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Oxidative Stress Evaluation in Sperm Samples from Fertile and Infertile Men

Andor Crippa[1], Maria C Magli[1], Anna P Ferraretti[1*], Antonino Pipitò[2], Edoardo Pescatori[1] and Luca Gianaroli[1]

[1]*Reproductive Medicine Unit, S.I.S.Me.R. (Società Italiana Studi di Medicina della Riproduzione) S.r.l., Bologna, Italy*

[2]*FerPhrarma S.r.l., Milan, Italy*

Abstract

Background: Transition metal ions, such as iron, can make electron donations to oxygen forming superoxide or hydrogen peroxide, which is further reduced to an extremely reactive hydroxyl radical that induces oxidative stress. The purpose of the present study was to design a system that could easily detect and reliably measure the ferrous oxidation associated to oxygen radical reactions in the sperm samples.

Methods: A total of 64 sperm samples from 11 men who had normal semen parameters and proven fertility and 53 male partners of couple experiencing primary infertility, were included in the study. The semen samples from oligoasthenoteratozoospermic patients was divided on the basis of spermatic parameters into moderate, when the sperm concentration was ≥5 × 10^6/ml and in severe when the concentration was <5 × 10^6/ml. The evaluation of the ferrous oxidation was performed measuring the formation of iron complexes between ferric ions and thiocyanate anions by spectrofluorimetry.

Results: The concentration of the ferric thiocyanate complex ions was significantly higher in pathological sperm samples (137.6 ± 10.8 µmol/l in moderate oligoasthenoteratozoospermic, 170.0 ± 25.4 µmol/l in severe oligoasthenoteratozoospermic and 155.4 ± 7.3 µmol/l in non-obtructive azoospermic men), when compared with both infertile noormozoospermic (92.4 ± 10.7 µmol/l) (P<0.015) and with samples from fertile men (76.3 ± 6.2 µmol/l) (P<0.005). No significant differences were found in the concentration of ferric thiocyanate complex among the different pathological groups when compared to each other and in infertile noormozoospermic patients when compared with the samples from men of proven fertility (P=0.168). Accordingly, an inverse correlation was found between the concentration of the ferric thiocyanate complex and total motility, progressive motility and morphology.

Conclusions: This preliminary study shows that the method proposed detect quickly and reliably measures the ferrous oxidation associated to oxygen radical reactions in the sperm samples.

Keywords: Oxidative stress; Sperm samples; oligoasthenoteratozoospermic; Polyunsaturated fatty acids; spermatozoa

Introduction

Free radicals are a group of extremely reactive species with one or more unpaired electrons that modify other biomolecules by capturing their electron(s). This makes the biomolecule to lose its electron(s) and to become a free radical itself triggering a chain reaction leading to a peroxidative damage disrupting living cells [1]. Human spermatozoa are particularly susceptible to this damage because they contain high concentrations of Polyunsaturated Fatty Acids (PUFAs), they have no capacity for membrane repair and they possess the ability to generate ROS themselves, mainly superoxide anion and hydrogen peroxide that are essential, in low quantity, to stimulate a number of events which are involved in capacitation, hyper activation, and sperm-oocyte fusion [2]. When for any reason the concentration of oxygen metabolites becomes elevated, sperm cells have a limited capacity to protect themselves from oxidative stress. A variety of defence mechanisms encompassing antioxidant enzymes (Superoxide Dismutase (SOD), catalase, glutathione peroxidase and reductase), vitamins (E, C, and carotenoids), and biomolecules (glutathione and ubiquinol) are involved in biological systems. A balance between the benefits and risks from ROS and antioxidants appears to be necessary for the survival and normal functioning of spermatozoa [3,4].

Transition metal ions can make electron donations to oxygen, forming superoxide or hydrogen peroxide, which is further reduced to an extremely reactive $OH^{\bullet}$ radical that induces oxidative stress. Transition metals ions, mainly iron, are involved in Fenton's reaction, which produces highly reactive hydroxyl radicals. The Fenton reaction, in the human, has its in vivo significance mainly in case of overload by iron, i.e., in the conditions of hemochromatosis, b-talassemia and hemodialysis. Apparently high amounts of "free available iron" can have deleterious effects [5].

Many studies documented that mutations in superoxide dismutase enzymes [6] and iron-uptake regulator [7] may lead to excess levels of superoxide anion radicals and iron overload. Such a condition leads to the possibility of redox active iron to participate in organic and inorganic oxygen radical reactions, such as stimulating Lipid Peroxidation (LP) and catalyzing the formation of damaging hydroxyl radicals with subsequent tissue damage [8].

The LP involves the continuous formation of hydro peroxides (LOOH) as primary oxidation products that may breakdown to a variety of nonvolatile and volatile secondary products [9,10]. The lipid hydroperoxide can be degraded by a Fenton-type reaction in presence of Fe^{2+} to another alkoxyl radical ($LO^{\bullet}$). This radical also promotes the

***Corresponding author:** Anna Pia Ferraretti, Reproductive Medicine Unit, S.I.S.Me.R. (Società Italiana Studi di Medicina della Riproduzione) S.r.l., Via Mazzini 12, 40138 Bologna, Italy, Tel: 0039-051307307; Fax: 0039-051302933; E-mail: annapia.ferraretti@sismer.it

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chain reaction of LP [11]. Therefore, the peroxide value is an indicator of the initial stages of oxidative change [12].

A number of methods have been developed for determination of peroxide value, among which the chemical method based on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) in an acidic medium and the formation of iron complexes have also been widely accepted. This method spectrophotometrically measures the ability of lipid hydroperoxides to oxidize ferrous ions to ferric ions, which are complexed by either thiocyanate or xylenol orange [13-15].

The purpose of the present study was to design a system that could easily detect and reliably measure the ferrous oxidation associated to organic and inorganic oxygen radical reactions, in the sperm samples from infertile patients and men of proven fertility.

Materials and Methods

The patient population included a total of 64 sperm samples from men with normal semen parameters and proven fertility (n=11, age 38.6 ± 6.0 years) and partners of couple experiencing primary infertility with normal (n=11, age 36.9 ± 4.3 years) and abnormal semen parameters (n=42, age 38.2 ± 6.2 years) undergoing assisted conception cycles in our clinic. Subjects whose ejaculates contained white blood cells concentration greater than 1.0×10^6/ml, infertility attributed to obstructive pathology, infection, medication or trauma, were excluded from the study. All patients signed a specific informed consent before entering this study that was approved by our Institutional Review Board (no. 20091123).

Microscopic sperm analysis

Standard sperm analysis was performed after sample's fluidification, by assessing viscosity, volume, concentration, motility, vitality, morphology, leucocyte and erythrocyte count [16]. Morphology was classified according to strict criteria [17]. On the basis of semen parameters, the patients were grouped into normozoospermic (n=22), Oligoasthenoteratozoospermic (OAT) (n=38), and non-obtructive azoospermic (NOA) men (n=4) (Table 1). The semen samples from OAT patients was divided on the basis of spermatic parameters (count, motility and morphology) into moderate-OAT (m-OAT) (n=28) referred to samples where the cell count was $\geq 5 \times 10^6$/ml, while in severe OAT (s-OAT) (n=10) the sperm concentration was $< 5 \times 10^6$/ml. All cell counts were performed by a Neubauer improved camera. Morphology was analyzed by using the Papanicolau staining method and the preparation was examined with an 100x oil-immersion brightfield objective.

Analysis of Ferrous Oxidation in the Samples

The evaluations of ferrous oxidation were performed 30 minutes after each sample ejaculation by measuring a complex between Fe^{3+} Ions and the thiocyanate anion (SCN^-) by spectrofluorimetry (kit provided by Ferpharma, Diacron International). The Fe^{3+} ion binds to the SCN^- anion and forms a coloured complex ($Fe(SCN)^{2+}$), whose absorbance is directly proportional to the Fe^{2+} oxidized product of oxygen radical reactions into the seminal plasma. The kit used was made by three solutions: R1 (thiocyanate salt alcoholic solution), R2 (Fe^{2+} ion solution) and the standard (4 meq/l Cumene hydroperoxide).

At each measurement, the spectrofluorimeter (Free Carpe Diem, Diacron International) was calibrated by the standard solution. The Standard was diluted with distilled water (1:10) to obtain a final solution of 400 μeq/l and 10 μl were added to the same volume of R1 followed by the addition of 100 μl of R2. The final solution was introduced into a specific plastic cuvette (provided with the kit by Ferpharma, Diacron International) that was inserted into the spectrofluorimeter. After 4 minutes of incubation to 37°C, the instrument gave the value of concentration of the standard solution, expressed in meq/l. In this way it was possible obtain the K Factor (multiplicative factor) for the evaluation of concentration of the samples. At each measurement a sample blank is also performed to account for background interferences. The sample blank contains 1 ml of R1 and 100 μl of R2 without seminal fluid.

The evaluations of ferrous oxidation in the sperm samples, was determined by adding 10 μl of seminal fluid to 1.5 ml eppendorf tube contain 1 ml of R1, followed by the addition of 100 μl of R2. The final preparation was incubated at 37 °C for 2 minutes. After centrifugation at 600g for 2 minutes, the sample was transferred into a plastic cuvette and the absorbance, due to production of a blood-red coloured complex, was measured by the spectrofluorimeter using an excitation wavelength of 505 nm. The values provided by the instrument are expressed in meq/l, and then converted to μmol/l by multiplying by 500. Each sperm sample was underwent at least three determinations.

Statistical Analysis

The Mann-Whitney U test was used to test whether the medians of two independent distributions were different. This test is non-parametric, which means that the distributions can be of any shape. The statistical package PAST (Øyvind Hammer, PAleontological STatistics Version 3.0, available at http://folk.uio.no/ohammer/past/), was used for numeric calculations. This statistics software was also used to evaluate the Pearson's correlation coefficient, r and the probability that two variables were not correlated [18-20]. Results were considered significant at $P<0.05$. For every group of study, the values of the concentration and the total quantity of ferric thiocyanate complex ions were presented as mean ± standard error of the medians of the determinations carried. The Cohen's d was used to measure the effect sizes between the groups [21]. Cohen proposes the following categories for the interpretation of magnitude of d: < 0.2, no effect; 0.2 ≤ to < 0.5, small effect; 0.5 ≤ to < 0.8, medium effect; ≥ 0.8, large effect.

Results

No significant differences were detected for any of the assessed parameters between infertile normozoospermic patients and patients of proven fertility ($P>0.05$) (Table 1).

As shown in Figure 1, the concentration of ferric thiocyanate complex ions was significantly higher in pathological sperm samples (137.6 ± 10.8 μmol/l, range 47.5-283.5 μmol/l in m-OAT; 170.0 ± 25.4 μmol/l, range 83.0-314.0 μmol/l in s-OAT; 155.4 ± 7.3 μmol/l, range 135.0-169.1 μmol/l in NOA) when compared with both infertile normozoospermic samples (92.4 ± 10.7 μmol/l, range 36.8-135.0 μmol/l) ($P<0.015$) and with samples from fertile men (76.3 ± 6.2 μmol/l, range 37.0-106.5 μmol/l) ($P<0.006$).

The Cohen's d, used to measure the effect sizes comparing the m-OAT, s-OAT and NOA groups with infertile normozoospermic and fertile groups, was >0.8 for all the groups. This means that the magnitude of effect size was large in all the groups compared.

No differences were found between OAT moderate samples and OAT severe samples, OAT moderate samples and NOA samples and

	Concentration No. sperm × 10^6/ml M ± SD (range)	Total motility (%) M ± SD (range)	Progressive motility (%) M ± SD (range)	Morphology (%) M ± SD (range)
Fertile n=11	111.4 ± 92.6[a] (20-305)	50.4 ± 10.6[b] (40-70)	36.8 ± 15.4[c] (15-60)	16.4 ± 2.5[d] (14-20)
Infertile normozoospermic n=11	72.8 ± 27.4[a] (35-135)	53.2 ± 9.0[b] (45-70)	39.5 ± 10.1[c] (30-60)	16.6 ± 1.9[d] (14-19)
Infertile moderate-OAT n=28	42.0 ± 36.2 (5-131.6)	36.4 ± 12.5 (20-65)	23.0 ± 10.4 (5-45)	9.8 ± 2.8 (4-16)
Infertile severe-OAT n=10	2.3 ± 1.6 (0.04-4.8)	22.5 ± 15.1 (10-55)	11.5 ± 13.8 (5-50)	5.6 ± 2.5 (2-10)
Infertile NOA n=4	0	0	0	0

OAT: Oligoasthenoteratozoospermic; NOA: Non-Obtructive Azoospermic; [a,b,c,d]P>0.05.

Table 1: Sperm samples parameters of the studied patients. All figures in each column are significantly different (P<0.05) with the exception of the comparisons between fertile and infertile normozoospermic men (P>0.05).

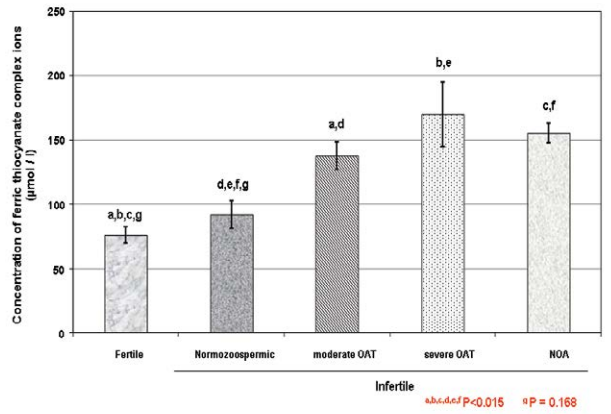

Figure 1 Concentration of ferric thiocyanate complex ions in fertile and infertile men. The values of the concentrations of ferric thiocyanate complex ions are presented as mean ± SE of the medians of the determinations carried.

Figure 1: Concentration of ferric thiocynate complex ions in fertile and infertile men. The values of the concentrations of ferric thiocynate complex ions are presented mean ± SE of the medians of the deterinations carried.

OAT severe samples and NOA samples (P=0.289, P=0.292, P=0.724 respectively) and in the infertile normozoospermic patients when compared with the samples from men of proven fertility (P=0.168). The Cohen's d between the infertile normozoospermic and fertile groups was 0.6. The magnitude of effect size between these groups with equal sample size was medium.

The total quantity of ferric thiocyanate complex ions in the ejaculate was also calculated. The trend was similar to the concentration of ferric thiocyanate complex ions per ml, with the highest levels in pathological sperm (436.1 ± 50.2 nmol, range 57.0-1132.6 nmol, in m-OAT; 514.2 ± 89.6 nmol, range 245.3-1099.0 nmol, in s-OAT; 339.4 ± 61.6 nmol, range 219.9-512.0 nmol, in NOA) when compared with both infertile normozoospermic samples (281.7 ± 84.7 nmol, range 55.5-1060.4 nmol) (P<0.02) and with fertile men (242.1 ± 50.1 nmol, range 55.5-614.3 nmol) (P<0.02). No differences were found between fertile and normal infertile sample (P=0.92), between OAT moderate samples and OAT severe samples, OAT moderate samples and NOA samples and OAT severe samples and NOA samples (P=0.48, P=0.71, P=0.29 respectively) (value not represent in the figure).

A significant inverse correlation was found between the concentration of ferric thiocyanate complex ions in the ejaculated samples and total motility (r=-0.39, P=0.0022, Figure 2A), progressive motility (r=-0.55, P=1.19E-05, Figure 2B) and morphology (r=-0.45, P=0.00034, Figure 2C).

Discussion and Conclusion

The possibility that the peroxidative damage to sperm plasma membrane might be involved in those cases of infertility characterized by fertilization failure was suggested by studies indicating the increased production of ROS by spermatozoa as a possible cause [22,23]. Under such conditions, the main defence mechanism of human spermatozoa, the enzyme SOD, would be overwhelmed and the resulting combination of hydrogen peroxide and excess superoxide anion would favour the production of hydroxyl radicals, via the Haber-Weiss reaction [24,25]. The rate constant for this reaction is considerably enhanced by the presence of transition elements such as iron, the availability of which has been demonstrated in human seminal plasma [26]. The hydroxyl radicals formed as a result of the Haber-Weiss reaction are powerful initiators of lipid peroxidation and would be expected to impair human sperm function through peroxidation-induced changes in membrane fluidity and integrity [27,28]. It is also known that leukocytes, particularly neutrophils and macrophages in the seminal plasma are a source of ROS, whose production is further increased by lifestyle factors such as smoking and pollution with derived damage on sperm DNA integrity [29].

A practical method to estimate the ferrous oxidation in a sperm sample is represented by the quantification of ferric ions through the formation of the ferric thiocyanate complex ions ($Fe(SCN)^{2+}$). The concentration of this substance, red in colour, is evaluated by measuring the absorbance via a spectrofluorimeter. By using this approach, we could confirm that the ferric thiocyanate complex ions concentration was increased in pathological samples when compared with normozoospermic, both infertile and fertile (Fig. 1), indicating a tight correlation between morphological and functional properties. This was furthermore confirmed by correlation studies that reported an inverse association of thiocyanate complex ions concentration with motility and morphology, although the correlation was moderate (Figure 2). However the Cohen's d, used to measure the effect sizes comparing the pathological samples with infertile normozoospermic and fertile groups, was >0.8 for all the groups, indicating that the magnitude of effect size between these groups with different sample size was large.

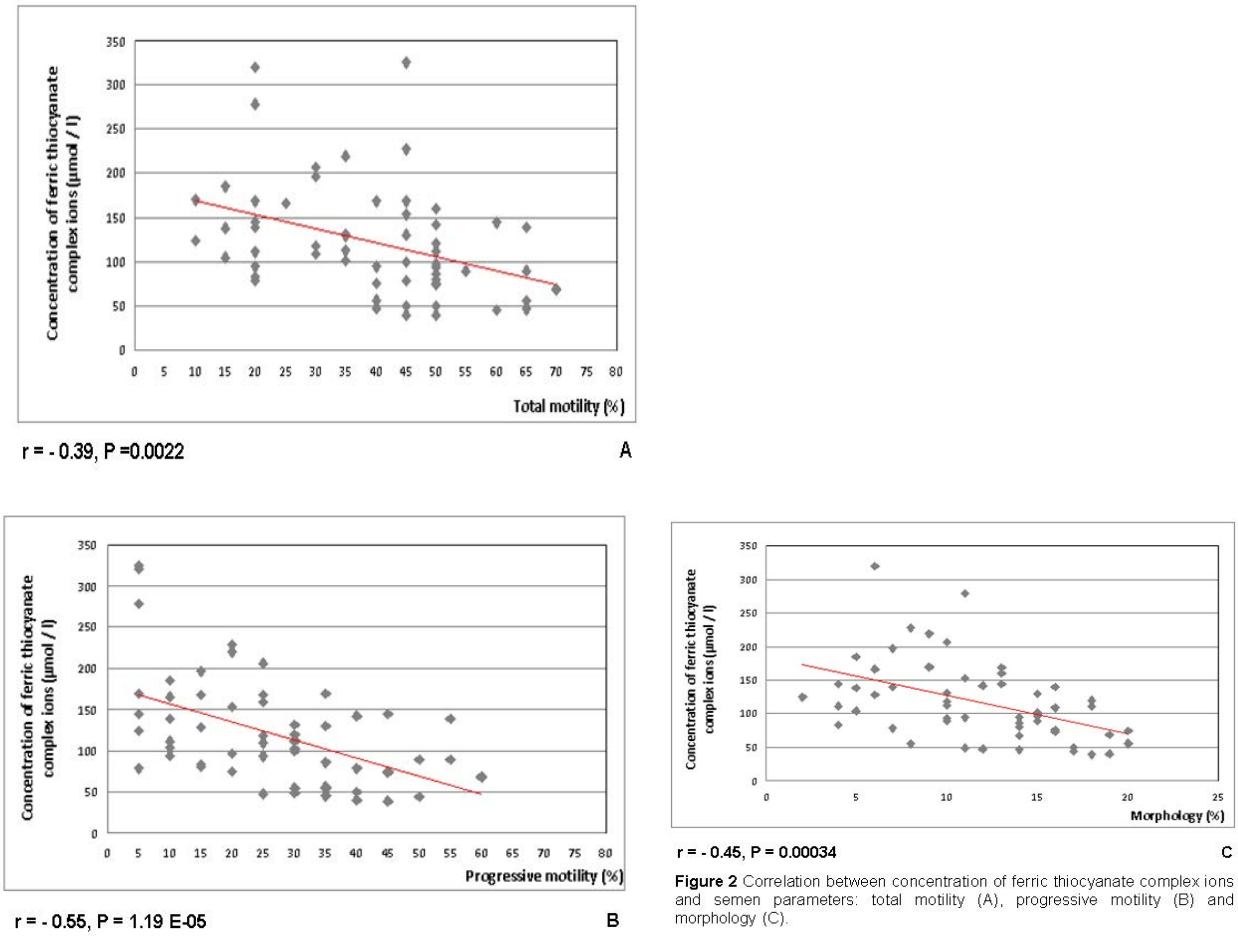

Figure 2 Correlation between concentration of ferric thiocyanate complex ions and semen parameters: total motility (A), progressive motility (B) and morphology (C).

Figure 2: Correalation between concentration of ferric thiocynate complex ions and semen parameters total mobility (A), progressive motility (B) and morphology (C).

This suggests that the statistical significance between these groups depend not only from the different concentration of ferric thiocyanate complex ions but also on the sample size. Furthermore also the absence of statistically significant differences between the concentrations of ferric thiocyanate complex ions in infertile normozoospermic and fertile groups could be depending on the sample size. In fact the Cohen's d between the infertile normozoospermic and fertile groups was 0.6. It might be necessary increase the sample size in both fertile and infertile groups.

Excessive ROS levels are related to an increase in lipid peroxidation of the sperm plasma membrane [25]. Lipid peroxidation results in loss of membrane fluidity, which is essential for sperm motility and sperm oocyte fusion. Several studies have noted that levels of ROS correlate with motility. Motility is impaired either because adenosine triphosphate depletion in axons or lipid peroxidation of the sperm plasma membrane [30,31].

The our results show that the concentration of ferric thiocyanate complex ions in ejaculated samples was inversely correlated with total motility and progressive motility (Figure 2A and 2B), indicating that the production of thiocynate complex has associated with the presence in the severe semen samples of a high concentration of ROS; in particular with the presence of the radicals $O_2^{-\bullet}$ and H_2O_2 that can generate the highly reactive hydroxyl radical, increasing the rate of cellular damage through peroxidation-induced change and in membrane fluidity and integrity, leading abnormal sperm function and infertility [32,33]. The concentration of ferric thiocyanate complex ions was also inversely correlated with the presence of morphologically abnormal spermatozoa (Figure 2C), another important source of ROS [31].

In the NOA samples the concentration of ferric thiocyanate complex ions was significantly higher with respect to fertile and normal infertile sample. Probably the origin of ROS in the NOA semen samples was associated to the presence of immature sperm cells, infiltrating macrophage and diminished antioxidant activity. Theoretically, cellular damage in the semen is the result of an improper balance between ROS generation and scavenging activities. The scavenging potential in gonads and seminal fluid is normally maintained by adequate levels of antioxidants Superoxide Dismutase (SOD), catalase, glutathione peroxidase and reductase [32,33]. This balance can be referred to as Oxidative Stress Status (OSS) and its assessment may play a critical role in monitoring sperm damage and infertility [34]. A situation in which there is a shift in this ROS balance towards pro-oxidants, because of either excess ROS or diminished antioxidants, can be classified in terms of positive oxidative stress status [34].

The progression of lipid peroxidation can be monitored by measuring products of LP such as conjugated dienes, lipid hydroperoxides, aldehydes, aldehyde-protein adducts, alkanes or the depletion of substrates like PUFAs or antioxidants, besides chemiluminescence. The measurement of several oxidation products enables assessment at different stages of the oxidative pathway, providing detailed information of this dynamic process [35].

The most convenient, and widely used, assay of lipid peroxidation is the Thiobarbituric Acid (TBA) reaction for Malondialdehyde (MDA), a small carbonyl compound which is produced as an end product of lipid peroxidation, probably from cyclic peroxides or endoperoxides. The generation of MDA can be significantly enhanced by the addition of a ferrous ion promoter to the incubation system [27]. Have been demonstrated that ferrous ions catalyse the breakdown of pre-existing lipid hydroperoxides in spermatozoa and the subsequent propagation of a lipid peroxidation chain reaction through the generation of peroxyl and alkoxyl radicals [36-38].

The oxidative stress evaluation using our method was determined by the addition of ferrous ions to the sperm samples and measuring the complex between Fe^{3+} Ions and the thiocyanate anion. The concentration of ferric thiocyanate complex produced could be proportional to the quantity of pre-existing lipid hydroperoxides in the sperm plasma membrane with which to initiate the peroxidative chain reaction, but this assay is not specific for estimate the lipid peroxidation.

In conclusion this preliminary study, show that the method proposed provide a quick (within 10 minutes) and reliable (overlapping values in the triplicates) method for the evaluation of the ferrous oxidation in the semen samples due to the oxygen radical reactions.

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