

Relationship between acrosin activity of human spermatozoa and oxidative stress

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Abstract

Aim: To study the association between seminal oxidative stress and human sperm acrosin activity.

Methods: It is a prospective study consisting of 30 infertile men and 12 fertile normozoospermic volunteers. A full history, clinical examination and scrotal ultrasound were done to exclude other related factors such as smoking and varicocele. Presence of white blood cells (WBCs) in semen samples was evaluated by peroxidase staining. Lipid peroxidation in spermatozoa was induced after incubating with ferrous sulphate (4 mmol/L) and sodium ascorbate (20 mmol/L). Induced peroxidation of spermatozoa was assessed by determining the production of thiobarbituric acid reactive substances (TBARS). Acrosin activity was measured using the gelatinolysis technique. The halo diameters around the sperm heads and the percentages of spermatozoa showing halo formation were evaluated. An acrosin activity index was calculated by multiplying the halo diameter by the halo formation rate. **Results:** A significant difference was observed in acrosin activity parameters and TBARS levels between samples with WBCs ($>1 \times 10^6$ /mL of ejaculate) and those without. This difference was also noted between the normozoospermic and the oligoastheno-teratozoospermic semen samples. The TBARS production by spermatozoa had a significant negative correlation with the acrosin activity index ($r = -0.89$, $P < 0.001$). **Conclusion:** The presence of oxidative stress in an individual with leukocytospermia and/or abnormal semen parameters is associated with impaired sperm function as measured by its acrosin activity.

1 Introduction

Acrosin is a trypsin-like serine proteinase found within the acrosome of human spermatozoa [1]. It is an important proteolytic enzyme that can hydrolyze the zona pellucida in oocytes. It also plays a vital role in the process of fertilization and is involved in acrosome reaction [2]. Its absence or reduced activity may be a clinical cause of male infertility [3]. Thus, measuring acrosin activity is a suitable approach for evaluating the fertilizing capacity of human spermatozoa.

Several techniques, including gelatinolysis, have been described to assess human sperm acrosin activity [4]. Using this technique, researchers found that smoking and varicocele affect sperm function [5]. The acrosin activity index was shown to predict the fertilizing potential of a sperm population [6].

Oxidative stress (OS) is defined as increased rate of cellular damage induced by oxygen-derived oxidants called reactive oxygen species (ROS). Oxidative stress occurs when there is an imbalance between the production of ROS and the scavenging ability of the antioxidants [7-9]. Irrespective of the clinical diagnosis, the presence of seminal OS in infertile men suggests its role in the pathophysiology via several mechanisms acting in synergism which can impair sperm characteristics and functional capacity [10, 11].

Spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids that are required to maintain the membrane fluidity. This fluidity regulates specific functions such as acrosome reaction and fusion with oocyte membrane [12]. The final end product of lipid peroxidation, malondialdehyde, estimated by thiobarbituric acid (TBA) assay can be used to gauge the level of OS in semen. In the male genital tract, ROS are generated by spermatozoa and leukocytes [13, 14]. Increased seminal leukocytes may stimulate human spermatozoa to produce ROS. Such stimulation may be mediated via direct cell-cell contact or by soluble products released by leukocytes [14]. In leukocytospermia, a growing body of evidence indicates that male infertility may be partly caused by OS, which negatively affects sperm function and quality [14, 15].

The aim of the present study was to detect the effect of OS (as measured by induced peroxidation) on sperm function (acrosin activity) and their relation with leukocytospermia and other sperm characteristics.

2 Materials and methods

This study consisted of 30 infertile patients attending the Dermatology and Andrology Department at Mansoura University Hospitals, Egypt for infertility evaluation and 12 fertile normozoospermic volunteers. A thorough medical and surgical history was obtained including fertility status. None of the participants, both the fertile and infertile study population had a history of smoking or exposure to radiation and gonadotoxins. Full general and local examinations and scrotal ultrasound using a high-resolution color flow duplex Doppler ultrasonography unit (Acuson 128XB, Seimens-Acuson, California, USA) were done to exclude other related factors such as varicocele.

2.1 Collection and analysis of semen samples

The samples were collected by masturbation after sexual abstinence for 2 - 3 days. A single sample provided by each subject was examined according to the WHO criteria and analyzed for appearance, volume, consistency and pH [16]. On microscopic examination, sperm concentration, percentage of motile sperm and motility characteristics (velocity and linear velocity) were objectively evaluated using a computer-assisted method (Autosperm, Fertipro, Beernem, Belgium) as described by Hinting *et al* [17]. Spermatozoa were classified according to their motility characteristics. Forward progression was assessed as follows: Grade A: linear velocity $\geq 22 \mu\text{m}/\text{sec}$; Grade B: linear velocity $\leq 22 \mu\text{m}/\text{sec}$ & curvilinear velocity $> 5 \mu\text{m}/\text{sec}$; Grade C: curvilinear velocity $< 5 \mu\text{m}/\text{sec}$ and Grade D: immotile spermatozoon. Sperm morphology was evaluated by a phase contrast microscopy of the native sample. In addition, air-dried smears were prepared and fixed in equal parts of ethanol and ether. Staining was performed using the simplified SpermMac stain (Autosperm, Fertipro, Lotenhulle, Belgium). Differentiation between WBCs and spermatogenic cells was performed using a peroxidase stain. Leukocytospermia is defined as more than 1 million white blood cells per milliliter of ejaculate [16]. Group 1 consisted of fertile volunteers with normal semen parameters and without leukocytospermia ($n = 12$). Group 2 included patients with OAT and without leukocytospermia ($n = 10$). Group 3 comprised of patients with normal semen parameters and leukocytospermia ($n = 11$). Group 4 consisted of patients with OAT and leukocytospermia ($n = 9$). The sperm characteristics exhibited by these groups are given in Table 1.

Table 1. Semen profiles of different study groups.

| perm parameters | Samples with $<1 \times 10^6$ WBC/mL | | Samples with $>1 \times 10^6$ WBC/mL | |
|--------------------------------|--------------------------------------|----------------------|--------------------------------------|---------------------|
| | Group I (n = 12) | Group II (n = 10) | Group III (n = 11) | Group IV (n = 9) |
| Concentration (10^6 /mL) | 65.0 (59.2-74.5) | 8.9 (2.3-17.4) | 64.1 (60.7-74.1) | 8.3 (2.8-16.4) |
| Motility - Grade A (%) | 55.1 (51.5-60.0) | 15.0 (8.0-25.0) | 54.2 (52.3-61.0) | 8.0 (3.0-14.3) |
| Motility - Grade A+B (%) | 65.5 (57.5-69.5) | 30.0 (18.0-40.0) | 64.5 (62.5-69.0) | 16.0 (12.5-29.2) |
| Velocity (μ m/sec) | 38.7 (33.0-45.9) | 30.8 (23.2-32.8) | 38.1 (36.0-45.9) | 22.9 (19.5-24.8) |
| Linear velocity (μ m/sec) | 34.4 (31.9-41.0) | 24.0 (22.1-29.0) | 33.3 (31.6-39.8) | 16.2 (12.9-20.8) |
| Normal morphology (%) | 62.0 (40.0-65.0) | 9.0 (4.0-10.0) | 52.0 (51.0-58.0) | 6.0 (3.5-10.0) |

Note: Values are median (25 % - 75 % interquartile range).

Group I: Fertile volunteers with normozoospermia and $<1 \times 10^6$ WBC/mL.

Group II: Infertile patients with oligoasthenoteratozoospermia (OAT) and $<1 \times 10^6$ WBC/mL.

Group III: Infertile patients with normozoospermia and $>1 \times 10^6$ WBC/mL.

Group IV: Infertile patients with OAT and $>1 \times 10^6$ WBC/mL.

2.2 Determination of thiobarbituric acid reactive substances (TBARS) production

Spermatozoa were pelleted by centrifugation and suspended to a concentration of 10×10^6 in Hanks balanced salt solution without Ca^{2+} and Mg^{2+} . Lipid peroxidation was induced by ferrous sulphate (4 mmol/L) and sodium ascorbate (20 mmol/L) to 1mL sperm suspension and the mixture was incubated for 2 h at 37 °C. The major lipid peroxidation product measured by this method was malondialdehyde. The reagent for TBARS determination was prepared by mixing 200 μ L 7 % SDS, 2 mL 0.1 mol/L HCL, 300 μ L 10 % phosphotungstic acid, 1mL 0.67 % of thiobarbituric acid and 100 μ L 0.2 mmol/L butylated hydroxytoluene. The latter was added to the reaction mixture to prevent propagation of the lipid peroxidation chain reaction during the course of the assay. The assay was performed by adding 360 μ L aliquots of this mixture to 200 μ L of the sperm suspension in which lipid peroxidation was induced and boiled for 30 minutes in a water-bath. The tubes were cooled, and the thiobarbituric acid adduct was extracted with 500 μ L butanol. TBARS standards were created by the acid hydrolysis of 1, 1, 3, 3 tetraethoxy propane by overnight incubation in the presence of 0.1 mol/L HCL. The thiobarbituric acid adduct was measured by spectrofluorometer (Shimadzu RF-5001, Shimadzu Corporation, Japan) with excitation and emission maxima of 510 nm and 553 nm, respectively. The results were calculated as picomoles TBARS produced by 10×10^6 spermatozoa/hour [18].

2.3 Preparation of gelatin-covered microslides and gelatinolysis

Gelatin-covered slides were prepared by spreading 20 μ L of 5 % gelatin (Merck, Darmstadt, Germany) in distilled water on the slides. The slides were then air-dried, stored at 4 °C overnight and fixed and washed in phosphate-buffered saline [6]. Semen samples of 20 μ L were diluted 1:10 in PBS containing 15.7 mmol/L α -D-glucose. Semen samples were smeared on prepared slides and incubated in a moist chamber at 37 °C for 2 h. The halo diameter around any 10 spermatozoa shown to be representative of sperm present in the ejaculate was measured in phase contrast with an eyepiece micrometer. The halo formation rate was calculated per slide as the percentage of spermatozoa showing a halo. One hundred spermatozoa were evaluated. An acrosin activity index was calculated by multiplying the halo diameter by the halo formation rate.

2.4 Statistical analysis

Statistical analysis was performed using the MedCalc program (MedCalc Software, Mariakerke, Belgium). The significance of differences between groups were assessed using Wilcoxon's test. Correlations were calculated using Spearman's rank order coefficient. Receiver operating characteristics (ROC) curves were used to determine the discriminative power between the groups and to identify criterion values. A *P* value of <0.05 was considered statistically significant.

3 Results

The present study consisted of 42 subjects with a mean age of 35.47±6.81 years. When compared by group, the patients' ages did not differ significantly (*P* >0.05).

Table 2 shows the WBC concentration and TBARS produced by 10×10^6 spermatozoa after incubation for 2 hours with promotor of peroxidation in Groups I through IV. The differences were statistically significant in all group compared. The gelatinolysis assay parameters are also shown in Table 2. The halo diameter, halo formation rate and acrosin activity index were significantly higher in the patients without leukocytospermia than in the matched groups with leukocytospermia. Also, the gelatinolysis parameters from the normozoospermic samples were significantly higher than those with OAT. Figure 1A (normozoospermic fertile volunteers samples without leukocytospermia) shows well-defined halos (>10 μm) with a good acrosin activity as measured by the phase contrast microscopy. Figures 1B & 1C (normo-zoospermic and OAT patient samples with leukocytospermia) show poorly defined halos and no halo formation respectively.

Figure 1. Photomicrograph (400 \times of human spermatozoa after incubation for 2 hours on gelatin slides showing: (A) good acrosin activity in normozoospermic samples without leukocytospermia, (B, C) poor and no acrosin activity in normozoospermic and OAT samples with leukocytospermia, respectively.

Table 2. White blood cells (WBCs), thiobarbituric acid reactive substances (TBARS) levels and gelatinolysis parameters in the study groups.

| Variables | Samples with $<1 \times 10^6$ WBC/mL | | Samples with $>1 \times 10^6$ WBC/mL | | P value | | | |
|---------------------------------|--------------------------------------|----------------------|--------------------------------------|---------------------|---------|----------|----------|-----------|
| | Group I (n = 12) | Group II (n = 10) | Group III (n = 11) | Group IV (n = 9) | I vs II | I vs III | II vs IV | III vs IV |
| WBCs ($\times 10^6$ /mL) | 0.3 (0.2-0.5) | 0.6 (0.1-0.8) | 2.6 (1.6-3.5) | 3.2 (2.8-4.3) | >0.05 | <0.001 | <0.001 | <0.001 |
| TBARS (picomoles) | 1.9 (1.2-2.7) | 4.8 (4.1-6.2) | 9.2 (7.5-11.0) | 16.1 (14.6-16.9) | <0.001 | <0.001 | <0.001 | <0.001 |
| Halo diameter (μm) | 16.2 (15.2-17.9) | 12.4 (11.6-14.5) | 12.1 (10.7-14.2) | 8.5 (7.3-10.8) | <0.001 | <0.001 | <0.01 | <0.01 |
| Halo formation rate (%) | 82.0 (78.5-84.5) | 71.0 (66.0-74.0) | 65.0 (53.5-70.3) | 44.0 (39.5-48.3) | <0.01 | <0.001 | <0.001 | <0.001 |
| Acrosin activity index | 13.6 (12.6-14.5) | 9.5 (7.8-9.8) | 8.1 (6.2-8.7) | 3.7 (3.1-4.7) | <0.001 | <0.001 | <0.001 | <0.001 |

Values are median (25 % - 75 % interquartile range); *P*<0.05 was considered significant by Wilcoxon's test.

Group I: Fertile volunteers with normozoospermia and $<1 \times 10^6$ WBC/mL.

Group II: Infertile patients with oligoasthenoteratozoospermia (OAT) and $<1 \times 10^6$ WBC/mL.

Group III: Infertile patients with normozoospermia and $>1 \times 10^6$ WBC/mL.

Group IV: Infertile patients with OAT and $>1 \times 10^6$ WBC/mL.

The correlations between the concentrations of WBCs, TBARS production after peroxidation, sperm parameters and acrosin activity index are shown in Table 3. Acrosin activity index was positively

correlated with different semen variables and negatively correlated with production of TBARS and WBC concentration. In addition, the TBARS production was negatively correlated with different semen variables and positively with the WBC concentration.

Table 3. Correlation between parameters of gelatinolysis, thiobarbituric acid reactive substances (TBARS) and different semen variables ($n = 42$). r - Spearman's rank-order correlation coefficient; $P < 0.05$ was considered significant.

| | Acrosin activity index | | TBARS | |
|--|------------------------|---------|-------|---------|
| | r | P value | r | P value |
| Concentration ($\times 10^6$ /mL) | 0.5 | <0.01 | -0.36 | <0.05 |
| Grade A (%) | 0.63 | <0.001 | -0.48 | <0.01 |
| Grade A+B (%) | 0.59 | <0.001 | -0.44 | <0.01 |
| Velocity ($\mu\text{m}/\text{sec}$) | 0.53 | <0.01 | -0.37 | <0.05 |
| Linear velocity ($\mu\text{m}/\text{sec}$) | 0.57 | <0.001 | -0.39 | <0.05 |
| Normal morphology (%) | 0.61 | <0.001 | -0.43 | <0.01 |
| WBCs ($\times 10^6$ /mL) | -0.71 | <0.001 | 0.79 | <0.001 |
| TBARS (picomoles) | -0.89 | <0.001 | - | - |

We calculated the cut off value of OS (TBARS = 4.1 Pmole) that discriminates between a fertile normozoospermic population without leukocytospermia and the infertile patients including those with 1) leukocytospermia but normozoospermic and 2) oligoasthenoterato-zoospermic but without leukocytospermia and with leukocytospermia. This cut-off value was determined by ROC curve analysis. The mean value of TBARS was significantly lower ($P < 0.01$) in the fertile group [1.9 (1.2-2.7) Pmole] than in the infertile group [8.8 (6.1-14.2) Pmole]. In regards to gelatinolysis parameters, the mean halo diameter, halo formation rate and acrosin activity index were significantly higher in the fertile group [16.2 (15.2-17.9) mm, 82 (78.5-84.5) %, 13.6 (12.6-14.5), respectively] versus that of the infertile group [11.5 (9.8-14.2) mm, 64.5 (46.0-71.0) %, 7.8 (4.8-9.4), respectively].

Based on the cut-off values for OS, we used ROC curve analysis of the acrosin activity index to discriminate between the samples of subjects with and without OS affecting sperm function (Figure 2). At an acrosin activity index 11.02, we could discriminate between the samples with and without OS with high sensitivity (100 %) and specificity (85.7 %). The area under the curve for the acrosin activity index was 0.97 (0.86 - 0.996). Only acrosin activity index was used as it includes the discriminatory power of both the halo diameter and halo formation rate.

Figure 2. Receiver operating characteristics curve for acrosin activity index to discriminate between samples with and without oxidative stress.

4 Discussion

In our study, the decreased gelatinolysis parameters associated with WBC infiltration could be the result of increased peroxidative damage to spermatozoa by ROS. In fact, all cellular components including lipids, proteins, nucleic acid and sugars are potential targets for ROS. The extent of OS-induced damage depends not only on the nature and amount of ROS involved but also on the timing and duration of ROS exposure and extracellular factors [19]. Zalata *et al* found that exposing spermatozoa to WBCs induces OS, which decreased the membrane fluidity and impairs the fusogenic capacity [20]. Indeed, this decrease in fluidity could affect the acrosomal membrane and impair the acrosin activity.

Even though the groups with WBCs (groups III & IV) and without (I & II) were comparable in terms of sperm variables (I & III, II & IV compared), there was a statistically significant difference between these groups in terms of TBARS production and gelatinolysis para-meters. This indicates the significance of

infiltrating WBCs in producing OS and impairing the functional capacity of sperm without affecting the basic semen parameters.

The significant negative correlation between both the production of TBARS from the spermatozoa and prevalence of WBCs and gelatinolysis parameters indicate the deleterious effect of OS on acrosin activity. We observed a statistically significant difference in terms of TBARS production and gelatinolysis parameters when we divided the WBC negative and positive patients based on normal and abnormal sperm parameters. This finding supports the notion that abnormal spermatozoa produce OS.

The highly significant negative correlation between TBARS production and gelatinolysis parameters was observed when all the subjects were analyzed as a single group. This indicates that irrespective of the source of OS, gelatinolysis parameters were truly representative of the OS status of an individual. Functional tests of spermatozoa (eg, acrosin activity test) or tests to document OS (TBARS levels, ROS levels) may be included in the routine evaluation of male infertility. Our findings differ from those of Ichikawa *et al*, who reported that ROS levels may exert a negative influence on acrosome reaction while ROS levels in semen have no relationship with acrosin activity [21].

El Mulla *et al* found that smoking and varicocele affected sperm function including acrosin activity as measured by gelatinolysis [5]. Indeed, this could be attributed to OS in their patients, as previous studies demonstrated OS in patients with history of smoking and varicocele [19, 22]. Acrosin activity as determined by the gelatinolysis technique has been shown to be predictive of the fertilizing capacity of human spermatozoa. A normal halo formation rate as well as a normal diameter (acrosin activity) indicate good fertilizing capacity of spermatozoa [6]. They reported that if an assay shows a halo formation rate <60 %, a halo diameter <10 mm and acrosin activity index <6, it is indicative of subfertility [6]. In our study, the acrosin activity index was shown to have a high sensitivity and specificity in discrimination between samples with and without OS.

In conclusion, our data suggest that OS associated with WBC infiltration and/or abnormal spermatozoa could affect sperm function – acrosin activity in particular. Gelatinolysis parameters could help to discriminate between patients with and without OS. Further investigations are needed to assess the relevance of these data in the clinical management of male infertility.

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