Research article

A microplate-based DCFH-DA assay for the evaluation of oxidative stress in whole semen

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ABSTRACT

Aims: The well-documented relationship between sperm oxidation and male infertility strongly encourages the development of assays for reactive oxygen species detection in semen samples. The present study aims to apply the microplate-based 2',7'-dichlorofluorescein diacetate assay to the evaluation of oxidative stress in unprocessed whole semen, thus avoiding sample centrifugations and other manipulations that may cause significant reactive oxygen species increments.

Main methods: The fluorescence assay consisted in the quantification of both intracellular and extracellular reactive oxygen species levels in unwashed semen specimens by using the probe 2',7'-dichlorofluorescein diacetate into a 96-well plate. The method was useful for the preliminary assessment of the oxidation levels of whole semen samples from men undergoing standard sperm analysis as well as to evaluate the effect of some pro-glutathione molecules on semen oxidative status.

Key findings: The 2',7'-dichlorofluorescein diacetate assay was successfully adapted to the evaluation of oxidative stress in whole semen, effectively revealing the perturbation of the redox homeostasis of the sample. Accordingly, specimens with abnormal sperm parameters (n = 10) presented oxidation indexes significantly higher than those with normospermia (n = 10) [7729 (range 3407–12769) vs. 1356 (range 470–2711), $p < 0.001$]; in addition, semen oxidation indexes negatively correlated to sperm motility and morphology. Noteworthy, whole semen exposure to pro-glutathione compounds led to reduced semen oxidation levels and sperm protection against oxidative damage.

Significance: Based on our pilot experimental data, the microplate-based 2',7'-dichlorofluorescein diacetate assay appears to be a convenient method for the detection of reactive oxygen species levels in whole semen samples, avoiding artifacts due to semen centrifugation steps. At the same time, the test could be a helpful tool for the basic and quick screening of antioxidant molecules able to preserve semen quality.

1. Introduction

The role of oxidative stress in male infertility has been widely recognized [1, 2, 3]. Low levels of reactive oxygen species (ROS) are essential for cellular physiological functions, including spermatogenesis and various steps preceding fertilization, such as capacitation and acrosome reaction [4]. On the contrary, elevated ROS levels may be cytotoxic for sperm cells through membrane lipid peroxidation, DNA damage, and apoptotic cell death, lastly resulting in a loss of sperm function [5, 6]. All these events negatively affect sperm parameters such as morphology and motility [7, 8]; in particular, oxidative stress has been linked to idiopathic male infertility (which represents approximately 30–50% of male infertility cases), leading to the concept of "Male Oxidative Stress Infertility" (MOSI) for many patients who were previously classified as having idiopathic infertility [9, 10].

In this context, the laboratory assessment of oxidative stress in semen samples has gained considerable attention, since it might be a helpful tool in the initial evaluation and follow-up of infertile male patients [11, 12]. For this reason, the availability of methods useful to easily measure general aspects of oxidative stress and redox status is recommended.

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Various direct and indirect ROS assays have been introduced [13], some of which focus on ROS production by isolated spermatozoa, using washed sperm cell suspensions [14, 15]. However, centrifugations and other manipulations trigger significant ROS production within cells, thus creating artifacts [16]; at the same time, the removal of seminal plasma during the washing process eliminates antioxidant molecules that would naturally protect sperm from ROS attack [17, 18].

It must also be considered that oxidative stress in the reproductive tract can be attributed to ROS production not only from sperm cells but also from polymorphonuclear leucocytes [19, 20]; therefore, for the determination of oxidative stress in diagnosing infertility, measuring ROS in unwashed, whole semen is most appropriate, reflecting both the pro- and anti-oxidant status of the sperm microenvironment. In this context, ROS assays using the chemiluminescent probe luminol have been validated in unprocessed semen in the last years [21, 22, 23], demonstrating negative correlations between ROS in whole semen and sperm motile parameters, the natural pregnancy rate, and the sperm motility index [8, 24, 25]. Recently, the evaluation of seminal oxidation-reduction potential (ORP), measuring the electron transfer from antioxidants to oxidants, has been proposed to provide information on the redox balance and to evaluate semen quality in association with the standard semen analysis [26, 27, 28].

2',7'-dichlorofluorescein diacetate (DCFH-DA) is one of the most widely used probes for oxidative stress evaluation, being easy to use, inexpensive, and sensitive to cellular redox state changes [29]. Upon oxidation, it is converted to highly fluorescent 2',7'-dichlorofluorescein (DCF), which can be detected cytometrically or by a microplate reader. To date, DCFH-DA has been mainly used for ROS detection in washed sperm cell suspensions by flow cytometry [14, 30, 31]. In the present paper, we proposed a microplate-based DCFH-DA assay for the evaluation of oxidative stress in unwashed semen specimens, thus quantifying both intracellular and extracellular ROS levels, avoiding artifacts due to the washing steps. The test was also used to assess the effect of different antioxidant molecules in reducing semen oxidative stress and applied preliminarily to whole semen samples from routine semen analysis thus exploring the clinical potential of the method.

2. Materials and methods

2.1. Microplate-based DCFH-DA fluorescence assay

To develop the DCFH-DA test in whole semen, five samples were collected on different days from the same fertile donor (age 43 years old), who had normal sperm parameters (concentration $65 \pm 8 \times 10^6$/ml, motility $60 \pm 4\%$, and morphology $12 \pm 1\%$). Samples were produced by masturbation after 3–4 days of sexual abstinence and maintained for approximately 20 min at 36 ± 1 °C to liquefy, accordingly to WHO guidelines [32].

ROS levels were measured in unwashed whole semen immediately after liquefaction using a DCFH-DA-based microplate fluorescence assay, consisting of 90 μl test sample and 10 μl probe 1 mM. Sterile black 96-well plates were from ThermoFisher Scientific, and the microplate fluorescence reader FluoStar Optima was from BMG Labtech. DCFH-DA (Sigma-Aldrich) was prepared as a 20 mM stock solution in 100% ethanol and maintained at -20 °C until used. Increasing probe concentrations (20, 50, 100, and 200 μM) as well as increasing semen dilutions (1:1, 1:2, 1:5, and 1:10) were tested in preliminary experiments (Supplementary material, Figures 1S and 2S, respectively). The working probe concentration was set at 100 μM, so as not to exceed the safe concentration of ethanol (0.5%) [33]. Probe dilution was carried out in phosphate-buffered saline (PBS). To monitor DCFH-DA oxidation upon incubation with liquefied semen samples, the fluorescence time course was recorded every minute up to 15 min of incubation at 37 °C in the microplate reader (excitation 485 nm, emission 520 nm). Fluorescence kinetic up to 60 min after probe addition to whole semen is shown in Supplementary material (Figure 3S). Based on probe oxidation rate

(fluorescence units/min), kinetic was linear after 10 min from probe addition, suggesting that a loading time of at least 10 min was necessary to allow DCFH-DA to enter within cells.

To evaluate the effect of time on semen oxidative status, ROS levels were also measured in whole semen incubated at 37 °C for up to 6 h. At each experimental time point (0, 1, 2, 3, 4, 5, and 6 h), 10 μl DCFH-DA (100 μM, final) were added to 90 μl semen sample and fluorescence was recorded every minute for 15 min after probe addition. Semen oxidation levels were evaluated by calculating the area under the curve (AUC) between 0 and 15 min of fluorescence emission.

2.2. Whole semen exposure to oxidative insults and antioxidant molecules

Whole semen samples (n = 3) from the same donor were exposed to the oxidant hydrogen peroxide (H_2O_2) in the presence or absence of glutathione (GSH, Sigma-Aldrich). GSH, present in millimolar concentrations inside cells, acts as an antioxidant, a free radical scavenger, and a detoxifying agent [34]. Briefly, 90 μl of whole semen were pre-treated for 15 min at 37 °C with 10 μl DCFH-DA (100 μM, final), and fluorescence emission was recorded (baseline evaluation, T0). Five μl PBS (untreated control, CTR) or H_2O_2 (500 μM, final) $\pm$ GSH (5 mM, final) were then added to semen samples. Fluorescence emission was recorded every 10 min up to 60 min of incubation at 37 °C.

The DCFH-DA assay was also employed to evaluate the action of different pro-GSH molecules on semen oxidation status. The molecules selected were GSH-C4, N-acetylcysteine (NAC), and I-152. GSH-C4 is the N-butanoyl GSH derivative, consisting of GSH carrying a hydrophobic group to make cellular entry easier [35]. NAC (Sigma-Aldrich) is the N-acetyl derivative of the natural amino acid L-cysteine, which is considered the rate-limiting factor in GSH synthesis. I-152 is a conjugate of NAC and S-acetyl-β-mercaptoethylamine designed to release the parent drugs (i.e., NAC and β-mercaptoethylamine, MEA, or cysteamine) [36]. I-152 was synthesized as previously described [37]. All these molecules were shown to be excellent GSH replenishing tools [35, 36, 37, 38].

Briefly, 90 μl of whole semen were pre-treated for 15 min at 37 °C with 10 μl DCFH-DA (100 μM, final). Five μl PBS (untreated control, CTR) or GSH, GSH-C4, NAC, and I-152 at different concentrations (0.01, 0.1, and 1 mM, final) were then added to semen samples. Fluorescence emission was recorded up to 135 min of incubation at 37 °C and AUC was calculated. Experiments were conducted in triplicates.

2.3. ROS detection in washed spermatozoa

Whole semen samples (n = 3) from the same donor were washed with G-MOPS™ PLUS (Vitrolife) and spermatozoa were resuspended in the same medium to the same initial volume. Ten μl DCFH-DA (100 μM, final) were then added to 90 μl washed cells and fluorescence emission was recorded every minute up to 15 min of incubation at 37 °C.

To investigate if antioxidants might protect spermatozoa from oxidation, 400 μl aliquots of liquefied semen samples were treated with I-152 (1 mM, final) or PBS (control) for 20 min at 37 °C. Samples were then washed with G-MOPS™ PLUS supplemented with I-152 (1 mM, final) or PBS (control). After centrifugation, cell pellets were resuspended in the same medium to the same initial volume (400 μl). Ten μl DCFH-DA (100 μM, final) were then added to 90 μl washed spermatozoa; fluorescence emission was recorded up to 30 min of incubation at 37 °C and AUC was calculated. Experiments were conducted in triplicates.

2.4. Sperm viability evaluation

Sperm viability was analyzed by the WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Sigma-Aldrich), which has been recently reported as an affordable and reliable method for the evaluation of sperm viability [39]. The assay was based on the cleavage of the tetrazolium salt WST-8 by cellular dehydrogenases in viable cells [40]. In whole semen

experiments, 10 μl WST-8 were added to 100 μl semen samples diluted 1:2 in PBS in a clear 96-well plate. After 60 min of incubation at 37 °C, color development was evaluated at 450 nm in a multiwell plate reader (Multiskan FC, ThermoFisher Scientific). The same procedure was applied to washed spermatozoa using 100 μl sperm cell suspension.

2.5. ROS evaluation in whole semen samples from routine semen analysis

After informed consent, ROS levels were measured in whole semen samples from subjects (n = 20, aged between 23 and 46 years old) undergoing standard semen analysis at the Clinica Nuova Ricerca, Rimini, Italy. Samples were produced on-site by masturbation after 3–4 days of sexual abstinence and maintained for approximately 20 min at 36 ± 1 °C to liquefy, accordingly to WHO guidelines [32]. Semen collection was carried out in accordance with the Declaration of Helsinki for experiments involving humans after approval by the Internal Review Board of Clinica Nuova Ricerca (Rimini, Italy) granted on January 11, 2021.

Semen oxidation levels were evaluated by the DCFH-DA microplate fluoresce assay immediately after sample liquefaction. Ten μl DCFH-DA 1 mM (100 μM, final) were added to 90 μl whole semen and fluorescence emission was recorded every minute up to 15 min of incubation at 37 °C in the microplate reader FluoStar Optima (ex/em: 485/520 nm). The area under curves was calculated and normalized by total sperm count to obtain the relative oxidation index (AUC/10^6 ml sperm). Analyses were performed in duplicate.

2.6. Data analysis

Comparisons between multiple means were performed via ANOVA followed by post hoc analysis for significance (Tukey test). Differences between the means of unpaired samples were analyzed using Student's t-test. Correlations were calculated using linear regression analysis. Differences were considered to be statistically significant when the p-value was <0.05. Statistics were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

The DCFH-DA microplate assay was developed employing normospermic whole semen samples collected from the same fertile donor on five different days. Fluorescence emission kinetics were monitored for 15 min after probe addition to semen samples immediately after liquefaction. As reported in Figure 1A, a time-dependent fluorescence increment was recorded upon DCFH-DA incubation with the sample. No fluorescence emission was observed with whole semen alone (negative control), evidencing no sample interference with probe emission.

This kinetic profile may vary considerably according to semen oxidative status, such as in the case of whole semen allowed to incubate at 37 °C for up to 6 h. As indicated in Figure 1B, an increment of probe oxidation was evidenced at each experimental time point; in particular, fluorescence emission profiles changed with time in response to
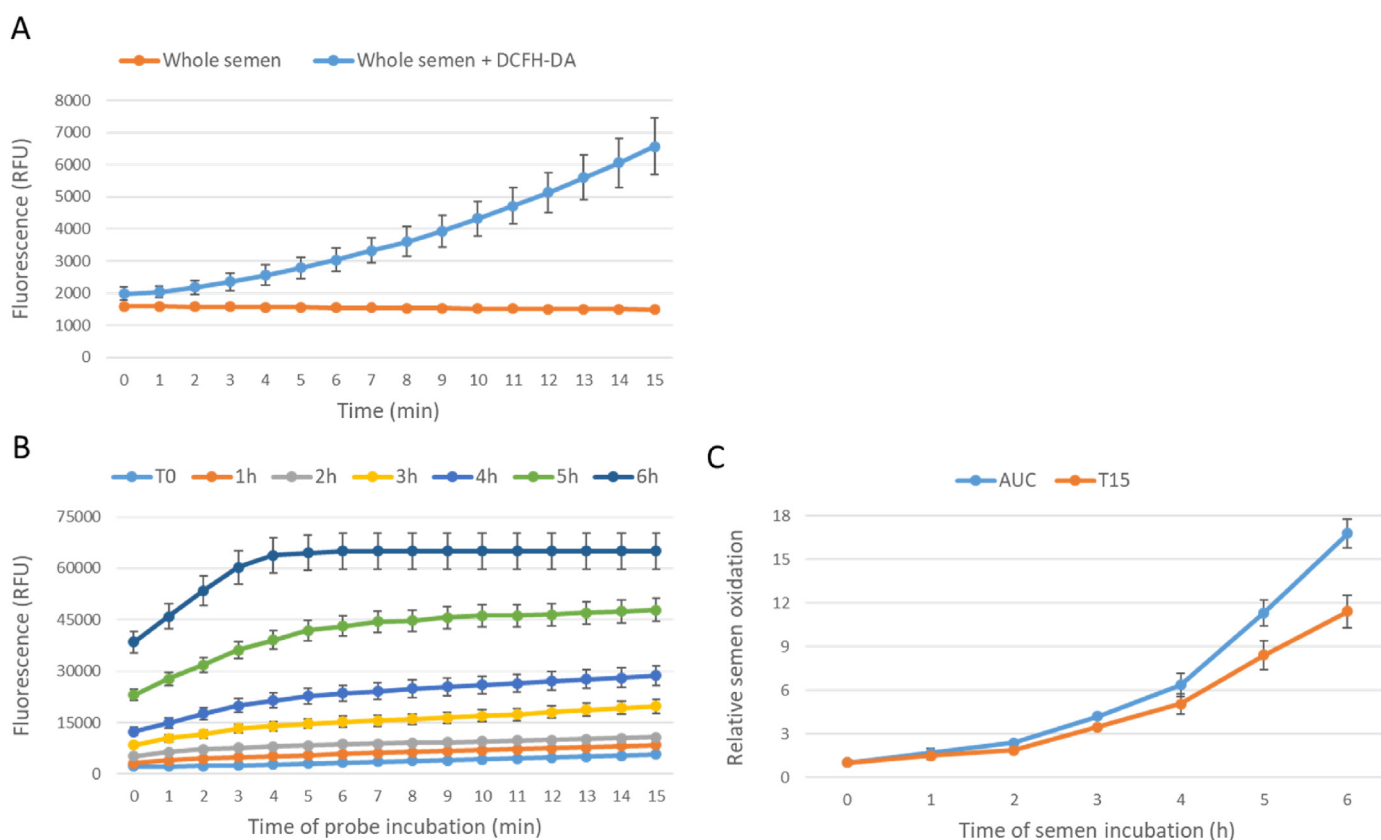
Figure 1. Determination of time-dependent ROS increase by DCFH-DA in whole semen. (A) Fluorescence emission kinetic upon DCFH-DA incubation with whole semen. Ten μl DCFH-DA (100 μM, final) were added to 90 μl of whole semen in a black 96-well plate. Fluorescence emission was recorded every minute up to 15 min of incubation at 37 °C in the microplate reader FluoStar Optima (ex/em: 485/520 nm, gain setting: 1300). Data are represented as mean ± SD (n = 5). [Whole semen: 90 μl whole semen plus 10 μl PBS]. **(B) Time-dependent increase of fluorescence emission upon DCFH-DA addition to whole semen incubated up to 6 h at 37°C.** 10 μl DCFH-DA (100 μM, final) were added to 90 μl whole semen in a black 96-well plate both immediately after liquefaction (T0) and after 1, 2, 3, 4, 5, and 6 h of semen incubation at 37 °C. Fluorescence emission was recorded every minute for 15 min after probe addition (ex/em: 485/520 nm, gain setting: 1300, upper limit: 65000 RFU). Data are represented as mean ± SD (n = 3). **(C) Relative increment of whole semen oxidation during sample incubation at 37°C up to 6 h.** Oxidation was evaluated at each experimental time point by calculating the area under the curve (AUC) between 0 and 15 min of fluorescence emission or by considering the fluorescence value after 15 min of incubation with the probe (T15). Data are represented as mean ± SD (n = 3).

increased seminal oxidative stress. Consequently, semen oxidative status could be better represented by the measurements of areas under the curve (AUC) between 0 and 15 min after probe addition rather than by fluorescence detection at a fixed time point (15 min, T15) (Figure 1C).

In the same set of experiments, the evaluation of sperm viability in whole semen samples incubated up to 6 h at 37 °C revealed a time-dependent decrease in cell viability (Figure 2A); in addition, a significant negative correlation was found between semen oxidation levels and sperm viability (R = -0.949, p = 0.001) (Figure 2B).

Fluorescence kinetic profiles may change also when the redox system of the sample is perturbed, such as in the case of semen exposure to oxidants or antioxidants. To this end, whole semen was pre-treated with DCFH-DA and then exposed to H_2O_2 and/or GSH for 1 h (Figure 3A). H_2O_2 led to a rapid increase in fluorescence emission as compared to untreated semen (control); on the contrary, GSH addition caused a decrement in fluorescence emission owing to reduced probe oxidation. When semen was incubated with both molecules, fluorescence initially increased due to DCFH-DA oxidation by H_2O_2, and then it started to decrease due to the antioxidant action of GSH, reaching fluorescence values comparable to the untreated control.

Unfortunately, GSH as it is, is rapidly oxidized and hardly crosses the cell membrane [41]. For this reason, several GSH derivatives or precursors, designed to be more stable and able to enter the cells, have been investigated as GSH-boosting molecules [42]. In this context, we used the DCFH-DA assay to evaluate the efficacy of different pro-GSH molecules in reducing semen oxidative status. The antioxidant activity of GSH-C4, NAC, and I-152 at the concentrations of 0.01, 0.1, and 1 mM was compared with that of GSH. The results reported in Figure 3B show that the hydrophobic derivative of GSH, i.e. GSH-C4, was more effective than GSH in decreasing the fluorescence emission in the range of concentrations tested and that I-152 caused a significant decrement in semen oxidation levels as compared to the untreated sample (CTR) at the concentration of 1 mM (Figure 3C).

We applied the DCFH-DA assay also to washed spermatozoa obtained from whole semen samples. As reported in Figure 4A, probe oxidation rapidly increased in spermatozoa as compared to whole semen; in fact, isolated cells were not protected from oxidation by the antioxidant defense system of seminal plasma [17, 18]. Based on this evidence and the results obtained by evaluating the antioxidant capacity of the pro-GSH molecules (Figure 3), we investigated if I-152 (1 mM) might protect spermatozoa from oxidation during semen manipulation. As indicated in Figure 4B, untreated spermatozoa (CTR) showed the highest oxidation

levels as compared to cells isolated from whole semen treated with I-152 at different stages of sample manipulation. The best results were obtained when I-152 was added to whole semen both before (20 min pre-incubation) and during centrifugation (Figure 4C). In fact, the antioxidant activity of I-152 was lower if the molecule was just pre-incubated for 20 min with semen before centrifugation, confirming that the centrifugation itself is a critical step favoring cell oxidation [16]. The evaluation of sperm viability in the same set of washed sperm cell suspensions revealed that cell viability significantly increased when whole semen samples were treated with I-152 both before and during centrifugation as compared to untreated semen samples (CTR) (Figure 4D). A significant negative correlation was found between sperm oxidation levels and sperm viability (R = -0.990, p = 0.01).

Finally, the DCFH-DA microplate assay was preliminarily used to evaluate the oxidation levels of whole semen samples from men undergoing standard sperm analysis thus to tentatively exploring possible future clinical applications. Seminal parameters (sperm concentration, motility, and morphology) are reported in Table 1.

After probe addition to liquefied samples, fluorescence emission was monitored for 15 min, and the oxidation index (AUC/total sperm count) was calculated (the coefficient of variation from duplicate measurements was less than 10%). As reported in Figure 5A, samples with abnormal sperm parameters had significantly higher oxidation indexes in comparison to normospermic samples. In detail, mean oxidation indexes were 1356 (range 470–2711) and 7729 (range 3407–12769) in samples with normospermia and abnormal semen parameters, respectively. Correlation analysis between oxidation indexes and sperm parameters was also conducted. A negative correlation was found between whole semen oxidation levels and sperm total motility (Figure 5B) as well as between semen oxidation and sperm morphology (Figure 5C).

4. Discussion

Taking advantage of our research experience in using the DCFH-DA probe for the assessment of intracellular ROS levels in cultured cell models [43, 44, 45, 46], in this study we explored the possibility to adapt the microplate-based DCFH-DA assay to unwashed whole semen samples, thus quantifying both intracellular and extracellular ROS levels. Indeed, the measurement of oxidative stress in whole semen is an important tool that may provide valuable information on the pathophysiology of male infertility and help to identify subgroups of infertile patients that may benefit from antioxidant supplementation [11, 12]. While low
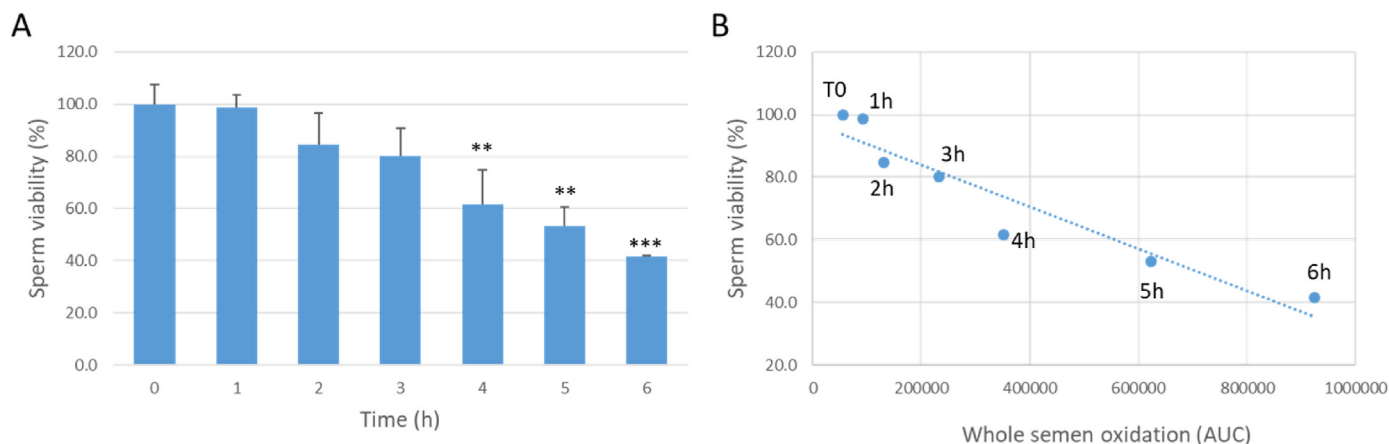

Figure 2. Determination of sperm viability in whole semen. (A) Decrease of sperm viability during whole semen incubation at 37°C up to 6 h. Ten μl WST-8 were added to 100 μl whole semen (diluted 1:2 in PBS) in a clear 96-well plate both immediately after liquefaction (T0) and after 1, 2, 3, 4, 5, and 6 h of semen incubation at 37 °C. Absorbance was evaluated at 450 nm after 60 min from WST-8 addition, and sperm viability (%) was calculated. Data are represented as mean ± SD (n = 3). **p < 0.01, ***p < 0.001 vs. T0 (Tukey post-hoc test). **(B) Negative correlation between whole semen oxidation and sperm viability during whole semen incubation at 37°C up to 6 h.** At each experimental time point (0, 1, 2, 3, 4, 5, and 6 h), semen oxidation levels (calculated as AUC from the DCFH-DA assay) were plotted against sperm viability (calculated as % from the WST-8 assay).
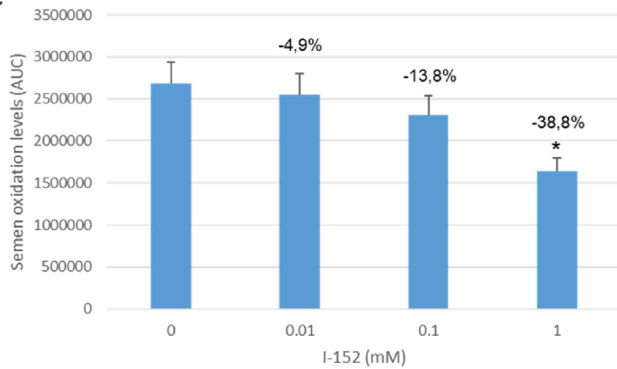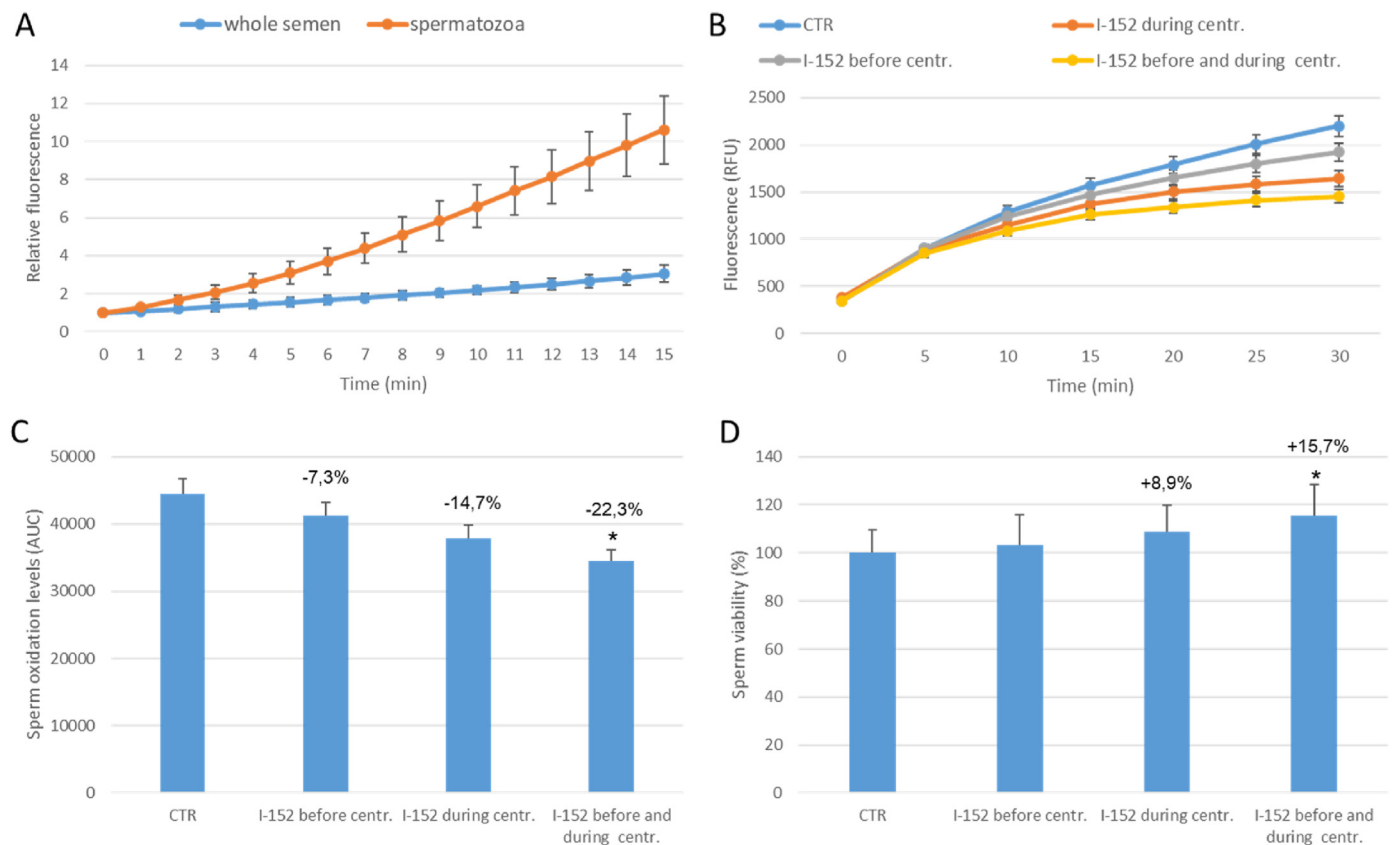
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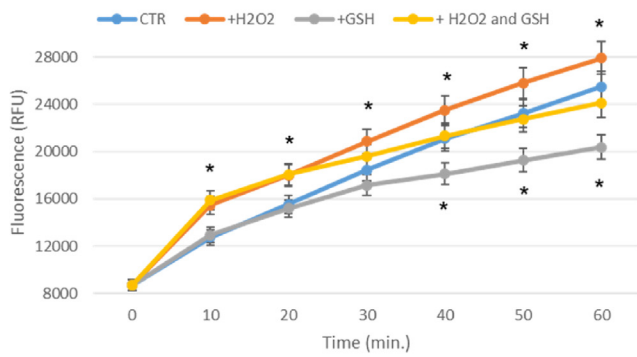
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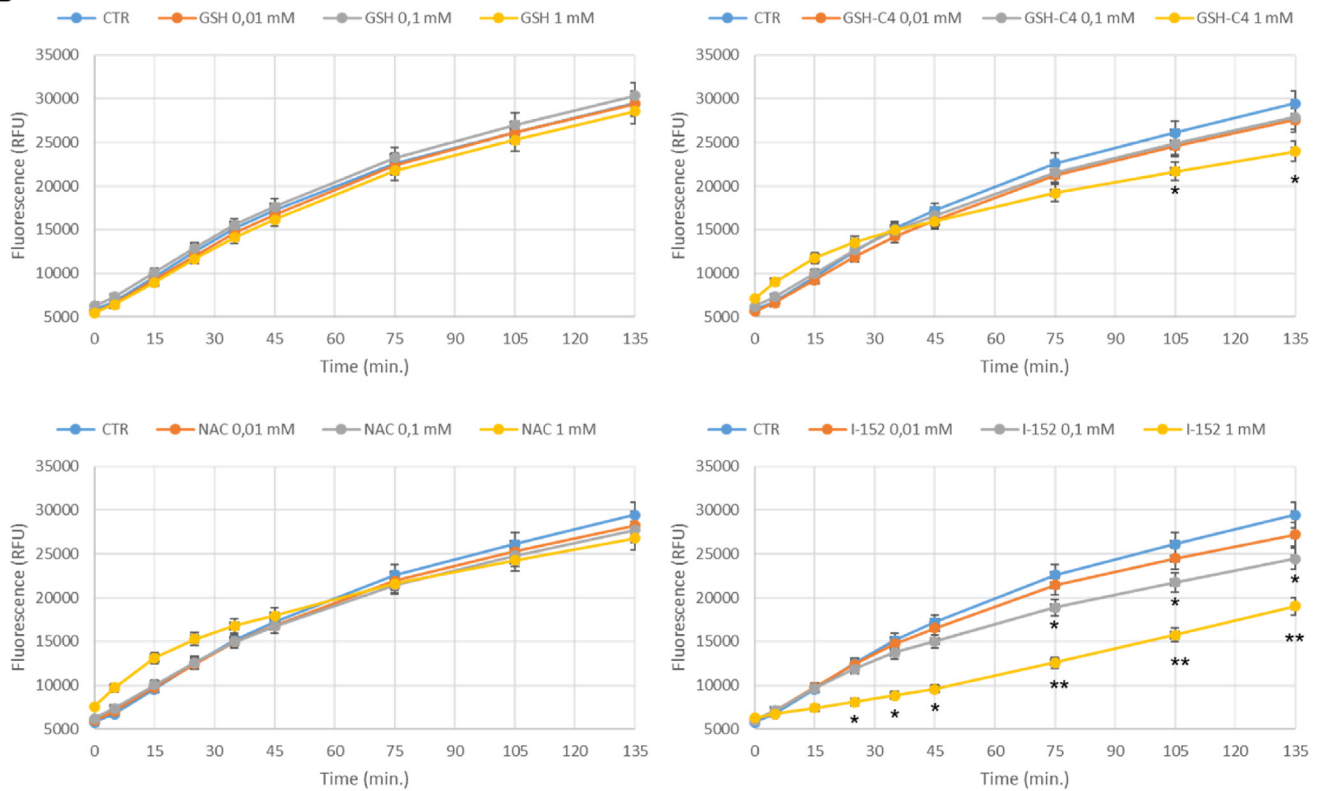
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Figure 4. Determination of ROS levels in washed spermatozoa in the absence or presence of I-152. (A) Relative fluorescence emission upon DCFH-DA addition to whole semen or washed spermatozoa. Ten μl DCFH-DA (100 μM, final) were added to 90 μl whole semen or 90 μl washed spermatozoa in G-MOPS™ PLUS. Fluorescence emission was recorded every minute up to 15 min of incubation at 37 °C. Data are represented as mean ± SD (n = 3). **(B) Fluorescence emission upon DCFH-DA addition to washed spermatozoa obtained from semen samples treated with I-152 1 mM at different stages of manipulation**. Ten μl DCFH-DA (100 μM, final) were added to 90 μl washed spermatozoa in G-MOPS PLUS. Fluorescence emission was recorded every 5 min up to 30 min of incubation at 37 °C. Data are represented as mean ± SD (n = 3). **(C) Oxidation levels in washed spermatozoa obtained from whole semen treated with I-152 1 mM at different stages.** Oxidation was evaluated for each condition by calculating the area under the curve (AUC) between 0 and 30 min of fluorescence emission. Data are represented as mean ± SD (n = 3). *p < 0.05 vs untreated semen (CTR) (Tukey post-hoc test). **(D) Cell viability of spermatozoa obtained from whole semen treated with I-152 1 mM at different stages**. Ten μl WST-8 were added to 100 μl washed sperm cell suspension in a clear 96-well plate. Absorbance was evaluated at 450 nm after 60 min from WST-8 addition, and sperm viability (%) was calculated. Data are represented as mean ± SD (n = 3). *p < 0.05 vs. CTR (Tukey post-hoc test).

Table 1. Seminal parameters.

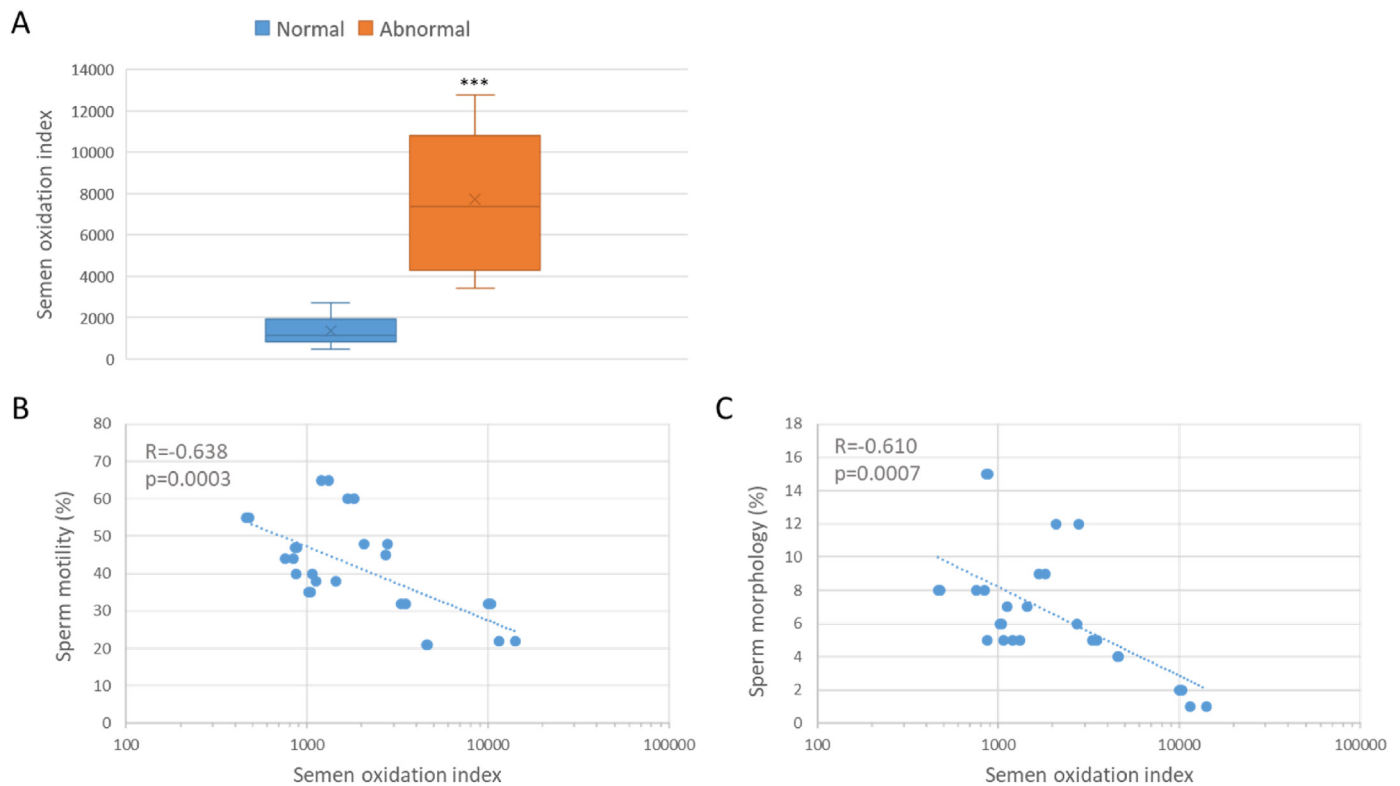|	Normospermic (n = 10)	Non-normospermic (n = 10)
Concentration (10^6/ml)	63 ± 22	22 ± 14**
Motility (%)	48 ± 8	23 ± 11**
Morphology (%)	8 ± 3	3 ± 2**

**p < 0.01 vs. normospermic samples (t-test for unpaired data).

physiological ROS concentrations are essential for sperm capacitation, hyperactivation, and spermatozoon-oocyte fusion, excessive ROS production can negatively impact sperm quality and function due to chemical and structural modifications to membrane lipids and sperm DNA [4, 5, 6]. In particular, it has been reported that Male Oxidative Stress Infertility (MOSI) affects about 37 million men with idiopathic male infertility and involves oxidative stress and altered semen characteristics [9, 10].

2',7'-dichlorofluorescein diacetate (DCFH-DA) is one of the most widely used probes for oxidative stress measurement, being very easy to use, inexpensive, and sensitive to cellular redox state changes [29]. To date, DCFH-DA has been mainly used for ROS detection in washed sperm samples by flow cytometry [14, 30, 31]. Herein, we employed a common microplate fluorescence reader to monitor the fluorescence time course upon DCFH-DA addition to unwashed whole semen samples, thus avoiding artifacts due to the washing steps that may trigger significant

Figure 3. Determination of ROS decrease induced by different pro-GSH molecules in whole semen. (A) Probe oxidation upon whole semen incubation with H_2O_2 and/or GSH. Ninety-μl of whole semen were pre-treated for 15 min at 37 °C with 10 μl DCFH-DA (100 μM, final) in a black 96-well plate, and fluorescence emission was recorded (baseline evaluation, T0). Five μl PBS (untreated control, CTR) or H_2O_2 (500 μM, final) or GSH (5 mM, final) or H_2O_2 plus GSH (500 μM and 5 mM, respectively) were then added to semen samples. Fluorescence emission was recorded up to 60 min of incubation at 37 °C. Data are represented as mean ± SD (n = 3). *p < 0.05 vs. untreated semen (CTR). **(B) Probe oxidation upon whole semen incubation with different antioxidant molecules.** Ninety-μl of whole semen were pre-treated for 15 min at 37 °C with 10 μl DCFH-DA (100 μM, final) in a black 96-well plate. Five μl PBS (untreated control, CTR) or GSH, GSH-C4, NAC, and I-152 at different concentrations (0.01, 0.1, and 1mM) were then added to semen samples. Fluorescence emission was recorded up to 135 min of incubation at 37 °C. Data are represented as mean ± SD (n = 3). *p < 0.05 and **p < 0.01 vs. untreated semen (CTR). **(C) Oxidation levels after I-152 addition to whole semen.** Oxidation was evaluated for each concentration by calculating the area under the curve (AUC) between 0 and 135 min of fluorescence emission. Data are represented as mean ± SD (n = 3). *p < 0.05 vs. untreated semen (CTR) (Tukey post-hoc test).

Figure 5. Determination of oxidation levels in normospermic and non-normospermic whole semen samples. **(A) Whole semen oxidation levels in samples with normal (n = 10) and abnormal (n = 10) sperm parameters.** Ten μl DCFH-DA (100 μM, final) were added to 90 μl of whole semen and fluorescence emission was recorded every minute up to 15 min of incubation at 37 °C. The area under curves was calculated and normalized by total sperm count to obtain the relative oxidation index (AUC/10^6 ml sperm). ***p < 0.001 vs. normospermic samples (t-test for unpaired data). **(B) Negative correlation between semen oxidation index (logarithmic transformed) and sperm motility. (C) Negative correlation between semen oxidation index (logarithmic transformed) and sperm morphology.**

ROS production within cells. In fact, the removal of seminal plasma during the washing process eliminates antioxidant molecules that would naturally protect sperm from ROS attack [16, 17, 18].

The term ROS covers a variety of oxygen metabolites, such as superoxide anions, hydroxyl radicals, and hydrogen peroxide (H_2O_2). Conflicting results are present regarding ROS specificity of DCFH-DA, but a definitive conclusion has not been reached. Some reports indicated that this probe is sensitive to H_2O_2 whereas others found no effects [47]. In our study, we observed that DCFH-DA could be directly oxidized by H_2O_2, as demonstrated by the time- and dose-dependent increments of probe fluorescence emission after H_2O_2 addition to the reaction mixture (Figure 4S, Supplementary material). Similarly, whole semen exposure to H_2O_2 led to a rapid increase in fluorescence emission due to probe oxidation (Figure 3A).

Different fluorescent probes have been employed to evaluate ROS levels in sperm samples, including MitoSOX™ Red and dihydroethidium, which detect superoxide anion, and RedoxSensor™ Red CC-1, CellROX® Orange Reagent, and MitoPY1, which seem to be mostly sensitive to hydrogen peroxide [47, 48]. In all cases, ROS production was detected by flow cytometry in viable spermatozoa and not in native semen samples; consequently, we could not compare the results obtained with the DCFH-DA assay in whole semen with those observed with the cited probes. In this context, it would be of interest for future applications to employ additional ROS-detecting probes with different ROS specificity to whole semen samples in a microplate-based system, thus exploring their potential in evaluating seminal oxidative stress in association with sperm parameters.

Herein, the preliminary application of the DCFH-DA microplate assay to ROS detection in whole semen samples from subjects undergoing standard semen analysis was useful to reveal higher oxidation indexes in specimens with abnormal sperm parameters as compared to those with

normospermia. Noteworthy, semen oxidation indexes negatively correlated to sperm motility and morphology, as previously evidenced using chemiluminescence-based ROS assays [7, 8]. These pilot experimental data demonstrate that the DCFH-DA microplate assay may effectively reveal the perturbations of the redox system of whole semen samples, thus sustaining clinical developments of the method. We are aware that the small sample size of whole semen specimens is a limitation that does not permit at this stage the clinical application of the test but only its experimental development. However, these preliminary results encourage future clinical validations employing a higher number of samples with different semen abnormalities, such as hyperviscosity, oligospermia, asthenospermia, teratospermia, and leukospermia.

Finally, the DCFH-DA assay was successfully applied in evaluating the reduction of semen ROS production by different pro-GSH molecules. We demonstrated that the addition of molecules able to increase GSH content was effective to reduce ROS levels and that I-152 was also capable to protect sperm cells from oxidative damage induced by semen centrifugation. It is essential to mention that I-152 increases the intracellular GSH concentration not only by providing a greater amount of precursors necessary for its synthesis as compared to other GSH-replenishing molecules such as NAC, but also by inducing the antioxidant response through the activation of the transcription factor Nrf2 [49]. Indeed, it has been previously demonstrated that I-152, at the concentrations used in this study (0.01–1 mM), differently from NAC and GSH-C4, can activate the Nrf2 pathway, which regulates the transcription of components of the glutathione and thioredoxin antioxidant systems [50]. Consequently, I-152-treated cells may counteract the detrimental effects of ROS by two different mechanisms of action, and this is the reason why GSH, GSH-C4, and NAC are generally used at higher concentrations [49]. Differences in I-152 antioxidant activity can be explained even by the rapid cellular uptake that makes it available early intracellularly at lower doses as

compared to NAC [36]. In this context, I-152 may represent an attempt to combine two pro-GSH molecules (NAC and MEA) into one to potentiate the cellular uptake of the parent molecules. Regarding NAC, it has been suggested that although it was designed to facilitate membrane permeability, its pharmacological activity might depend on the reduction of plasma cystine to cysteine, which then enters the cells and supports GSH synthesis [51].

Interestingly, the reduction of sperm oxidation levels by I-152 was related to a significant increment of sperm cell viability, thereby encouraging future research on the possible benefits of I-152 supplementation (or other thiol species). On one hand, it might be helpful as an antioxidant supplement in subjects with high ROS seminal levels [52]; on the other, I-152 addition to the culture medium during human in vitro fertilization procedures might be beneficial to preserve sperm cells from oxidation [53]. Accordingly, the improvement of culture conditions still represents one of the main goals of human-assisted reproductive technology research.

5. Conclusions

The microplate-based DCFH-DA assay appears to be a convenient method for the evaluation of ROS levels in whole semen, not including centrifugations or other sample manipulations. Moreover, the test could be a helpful tool for basic and quick screening of antioxidant molecules able to preserve semen quality.

Declarations

Author contribution statement

Serena Benedetti and Alessandra Fraternale: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Simona Catalani; Silvia De Stefani; Mariangela Primiterra: Performed the experiments.

Francesco Palma: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Simone Palini: Conceived and designed the experiments; Analyzed and interpreted the data Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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