Evaluation of the impact of leucocytospermia on semen oxidative status by chemiluminescence technique in infertile men

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ABSTRACT

The presence of high reactive oxygen species (ROS) levels in semen is a major factor involved in the decline of male fertility. In seminal plasma, ROS are mainly produced by activated leukocytes. Spermatozoa were the first cell type reported to show a potential susceptibility to oxidative damage. The aim of our study was to evaluate the impact of leucocytospermia on basal and FMLP (Formyl-Methionyl-Leucyl-Phenylalanine) induced oxidative status in semen of infertile men. We also analyzed the correlations of the spermatic parameters with amounts of ROS in semen. Our study included 50 semen samples of infertile men. Sperm analysis was performed using WHO standardized method. Seminal leukocytes were quantified using peroxidase technique. The measurement of ROS levels in semen was made by chemiluminescence assay. We measure respectively ROS amounts in neat semen and in washed sperm cells suspension from the same ejaculate. We also applied the test of provocation of leukocytes by FMLP on neat and washed samples to assess the spermatic oxidative status after leucocyte stimulation. Our results showed significant correlations between ROS levels in neat semen and many sperm parameters: motility, sperm concentration, leucocytes concentration and the rate of sperm cytoplasmic droplets. The studied samples were divided into 2 groups: (G1) composed of 36 samples without leucocytospermia and (G2) composed of 14 leucospermic samples. ROS levels were significantly lower in G1 than in G2 (p = 0.002). ROS production was significantly increased after application of FMLP in washed leucospermic samples (p = 0.001). The measurement of ROS in neat semen is a considerable contribution to explore the impairment of semen quality in infertile men. ROS levels in washed semen reflect the oxidative status generated by sperm preparation techniques used in assisted reproductive procedures. Levels of ROS are highly influenced by the presence of leukocytes and associated with decreased seminal parameters.

Keywords: Semen; Oxidative Stress; Leucocytospermia; Chemiluminescence; ROS; FMLP

1. INTRODUCTION

Oxidative stress induced by reactive oxygen species (ROS) plays crucial roles in a wide range of physiological processes and is also implicated in various diseases, such as cancer, cardiovascular pathology, neurodegenerative disorders, and other chronic conditions [1,2]. In semen, the controlled generation of very low amounts of ROS appears to regulate sperm normal functions [3,4]. Oxidative stress appears in semen once an imbalance between the production of ROS and their destruction by different enzymatic and non-enzymatic seminal antioxidant systems is created [5]. Interestingly, spermatozoa were the first cell type reported to show a potential susceptibility to oxidative damage [6]. While the presence of high levels of ROS in the ejaculate is among the risk factors involved in reducing male fertility [7,8]. It has evolved that three inter-related mechanisms account for
oxidative stress-mediated male infertility: impaired motility, impaired fertilization and oxidative DNA damage [9,10].

The principle sources of excessive generation of ROS in semen are activated leucocytes and abnormal spermatozoa [11,12]. Indeed, the prevalence of leucocytospermia in infertile men varies from 2% to 40% depending on the patient population, the detection method and threshold values used [13]. Moreover, it has been shown that depending on their activation status, leucocytes are capable of producing ROS and cytokines and there seems to be a relation between ROS formation and cytokine production [14,15].

The importance of seminal ROS production has been already stressed in the World Health Organization manuals (WHO 1999 and WHO 2010) [16,17]. The chemiluminescence method is the most commonly employed technique as a direct measurement of ROS generation in semen according to the standardized method recommended by the WHO 1999 [16]. This assay is capable of quantifying both intracellular and extracellular ROS [18]. Furthermore, the use of leukocyte-specific stimulant (FMLP: formyl-methionyl-leucyl-phenylalanine) can enhance the chemiluminescent signal to measure low amounts of light generated by leucocytes [16,19].

The degree of sperm damage by leucocytes products depends on the location of the inflammatory reaction, the duration of exposure of sperm to these products and the ability of spermatozoa to activate its intrinsic anti-lipoperoxidative defense systems [20]. The clinical significance of both ROS and leucocytes levels continues to be debated. Some authors have found ROS levels or leucocyte counts to be of little prognostic help in either in vivo or in vitro reproduction [21]. These findings are in disagreement with other in vivo [22] and in vitro studies [19,23] that reported the significant prognostic value of semen ROS levels in reproduction. Much of the controversy centers on the best definition of pathological leucocytospermia and the correlation of leucocytes with seminal oxidative stress are unclear [15,24,25].

The aim of our study was to evaluate the impact of leucocytospermia on basal and FMLP induced semen oxidative status using the chemiluminescence technique in infertile men. We also analyzed the correlations of the routine spermatic parameters with amounts of ROS generation in semen.

2. MATERIAL AND METHODS

2.1. Patients

Our study was carried out in 50 semen samples from male partner of infertile couple attending the Histology-Embryology laboratory of Sfax medical school (Tunisia) for semen investigations. The patients were aged between 27 and 51 years old with a mean age (± Standard Deviation (SD)) of 36.16 ± 0.57 years.

2.2. Collection of Semen Samples

Semen samples were collected by masturbation after 3 - 5 days of sexual abstinence and allowed to liquefy for 30 minutes at 37°C.

2.3. Semen Analysis

Basic semen analysis consisted in the measurement of the following semen parameters: volume, sperm concentration, percentage of motile spermatozoa, sperm vitality and percentage of normal spermatozoa. For sperm concentration, diluted semen samples were mixed before transferring a drop to the chamber of the hemocytometer. The spermatozoa were counted under a light microscope at 400× magnification. To determine the percentage of motile spermatozoa, a 10 µl drop of mixed semen was placed on a heated glass slide (37°C) under a square cover glass (22 mm) and observed at 400× magnification. The percentage of motile spermatozoa was evaluated immediately and four hours after semen liquefaction, we evaluated total motility, rapid progressive motility (type a), slow progressive motility (type b), and no progressive motility (type c) according to WHO guidelines [16]. Sperm vitality was assessed using eosin-nigrosin staining technique. A 20 µl of liquefied semen was mixed with 20 µl of eosin (1%) and 20 µl of nigrosin (10%). The suspension was incubated for 30 s at room temperature. Then, a 20 µl of the solution was smeared on a microscope slide. The smear was air dried and examined at 1000× magnification under oil immersion. Unstained sperm (white) were classified as viable and those that showed any pink or red coloration were classified as dead. Sperm morphology was assessed in Shorr-stained semen smears. All parameters were carried out according to the standardized methods recommended by the WHO [16].

2.4. Assessment of Leucocytes in Semen by Peroxidase Method

A leukocyte count was carried out by using the cytochemical peroxidase method, as described in the WHO laboratory manual [16]. This assay identifies polymorphonuclear granulocytes, the most prevalent leucocyte type in semen, as peroxidase-positive cells. A working solution was prepared by combining 250 µl of Benzidine with 50 µl of Hydrogen peroxide (H2O2). The procedure consisted of mixing 50 µl of neat semen with 50 µl of the working solution. This mixture was allowed for 30 minutes at room temperature. We transferred 20 µl of the mixture onto a hemocytometer chamber and the number of peroxidase-positive leucocytes which stained brown
was counted at 400× magnification. Leucocytospermia was defined as the presence of more than \(1 \times 10^6\) leucocytes per milliliter of semen [16].

2.5. Semen Preparation

From each sample, one aliquot of 0.5 ml of liquefied neat semen was used for immediate ROS measurement and another 0.5 ml aliquot of semen was centrifuged at 300 g for 7 minutes. Seminal plasma was removed and the pellet of cells was washed twice in 3 ml of PBS (Phosphate Buffer Saline, isotonic solution, pH = 7.4). The supernatant was then removed and the cells pellet was suspended in a volume of 0.5 ml of PBS. Sperm concentration in the final solution was evaluated before ROS measurement.

2.6. Measurement of Reactive Oxygen Species (ROS) in Semen by Chemiluminescence Technique

The measurement of ROS in semen was made by chemiluminescence using a microplate luminometer (Lumineskan Ascent, Thermo Electron Corporation). Chemiluminescence probe used is luminol (5-amino-2, 3-dihydro 1, 4-phthalazinedione; Sigma chemical Co) which is a redox-sensitive, light emitting probe [26]. A 10 mM stock solution of Luminol was prepared in DMSO (Dimethyl Sulfoxide; Sigma-Aldrich). For each semen sample, we measured the basal levels of ROS in neat semen and in a suspension of washed semen cells obtained from the same ejaculate.

The basal ROS production was measured respectively in 200 µl of liquefied neat semen and 200 µl of washed cells suspension after addition of 5 µl of the working solution of Luminol (0.1 mM) obtained by dilution of the stock solution with the HBSS (Hank’s Balanced Salt Solution). Negative controls for estimation of background signals were prepared for each assay by adding 5 µl of 0.1 mM Luminol to 200 µl of PBS. Also, 200 µl of PBS served as a blank.

We also applied the test of specific provocation of leucocytes by FMLP (Formyl-Methionyl-Leucyl-Phenylalanine; Sigma Aldrich) on all our samples (neat and washed). The FMLP is used to stimulate a chemiluminescent signal from any polymorphonuclear granulocytes that are present in the sperm suspension [16]. Since FMLP receptors are not present on the surface of human spermatozoa, this signal is specific for the leucocyte population [16]. A 0.1 mM stock solution of FMLP in DMSO was prepared.

The ROS production was measured respectively in 200 µl of liquefied neat semen and 200 µl of washed cells suspension after addition of 5 µl of the working solution of Luminol (0.1 mM) and stimulation with 2 µl of FMLP working solution (0.2 µM) obtained by dilution of the FMLP stock solution with the HBSS. The chemiluminescence signal was monitored for 15 minutes. Results were expressed as relative light units (RLU) per minute and per \(20 \times 10^6\) spermatozoa.

3. STATISTICAL ANALYSIS

A statistical analysis was performed using SPSS 13.0 software. Statistical tests including Student’s t test, Pearson’s and Spearman’s correlations, linear regression were used. The statistical significance was considered for p values < 0.05.

4. RESULTS

The mean values (± SD) and ranges of semen parameters, basal and after FMLP stimulation ROS levels in neat and washed semen are summarized in Table 1.

The levels of basal ROS in neat semen was negatively correlated with the immediate and late total motility, the immediate and late rapid progressive motility and the sperm concentration (Table 2; Figures 1(a)-(c)). We also found significant and positive correlations of levels of basal ROS in neat semen with the leucocytes concentration in semen firstly and with the rates of cytoplasmic droplets in the mid piece area of spermatozoa secondly (Table 2; Figures 1(d) and (e)).

There was a marked increase of ROS in semen after washing (Table 1) with significant correlations of the

### Table 1. Means and ranges of semen parameters, basal ROS levels in neat and washed semen and ROS levels in neat and washed semen after FMLP stimulation (n = 50).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Neat Semen</th>
<th>Washed Semen</th>
</tr>
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<tbody>
<tr>
<td>Volume (ml)</td>
<td>3.9 ± 1.5</td>
<td>1.9 - 8.5</td>
</tr>
<tr>
<td>Total motility (%)</td>
<td>38.7 ± 14.6</td>
<td>0 - 60</td>
</tr>
<tr>
<td>Rapid progressive mobility “a” (%)</td>
<td>10.6 ± 7.1</td>
<td>0 - 25</td>
</tr>
<tr>
<td>Sperm concentration (Millions/ml)</td>
<td>45.9 ± 39.8</td>
<td>0.02 - 189.6</td>
</tr>
<tr>
<td>Vitality (%)</td>
<td>68.9 ± 14.9</td>
<td>26 - 89</td>
</tr>
<tr>
<td>Leucocytes (millions/ml)</td>
<td>0.9 ± 2.1</td>
<td>0.01 - 9.5</td>
</tr>
<tr>
<td>Normal morphology (%)</td>
<td>7.2 ± 5.3</td>
<td>0 - 22</td>
</tr>
<tr>
<td>Cytoplasmic droplets (%)</td>
<td>5.1 ± 4.9</td>
<td>0 - 20</td>
</tr>
<tr>
<td>Basal ROS (RLU/mm/20.10^6 spz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In neat semen</td>
<td>3.9 ± 21.4</td>
<td>0.01 - 150.5</td>
</tr>
<tr>
<td>In washed semen</td>
<td>164.6 ± 1136.2</td>
<td>0.03 - 8037</td>
</tr>
<tr>
<td>ROS after FMLP stimulation (RLU/mm/20 × 10^6 spz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In neat semen</td>
<td>3.9 ± 21.7</td>
<td>0.01 - 153.0</td>
</tr>
<tr>
<td>In washed semen</td>
<td>222.7 ± 1542.7</td>
<td>0.02 - 10912.5</td>
</tr>
</tbody>
</table>
Figure 1. Correlations of basal ROS levels (log transformed) in neat semen with the following semen parameters: immediate total motility (a), immediate progressive motility (b), sperm concentration (c), leucocyte concentration (d), and cytoplasmic droplets (e). Empirical distributions of the ROS levels were highly skewed on the original scale, and so log-transformed data were used for all statistical analyzes. Basal ROS in neat semen was positively correlated with the leucocytes concentration in semen and with the rates of cytoplasmic droplets in the mid-piece area of spermatozoa.
ROS levels in neat semen with those in sperm suspension after washing (Figure 2).

The studied samples were divided into 2 groups: (G1) composed of 36 samples without leucospermia (leucocytes < 10^6/ml) and (G2) composed of 14 leucospermic samples (leucocytes ≥ 10^6/ml). ROS levels were significantly higher in the group of leucospermic samples compared with the group of leucocyte free samples (Table 3) and a significant and positive correlation of basal

Table 2. Correlations between ROS levels in neat semen and semen parameters (n = 50). The levels of basal ROS in neat semen were negatively correlated with the immediate and late total motility, the immediate and late rapid progressive motility and the sperm concentration.

<table>
<thead>
<tr>
<th>Correlation coefficient</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Sperm concentration</td>
<td>−0.77</td>
</tr>
<tr>
<td>Immediate total Motility</td>
<td>−0.45</td>
</tr>
<tr>
<td>Immediate rapid progressive motility “a”</td>
<td>−0.27</td>
</tr>
<tr>
<td>Late total Motility</td>
<td>−0.46</td>
</tr>
<tr>
<td>Late rapid progressive motility “a”</td>
<td>−0.29</td>
</tr>
<tr>
<td>Sperm vitality</td>
<td>+0.02</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>+0.32</td>
</tr>
<tr>
<td>Sperm morphology</td>
<td>−0.02</td>
</tr>
<tr>
<td>Cytoplasmic droplets</td>
<td>+0.51</td>
</tr>
</tbody>
</table>

NS = No significant. Pearson’s and Spearman’s correlations tests was used and statistical significance was assessed at p < 0.05.

Figure 2. The correlation of basal ROS levels (log transformed) in neat semen with basal ROS levels (log transformed) in washed semen was positively significant.

ROS levels in washed semen with seminal leucocytes concentrations was noted (Figure 3). ROS production was significantly increased after FMLP stimulation in leucospermic washed samples (Figure 4).

Figure 3. Correlation of basal ROS levels in washed semen with seminal leucocytes concentrations in neat semen. Positive correlation was noted between basal ROS levels (log transformed) in washed semen with seminal leucocytes concentrations (log transformed) in neat semen.

Figure 4. Correlation of FMLP stimulated ROS levels in washed semen with leucocytes concentrations in neat semen. Positive correlation was noted between FMLP stimulated ROS levels (log transformed) in washed semen with leucocytes concentrations (log transformed) in neat semen.
peroxidation reaction affects membrane structure and predisposing them to lipid peroxidation. Sperm membranes are characterized by the abundance of the reduction of ATP production.

ROS on sperm mitochondrial membrane potential and dysfunctional spermatozoa.

leucospermic samples (G2); the ROS levels were significantly enhanced in washed semen and after FMLP application in leucospermic group.

5. DISCUSSION

It is well established that the production of excessive quantities of reactive oxygen species in the male genital tract, essentially by leucocytes, can overwhelm the seminal antioxidant defenses and give rise to a harmful oxidative stress altering the microenvironment in which spermatozoa develop and mature. Currently, there are growing evidences that seminal oxidative stress is involved in the pathogenesis of many reproductive processes through sperm damage, deformity and eventually male infertility. We used chemiluminescence technique in our study because it is the most commonly described assay to detect ROS production within semen. It is very sensitive and has the advantage of relatively well established reported ranges for both the fertile and infertile population.

As regards the relationship between semen quality and its oxidative status evaluated by chemiluminescence assay, we found significant correlations between basal ROS levels produced in neat semen of infertile men and conventional sperm parameters such as motility, sperm concentration and one of sperm morphological abnormalities (cytoplasmic droplets in mid piece area of spermatozoa).

The decrease of sperm motility associated to an oxidative stress in infertile men semen was reported previously and was related to the negative effect of ROS on sperm mitochondrial membrane potential and the reduction of ATP production. In addition, the sperm membranes are characterized by the abundance of unsaturated fatty acids having a double-bonded nature and predisposing them to lipid peroxidation. This peroxidation reaction affects membrane structure and fluidity and causes damage to axonemal proteins leading to a permanent impairment in sperm motility.

In the present study, we founded a significant negative correlation between sperm concentration and the levels of ROS in semen. Indeed, it was widely reported that seminal concentrations of ROS in oligospermic patients were higher than that of normospermic patients. ROS and their metabolites attack DNA, lipids and proteins, alter enzymatic systems and cell signaling pathways, producing irreparable alterations and may accelerate the process of germ cell apoptosis. The latter process can lead to decline in sperm counts and may explain the apparent deterioration of semen quality. Also, the decrease in the sperm concentration could be explained by the presence of a genital inflammation process which often results in the presence of high number of leucocytes, principle source of ROS in semen. Indeed, our results showed a significant increase of ROS in leucospermic samples compared with the samples without leucospermia. These results are consistent with other reports indicating that seminal leucocytes have the potential to cause oxidative stress through their degranulation and the formation of free radicals. In fact, it was also reported that the presence of leucocytes in semen is associated with a high rate of production of ROS with reduction of sperm motility and its fertilizing ability. The activation state of leucocytes must play an important role in determining final ROS output. Pro-inflammatory seminal plasma cytokines such as IL-6, IL-8 and tumour necrosis factor TNF α seem to be produced locally by activated leucocytes in semen and a marked relationship between these cytokines, leucocytes and seminal ROS production was
reported [14,50,52]. During epididymal transit, the sperm are not in contact with seminal plasma antioxidants, and are therefore vulnerable to oxidative damage, especially when there is an epididymal inflammation [49]. It was also demonstrated that leucocytospermia increased the production of ROS by human spermatozoa [53].

The application of a leucocyte-specific stimulant (FMLP) test showed an increase in ROS production especially in semen samples of leucospermic group. Leucocytes are the only cells present in the human ejaculate possessing detectable receptors for FMLP and capable of generating reactive oxygen species in response to this reagent [54]. Our results confirm that leucocytes are the major source of ROS in semen and present an additional argument that the presence of leucocytes in semen is associated with a high rate of production of ROS. Moreover, the results of the FMLP provocation test have an important bearing on the fertilizing capacity of the spermatozoa in vitro [19,55].

The leucocytes are the principle but not the exclusive source of ROS in semen and in concordance with our findings, Aitken [44] and Gomez [56] showed a significant production of free radicals by cytosplasmic droplets present at the midpiece of the defective morphologically abnormal spermatozoa. These residues are rich in Glucose-6-phosphate dehydrogenase (G6PD), an enzyme which controls the rate of glucose flux and intracellular production of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) [57]. The latter is used to fuel the generation of ROS via NADPH oxidase located within the sperm membrane [56-58]. As a result, teratozoospermic sperm produce increased amounts of ROS with a reduced antioxidant capacity compared with morphologically normal sperm [9,59]. All these relationships underpin the well established observation that higher ROS have a negative impact on sperm quality but even men with normozoospermic idiopathic infertility exhibit significantly higher seminal ROS production and lower antioxidant capacity than fertile men for as yet unknown reasons [28,60].

Besides the measure of ROS level in neat semen, we quantified ROS in semen after washing and removal of seminal plasma. We apply this assay in our study to evaluate the oxidative status of semen during sperm preparation techniques for assisted reproductive technologies. Our results are similar to those reported in other studies [61-63] that showed a significant increase of ROS production in semen by repeated cycles of centrifugation and aggravated by the ablation of the natural antioxidant environment. Some studies [64-66] showed that the addition of antioxidants such as EDTA, catalase, ascorbate and tocopherol in sperm preparation medium may scavenge ROS and decrease their deleterious effects on spermatozoa but these findings are discussed [67,68].

Another point for consideration is the implication of genital oxidative stress in the sperm DNA fragmentation commonly observed in spermatozoa of infertile men [69]. In fact, significant correlations of seminal ROS levels with DNA damage were reported in many studies [43,70,71]. Moreover, other studies reported an increase in DNA damage in sperm from leucocytospermic samples [53,72,73]. Recently, it was suggested a direct implication of leucocytes, as an exogenous factor, in generating DNA base modifications evaluated by the formation of 8-oxoguanine, a key biomarker of the oxidative DNA damage [74]. Oxidized DNA bases have increased susceptibility to ROS action which reflects a direct and specific action of ROS on sperm DNA [9,75]. The most recent studies on the origin of sperm DNA damage suggested that there might be a cascade of changes that progress from the induction of oxidative stress and DNA base-pair oxidation to DNA fragmentation and cell death [76].

Nevertheless, it seems that the plasma membrane is less vulnerable to oxidative damage than DNA and sperm with significant oxidative DNA damage and intact membranes could preserve its ability to fertilize oocyte [70]. Many of these embryos developed from spermatozoa with fragmented DNA will unfortunately fail at the blastocyst or early fetal stage [77,78]. Thereby, the currently use of mechanical techniques such as Intra Cytoplasmic Sperm Injection (ICSI) to bypass some male factor infertility is unlikely to be the ‘best practice’ since ROS damaged DNA, frequently induced by seminal leucocytes, will result in poor quality blastocysts and an increase in miscarriage [9,25]. Additionally, the patients may be urged to consider antioxidant therapy before undergoing reproductive assistance technique that may reduce DNA damage levels and improve sperm fertility potential [9].

6. CONCLUSIONS

Oxidative stress is a major factor in male reproductive disorders because it may impair the physiological function of spermatozoa at the molecular level. Understanding the exact mechanisms, by which oxidative stress develops in semen, will improve the management of semen quality impairment by potential toxic ROS. To establish a treatment strategy of genital oxidative stress in infertile men, we must first specify its origin: seminal leucocytes and/or sperm cells themselves, to guide thereby the optimum therapeutic modalities for male infertility particularly in the context of leucocytospermia.

Progress in assisted reproductive technologies continues to be offered to infertile men, who would otherwise be unable to conceive chances to have their own offspring. However, these “mechanical” procedures are unable to compensate for oxidative damage to sperm DNA.
In addition, direct treatment of oxidative stress and its source may allow for natural conception.

7. ACKNOWLEDGEMENTS

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**ABBREVIATIONS**

ROS: Reactive Oxygen Species  
DNA: Deoxyribonucleic Acid  
FMLP: Formyl-Methionyl-Leucyl-Phenylalanine  
WHO: World Health Organisation  
H₂O₂: Hydrogen Peroxide  
SD: Standard Deviation  
RLU: Relative Light Units  
HBSS: Hank’s Balanced Salt Solution  
NADPH: Nicotinamide Adenine Dinucleotide Phosphate  
G6PD: Glucose-6-Phosphate Dehydrogenase  
ICSI: Intra Cytoplasmic Sperm Injection