Relationship between reactive oxygen species production in human semen and sperm DNA damage assessed by Sperm Chromatin Structure Assay

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Aim. The aim of this prospective study was to find possible relationship between ROS production measured by chemiluminescence and flow cytometry in human semen and sperm DNA damage estimated by Sperm Chromatin Structure Assay.

Methods. Study included 39 men from infertile couples and 23 fertile volunteers who served as a control group. Aliquot of neat semen was used for ROS detection by chemiluminescence. Aliquot of sperm suspension in phosphate buffered saline was used for the detection of ROS by flow cytometry. Another aliquot of sperm suspension was used for SCSA to measure DNA fragmentation index and High DNA stainability.

Results. DNA fragmentation index correlated negatively with sperm morphology and motility. High DNA stainability correlated positively with ROS production and negatively with sperm morphology and concentration. Although there were similar trends of rising DNA fragmentation index and ROS production among the three groups of men, the relationship did not reach statistical significance.

Conclusions. Higher values of DNA fragmentation index and high DNA stainability may also reflect developmental and/or environmental problems and not only oxidative stress.

Key words: human semen, ROS, SCSA, DNA damage

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INTRODUCTION

Sperm chromatin integrity is essential for the transmission of paternal genetic information. DNA damage also has significant negative impact on *in vitro* fertilization¹. Infertile men have been found to have substantially higher sperm DNA damage than fertile men¹⁻³. In addition, numerous studies reported significant, negative correlations between sperm DNA damage and the outcome of assisted reproductive technologies (ART). However, this view is not universally supported⁴. Sperm DNA damage may occur at any of the stages of spermatogenesis. At least three molecular mechanisms are suggested to cause sperm DNA damage: (i) alterations of sperm chromatin packaging^{5,6}, (ii) aborted apoptosis^{1,15} and (iii) oxidative stress. Reactive oxygen species (ROS) produced in small amounts are necessary for sperm functions such as capacitation and acrosome reaction⁸. Excessive ROS production however is negatively linked to sperm function and morphology⁹⁻¹⁴ and may be secondary to urogenital tract infections, neutrophils and macrophages infiltration¹⁵, radiation, chemotherapy and smoking.

The aim of our study was to compare levels of ROS production in human semen measured by chemiluminescence and flow cytometry with the levels of sperm DNA damage estimated by SCSA.

MATERIALS AND METHODS

The study was approved by the Ethics Board of the Palacky University in Olomouc, Czech Republic. All assessed males were healthy non-smokers without any dietary restrictions and signed the written consent. The study sample included 23 volunteers with proven natural fertility and sperm donors (control group) and 39 men from couples suffering from infertility who were divided into two groups: 16 normozoospermic males (NSI group) and 23 males with semen abnormalities (SA group).

Semen collection and analysis

Semen samples were collected by masturbation after a period of 3 to 5 days of sexual abstinence. After liquefaction (37 °C, 30 min), standard semen analysis was

performed according to WHO 2010 guidelines¹⁶. Aliquot of liquefied semen sample (400 μ L) was used for direct ROS estimation in neat semen by chemiluminescent (CL) assay. Another aliquot of liquefied semen (500 μ L) was centrifuged at $300 \times g$ for 7 min. The pelleted cells were resuspended in isotonic phosphate buffered saline (PBS) and sperm concentration was adjusted to 1×10^6 /mL. One aliquot of this sperm suspension was immediately used for ROS detection by CL flow cytometry and another aliquot was used as a blank control. For SCSA assay, another aliquot was centrifuged and resuspended in TNE buffer (15 mmol L⁻¹ NaCl, 10 mmol L⁻¹ Tris and 1 mmol L⁻¹ EDTA, pH = 6.8) and frozen in liquid nitrogen.

ROS detection by CL assay

ROS production was measured using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St. Louis, MO, USA) as a probe. Luminol (10 μ L, 5 mmol L⁻¹) prepared in dimethylsulphoxide (DMSO) was added to 400 μ L of the neat semen and data were immediately recorded. Blank samples for estimation of background signal were prepared by pipetting 10 μ L of 5 mmol L⁻¹ luminol to 400 μ L of PBS. The chemiluminescent signal was monitored for 15 min using a Digene DCR-1 single tube luminometer (Digene Diagnostics, Inc., Gaithersburg, MD, USA). Results were expressed as relative light unit (RLU) per minute per 20×10^6 spermatozoa¹⁷.

ROS detection by flow cytometry

Dihydrorhodamine 123 dye (DHR123; Life Technologies Corporation Carlsbad, CA, USA) was used to detect ROS as previously described¹⁵. DHR123 (1 μ L,

1 mmol L⁻¹ solution in DMSO) was added to 1 mL aliquot of sperm suspension containing 1×10^6 spermatozoa and incubated at 37 °C for 20 min. Unstained aliquot of each sperm sample was used as a blank control to estimate autofluorescence background. Flow cytometer FACS Calibur (BD Biosciences, San Jose, CA, USA) was used and data were analyzed using BD CellQuestPro software (BD Biosciences, San Jose, CA, USA). 25,000 cells were analyzed in each sample. Flow cytometric results were expressed in relative fluorescence unit (RFU) per 20×10^3 spermatozoa¹⁵.

Sperm chromatin structure assay (SCSA)

Sperm DNA damage was assessed by the SCSA as previously described². Frozen samples were rapidly thawed in a 37 °C water bath and 200 μ L of sample was treated with 400 μ L acidic detergent solution (80 mmol L⁻¹ HCl, 0.1% Triton-X 100, pH = 1.2) for exactly 30 s to induce DNA denaturation. Acridine Orange (AO) staining solution (1.2 mL, 6 μ g mL⁻¹) was added to sperm suspension. Flow cytometer FACS Calibur (BD Biosciences, San Jose, CA, USA) was used and data were analyzed using BD CellQuestPro software (BD Biosciences, San Jose, CA, USA). The extent of DNA denaturation was expressed in terms of DNA fragmentation index (DFI) and the abnormally high DNA stainability (HDS) using SCSA-Soft software (SCSA® DIAGNOSTICS, INC, Brookings Research & Technology Center, Brookings, SD, USA). 5,000 cells were analyzed in each sample. Duplicate measurements were performed; the sample for duplicate measurement was taken from the same thawed aliquot, diluted appropriately, processed for the SCSA and measured.

Table 1. Semen parameters and ROS production.

Parameter	Study groups			P values
	control group (n = 23)	NSI (n = 16)	SA (n = 23)	
Age (years)	32 (29, 35)	34 (29, 38)	34 (30, 39)	N.S
Sperm concentration (10^6 sperm/mL)	65 (37; 78) ^a	41 (26; 65) ^b	10 (5; 19) ^{a, b}	^a : $P < 10^{-6}$, ^b : $P < 0.002$
Sperm normal morphology (%)	35 (30.5; 40) ^a	31.5 (30.5; 33.5) ^b	17 (8.5; 24) ^{a, b}	^a : $P < 10^{-6}$, ^b : $P < 2.5 \times 10^{-4}$
Sperm motility (%)	56 (43.5; 61.5)	51 (44; 55)	50 (34.5; 57.5)	N.S
CL (log RLU/min/ 20×10^6 sperm)	2.92 (2.32; 3.60) ^{a, b}	3.78 (3.09; 4.40) ^a	4.02 (3.79; 4.29) ^b	^a : $P < 0.05$, ^b : $P < 1.5 \times 10^{-4}$
DHR123 (log RFU/ 20×10^3 sperm)	4.86 (4.78; 5.28)	4.97 (4.85; 5.22)	5.20 (4.81; 5.34)	N.S
Sperm DFI (%)	16.5 (11.5; 27.7) ^a	22.6 (11.8; 42.3)	35.0 (24.8; 41.3) ^a	^a : $P < 0.0035$
High DNA stainability (%)	12.1 (8.4; 17.9) ^a	12.4 (10.3; 17.1) ^b	24.4 (14.8; 31.6) ^{a, b}	^a : $P < 0.007$, ^b : $P < 0.035$

Legend: Data are expressed as medians (25th, 75th percentiles). Kruskal-Wallis test was used to compare sperm variables among the three groups. Letters ^a and ^b mark significant differences in evaluated parameters between groups.

Statistical analysis

Summary statistics are presented as median (25th and 75th percentiles). Kruskal-Wallis test was used to compare sperm variables among the three groups. Spearman's rank correlation was used to test the relationship between ROS production and DNA damage and between semen parameters and sperm DNA damage. All hypotheses testing was two-tailed; $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using software Statistica 8 (StatSoft, Inc.).

RESULTS

The median (25th and 75th percentiles) of age, semen parameters and ROS production in the three groups are given in Table 1. Sperm concentration and percentage normal sperm morphology were significantly lower in both SA and NSI groups compared to control group. Sperm motility was not significantly different among all three groups.

The median ROS production estimated by CL assay was lowest in the control group and highest for the SA group. The difference between the three groups was significant (Table 1). There was also a similar trend of progressively higher median of ROS production estimated by DHR123 from control group to NSI group to SA group (4.86, 4.97 and 5.20, respectively). However, the difference between groups was not significant. The median DFI % was lowest in the control group, non-significantly higher in NSI group and significantly higher in SA group ($P < 0.0035$). High DNA stainability was found to be significantly higher in SA group than in control group ($P < 0.007$) and in NSI group than in control group ($P < 0.035$).

Association between DNA damage and ROS production

DFI did not correlate with any measure of ROS production ($r = 0.04$ for CL assay and $r = -0.04$ for DHR123). High DNA stainability correlated positively with ROS production estimated by CL assay ($r = 0.27$, $P < 0.03$) and DHR123 ($r = 0.27$, $P < 0.041$).

Association between DNA damage and standard semen parameters

Sperm DNA fragmentation correlated negatively with normal sperm morphology ($r = -0.44$, $P < 0.0004$) and sperm motility ($r = -0.30$, $P < 0.017$). Correlation between sperm DNA fragmentation and sperm concentration was not significant ($r = -0.23$, $P < 0.071$). High DNA stainability correlated negatively with normal sperm morphology ($r = -0.41$, $P < 0.0008$) and concentration ($r = -0.60$, $P < 10^{-6}$). Correlation between high DNA stainability and sperm motility was not found ($r = -0.02$).

DISCUSSION

Some authors have reported a positive correlation between ROS production and sperm DNA damage^{18,19}. The results of our study demonstrated that there was a trend of rising DFI %, from control group to NSI group to infertile group with abnormal semen parameters. There was also a similar trend of raising ROS production measured with the CL method and DHR123 in the same group order. However, the relationship between DFI and ROS production did not reach statistical significance. The magnitude of DNA fragmentation is influenced by a diversity of developmental (apoptosis), biological (varicocele, high prolonged fever, advanced age) and environmental (radiation, air pollution, smoking, pesticides, and chemicals) factors. Because of this multifactorial origin of DNA damage, some other studies that examined the relationship between other factors such as apoptosis and sperm DNA fragmentation failed to demonstrate a significant positive relationship²⁰. In our study, there were even ejaculate samples showing high level of ROS production but negligible sperm DNA damage. On the other hand, there were also samples showing negligible ROS production but high percentage of spermatozoa with fragmented DNA. It was previously suggested that ROS measurement provided information about current status of ejaculate but it did not necessarily correspond with oxidative stress acting on spermatozoa during sperm maturation and its epididymal transit²¹.

Flow cytometry provides an estimate of ROS production in the intracellular space only, while CL assay provides a global estimate in both extracellular and intracellular space. Our study demonstrated stronger association between DFI and semen parameters than between DHR123 and semen parameters. This may be due to the inclusion of healthy fertile volunteers in our study population. Desai et al. demonstrated that reactive oxygen species levels are independent of sperm concentration, motility, and abstinence in a normal, healthy, proven fertile man²².

In our study, semen samples from males with semen abnormalities showed significantly higher values of both SCSA parameters (DFI and HDS) in comparison to control group (healthy, proven fertile men). This is in accordance with other studies^{1,2,3,22,23}. We found significant negative correlations between DFI and sperm morphology as well as between DFI and sperm motility. Furthermore, we found significant negative correlations between HDS and sperm concentration as well as between HDS and sperm morphology. These results are in accordance with previous findings that spermatozoa from patients with abnormal sperm concentration, morphology and motility showed increased levels of DNA damage¹. Significant relationship between SCSA and basic semen parameters² and between sperm head morphology and SCSA were previously reported²⁴. It seems that higher values of DFI and HDS reflect more developmental and/or environmental problems which have impact on semen quality.

ABBREVIATIONS

AO, Acridine Orange; ART, Assisted reproductive technologies; CL, Chemiluminescence; DFI, DNA fragmentation index; DMSO, Dimethylsulphoxide; DNA, Deoxyribonucleic acid; HDS, High DNA stainability; NSI, Normozoospermic infertile men; PBS, Phosphate buffered saline; ROS, Reactive oxygen species; SA, Semen abnormalities; SCSA, Sperm Chromatin Structure Assay; RLU, Relative light unit.

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CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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