome reaction. Furthermore, the use of overconsumption of antioxidants can be toxic. Historically, measurement of seminal OS has relied on single biomarkers. For instance, ROS by the chemiluminescence assay measures the quantity of reactive oxygen species whereas the colorimetric total antioxidant capacity (TAC) assay estimates the amount of antioxidants in a biological specimen. Similarly, the malondialdehyde (MDA) assay measures lipid peroxidation (i.e., post hoc damage; Grotto et al., 2007; Roychoudhury, Sharma, Sikka, & Agarwal, 2016). A combined ROS-TAC score was shown to provide a better measure of OS in semen compared to individual ROS and TAC parameters (Sharma, Pasqualotto, Nelson, Thomas, & Agarwal, 1999). Still, these methods are limited, as they do not assess the overall redox status in real time. Hence, the accurate and quick estimation of the seminal redox activity taking into account all known and unknown oxidants as well as antioxidant activity would be advantageous. The oxidation-reduction potential (ORP) provides a comprehensive measure of OS as it represents the potential for electrons to move from one chemical species to another. In a recent study, we investigated and confirmed the usefulness of the method to distinguish a population of infertile men from healthy donors based on ORP levels (Agarwal et al., 2016). The ORP cutoff of 1.48 mV/10^6 sperm/ml in semen and of 2.09 mV/10^6 sperm/ml in seminal plasma was able to distinguish subjects with normal semen quality from those with abnormal semen quality (Agarwal et al., 2016). In the semen, at a cutoff of 1.48 mV/10^6 sperm/ml, the sensitivity was 60.0% and specificity 75.0%. The real-time measurement of redox capacity by ORP in semen specimens offers potential advantages compared to the existing methods, but further validation studies are still needed.

The potential value of MiOXSYS and its application as a simple alternative to multiple individual markers of oxidative stress such as ROS (chemiluminescence), antioxidants (total antioxidant capacity and individual enzymatic and nonenzymatic antioxidants) and lipid peroxidation (MDA) which are cumbersome, and require expensive and complicated equipment such as in the measurement of ROS by the gold standard (chemiluminescence assay; Bertolla, 2016).

With regard to sperm motility, a previous study showed a decline in progressive motility, average path velocity, curvilinear velocity, straight-line velocity, and linearity after supplementation with ascorbic acid at 600 μmol/L concentration (Donnelly et al., 1999). However, this aforementioned study utilised asthenozoospermic samples and therefore differs to ours where only normozoospermic specimens were enrolled. In another in vitro study, ascorbic acid in a similar concentration (600 μmol/L) than ours was shown to increase sperm motility after 1 hr of incubation at 37°C (Fanaei et al., 2014). The increase in motility in this study was mainly attributed to the hyperactivation of the sperm population assessed by computer-assisted semen analysis (CASA), whose metabolism is still high within 1 hr of incubation and was further protected by the supplementation of ascorbic acid. On the contrary, we assessed motility manually after 2 and 4 hr incubation. Our results showing decline in motility as a function of time might be explained by a decrease in sperm energy as our washed specimens lacked any external energy sources. Higher concentrations of ascorbic acid might add additional benefit with regard to sperm motility in specimens exposed to heat stress in the presence of oxidative stress induced by H₂O₂, but this hypothesis needs to be investigated further.

Choosing the dosage of AA was a challenge because of disparity in the published literature. Kandar, Drabkova, and Hampl (2011) and Guz et al. (2013) used 399.32 and 444.8 μmol/L, respectively, in normozoospermic men (Guz et al., 2013; Kandar et al., 2011). Colagar and Marzony (2009) and Lewis, Sterling, Young, and Thompson (1997) have added 448.71 and 481 μmol/L, respectively, to the seminal plasma of fertile men (Colagar & Marzony, 2009; Lewis et al., 1997). Only one study, Thiele, Friesleben, Fuchs, & Ochsendorf (1995), reported relatively higher values (686 μmol/L) of ascorbic acid in seminal plasma of donors. As the majority of recent studies reported a maximum of 481 μmol/L in fertile men, we selected 400 μmol/L as the lowest concentration and 600 μmol/L as the highest concentration. Our results showed that the higher dose of AA was more efficient against OS compared to the lower dose, which is considered as
a physiological dose in the seminal plasma of healthy men (Colagar & Marzony, 2009; Kandar et al., 2011). Based on these results, the dose of 600 μmol/L may be suggested in patients with elevated ROS levels.

Although our results are reassuring with regard to the utility of ascorbic acid in reducing OS when added to the semen, our study has some limitations. The sample size was relatively small and the motility was assessed manually, which might have added subjectivity. Our method of OS analysis is new and not fully validated. Nevertheless, the instrument is very user-friendly, cost-effective and requires a simple procedure that gives the results immediately. Moreover, the instrument was shown to be sensitive enough to detect differences in sORP in sperm specimens challenged with ascorbic acid and H$_2$O$_2$.

In our study, ascorbic acid was added directly to the semen specimens rather than given orally to the patients. Despite not testing oral ascorbic acid supplementation, our design is advantageous because the same specimen was used for all experiments. Doing this we avoided bias, which could be attributed to the nature of samples, handling conditions during incubation and antioxidant distribution in the serum and well as potential interactions with other nutrients. Furthermore, semen specimens may differ with regard to their seminal plasma antioxidant capacity. By removing the seminal plasma, we controlled this important confounding factor.

In conclusion, ascorbic acid is effective in reducing oxidative stress in vitro as measured by MIOXSYS. Ascorbic acid supplementation may offer a protective role during semen preparation for IUI, IVF and ICSI.

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COMPETING INTERESTS

None of the authors declare competing financial interests.

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