CHAPTER 3

A Novel Association between Sperm Deformity Index and Oxidative Stress-Induced DNA Damage in Infertile Male Patients

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ABSTRACT

Aim: Sperm deformity index (SDI) is a novel quantitative expression of sperm morphology. The objective of our study was to investigate the impact of abnormal sperm morphology using SDI on reactive oxygen species (ROS) production and its correlation with sperm DNA damage.

Materials and Methods: Semen samples were collected from men undergoing infertility screening (n = 7) and healthy donors (n = 6). Mature spermatozoa were isolated and incubated with 5 mM NADPH up to 24 hours to induce ROS. Sperm morphology was evaluated using strict Tygerberg's criteria and the SDI. ROS levels and DNA damage were assessed using chemiluminescence and TUNEL assays respectively.

Results: SDI values [median (interquartiles)] were higher in patients compared to donors [2 (1.8, 2.1) vs. 1.53 (1.52, 1.58), $P = 0.008$]. Aliquots treated with NADPH showed higher ROS levels [1.22 (0.3, 1.87) vs. 0.39 (0.1, 0.57), $P = 0.03$)] and higher incidence of DNA damage than those not treated [10 (4.69, 24.85) vs. 3.85 (2.58, 5.1), $P = 0.008$]. Higher DNA damage was also seen following 24 hour incubation in patients compared to donors. SDI correlated with the percentage increase in sperm DNA damage following incubation for 24 hours in samples treated with NADPH ($r = 0.7$, $P = 0.008$) and controls ($r = 0.58$, $P = 0.04$).

Conclusions: SDI may be a useful tool in identifying potential infertile males with abnormal prevalence of OS-induced DNA damage. NAPDH plays a role in ROS-mediated sperm DNA damage, which appears to be more evident in infertile patients with semen samples containing high incidence of morphologically abnormal spermatozoa.

Key words: NADPH; oxidative stress; sperm deformity index; sperm DNA damage.

INTRODUCTION

Semen analysis including sperm morphology remains the main pillar for male infertility work-up. However, different methodologies for sperm morphology assessment have remained controversial for lack of a universally acceptable method. One drawback of attempts to classify sperm into morphological subgroups as proposed by WHO [1] is that each individual sperm is classified only once but may have several deformities. Tygerberg's strict criteria has been proposed to correlate with IVF outcome results [2]. However, it may not serve as the best discriminator between normal and functionally impaired samples due to lack of a cut-off point for normal values. In a report by Menkveld *et al*. [3], the average percentage of normal forms in fertile population was 6.5%, while in subfertile population it was 3%. On the other hand, successful oocyte fertilization and pregnancies have been reported in couples with 0% normal sperm morphology [4].

The sperm deformity index (SDI) is a novel expression of sperm morphological assessment by the strict Tygerberg's criteria for normal sperm morphology that was reported to correlate with fertilization rates [5]. SDI is a useful predictor in the identification of fertile and infertile semen, and is more reliable than the multiple anomalies index, which involves the assessment of only abnormal sperm [6, 7]. The fertilizing potential of the semen sample may be compromised at sperm deformity index > 1.6 despite the presence of normal forms [5].

In defective spermiogenesis, there is failure of the remodeling of sperm membrane components, which results in morphologically abnormal spermatozoa that exhibit cytoplasmic residues [8]. The enzyme glucose-6-phosphate dehydrogenase (G6PD) is excessively present in sperm residual cytoplasm and generates β-nicotinamide adenine dinucleotide phosphate (NADPH). In turn, NADPH is used as a source of electrons by spermatozoa to fuel the generation of reactive oxygen species (ROS) production [9-14].

A significant positive correlation was observed between sperm ROS production and the proportion of sperm with abnormal morphology characterized by high SDI scores [15]. High levels of ROS lead to oxidative stress (OS), which is one of the leading causes for sperm DNA damage [14, 16-19]. Despite the protective tight packaging of the sperm DNA [20], deoxyribonucleic acid bases and phosphodiester backbones are susceptible to peroxidation [21]. Moreover, spermatozoa are particularly susceptible to OS due to their limited antioxidant defenses and the presence of large quantities of polyunsaturated fatty acids in their plasma membranes [22].

The prevalence of spermatozoa with fragmented DNA is considered among the most common causes for male infertility that may pass undetected [19]. The correlation between sperm morphology and DNA integrity remains controversial [23, 24]. The objective of our study was to investigate the impact of abnormal sperm morphology using SDI on NADPH-mediated ROS

production and its correlation with sperm DNA damage. The results of this study may provide a predictor for the presence of OS-mediated sperm DNA damage where laborartory facilities for direct assessment are not available.

MATERIALS AND METHODS

Subject selection

The study was approved by the Institutional Review Board of the Cleveland Clinic Foundation. Semen samples were collected from men undergoing infertility screening (n = 7) and healthy donors (n = 6). Samples with a sperm concentration < 20 X 10^6/mL and < 2.0 mL volume were excluded from our study to ensure the presence of sufficient spermatozoa for all our planned evaluations.

Semen collection and evaluation

Semen specimens were collected by masturbation after 48 to 72 hours of abstinence. After liquefaction at 37°C for 20 minutes, 5 µL of each specimen was loaded on a 20 micron Microcell chamber (Conception Technologies, San Diego, CA) and analyzed for sperm concentration and motility. All specimens were examined for white blood cell (WBCs) contamination by using myeloperoxidase (Endtz) staining [25]. Semen samples containing > 1 X 10^6 WBCs/mL were excluded to avoid ROS generation from potentially non-spermatozoal cells.

Assessment of sperm morphology

For morphological evaluations, seminal smears were stained with Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL). Slides were coded (Andrology Laboratories, Cleveland Clinic Foundation) and evaluated by the investigator (N. Aziz, Liverpool Womens Hospital, UK). A total of 100 spermatozoa were scored per slide using bright field illumination and an oil immersion objective with a total magnification of X 2000. At least ten high-power fields selected at random from different areas of the slide were examined. A calibrated micrometer on the eyepiece of the light microscope was used to measure sperm dimensions.

All slides were assessed using a morphological classification based on applying the strict Tygerberg's criteria for normal sperm morphology [26]. Sperm was considered normal when the head had a smooth oval shape with a well-defined acrosome covering 40% to 70% of its apical part. The head length was required to be 3 to 5 µm and width 2 to 3 µm. In addition, the midpiece was required to be axially attached to the sperm head, smooth in outline, 1 µm in width and approximately 1.5 times the head length. Any cytoplasmic droplet present in contact with the head base or exceeding half the head area was considered abnormal. The tail also was required to be uniform, slightly thinner than the midpiece, uncoiled, free from kinks, and

approximately 45 µm in length. The morphologically abnormal sperms were classified into subgroups that included pyriform, tapered, large-headed, small-headed, acrosomal defects, amorphous, cytoplasmic droplet > 0.5 the head size, midpiece defects, tail anomalies, and double sperms (double head or tail in any combination).

A multiple entry scoring technique was adopted in which an abnormal sperm was classified more than once if more than one deformity was observed. The SDI was calculated by dividing the total number of deformities observed by the number of sperm randomly selected and evaluated, irrespective of their morphological normality [5]. Therefore, the ratio of the number of deformed sperm to the number of deformities in each sperm should not affect the final results of the SDI. Borderline forms were considered abnormal and included: 1) Spermatozoa with slightly elongated head with loss of its oval shape; 2) Those with rounded heads and intact acrosome; 3) Those with normal heads and a thickened midpiece. Quality control of sperm morphology assessment revealed no significant difference in repeated estimation different sperm morphological forms.

Sample preparation and induction of ROS by exogenous NADPH

In order to separate predominantly mature spermatozoa, the liquefied semen was loaded onto a 47% and 90% discontinuous ISolate gradient (Irvine Scientific, Santa Ana, CA) and centrifuged at 500*g* for 20 minutes. The resulting 90% pellet (mature spermatozoa) was aspirated, re-suspended in Biggers, Whitten-Whittingham media (BWW, Irvine Scientific, Santa Ana, CA) and the assessment of the sperm parameters including morphology was repeated. The mature sperm suspension was further subdivided into 2 aliquots and each aliquot was incubated with 5mM NADPH (Sigma, St Louis, MO) for 0 and 24 hours respectively at 37°C and 5% CO_2. Each aliquot had its corresponding control without NADPH.

Measurement of ROS

ROS levels in all fractions were measured in 400 µL aliquots containing > 2 million sperm/mL using 4 µL of 25 mM lucigenin (bis-N-methylacridnium nitrate, Sigma, St Louis, MO) at final concentration of 0.25 mM. Negative controls were prepared by adding equal volume of lucigenin to 400µL of PBS. ROS levels were determined by chemiluminescence assay using a luminometer (model: LKB 953, Berthold Technologies, Bad-Wilbad, Germany) for 15 min, and expressed as X 10^6 counted photons per minute (cpm) per 20 million sperm.

Evaluation of DNA fragmentation

Sperm DNA strand breaks were evaluated using a flow cytometric terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assay kit (Apo-Direct™, BD

Biosciences, Mississauga, ON) as established earlier [27]. Briefly, spermatozoa were washed twice in Dulbecco's phosphate buffered saline (PBS), re-suspended in 1% paraformaldehyde at a concentration of 1-2 X 10^6 sperm/mL and placed on ice for 30-60 min. These spermatozoa were again washed and re-suspended in 70% ice-cold ethanol by centrifugation at 300 *g* for 5 minutes as per the kit instructions. The ethanol supernatant was removed and the cell pellets were washed twice in wash buffer and re-suspended in 50 µL of the staining solution for 60 min at 37°C. The staining solution contained terminal deoxytransferase (TdT) enzyme, TdT reaction buffer; fluorescein tagged deoxyuridine triphosphate nucleotides (FITC-dUTP) and distilled water. All cells were further washed using rinse buffer, resuspended in 0.5 mL of propidium iodide (PI)/RNase solution and incubated for 30 minutes in the dark at room temperature.

Data acquisition was performed within 3 hours on a flow cytometer equipped with 488 nm argon laser as a light source (Becton Dickinson FACScan, San Jose, CA). A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of < 100 cells/second. FITC (log green fluorescence) was measured on FL1 channel (Y-axis) and the PI (linear red fluorescence) on the FL2 channel (X-axis). Data were processed using FlowJo v4.4.4 software (Tree Star Inc., Ashland, OR).

Statistical analysis

Patient and donor groups were compared using Mann Whitney test. Within-group differences between samples and controls were assessed using the Wilcoxon matched-pairs test. Correlation between variables was assessed using non-parametric Spearman's (*r*). Sample size was sufficient to detect significant difference between groups. Summary statistics are presented as median and interquartiles (25^{th} and 75^{th} percentile). All hypothesis testing was 2-tailed, with a significance level of 0.05.

RESULTS

In the neat semen samples, sperm count, motility and morphology were comparable in both patient and donor groups. The median and interquartile values (25%, 75% percentiles) of sperm count, motility, percentage sperm with normal morphology, prevalence of cytoplasmic droplets and SDI scores in mature spermatozoa isolated by double density centrifugation are illustrated in Table 1. In this isolated fraction, patients had higher SDI scores compared to donors (P = 0.008). Patients also had higher number of cytoplasmic residues compared to donors (P = 0.004) while the median percentages of sperm with normal morphology applying the strict Tygerberg's criteria showed no significant difference in both groups. Only one sample in the donor group (n = 6) had SDI >1.6, while 6 samples in the patient group (n = 7) had SDI>1.6.

The increase in ROS levels following incubation was calculated as the difference between 24 hours and 0 hour values. The median increase in ROS levels was significantly higher in aliquots exposed to NADPH compared to the unexposed aliquots [1.22 (0.3, 1.87) vs. 0.39 (0.1, 0.57), *P* = 0.03). However, ROS levels were comparable between patient and donor groups before and after 24 incubation regardless of NAPDH exposure.

Similarly, the increase in DNA damage levels following incubation was calculated as the difference between 24 hours and 0 hour values. Aliquots treated with NADPH (from patients and donors) showed significantly higher incidence of increased DNA damage than those not treated [10 (4.69, 24.85) vs. 3.85 (2.58, 5.1), *P* = 0.008]. The increase in DNA damage seen after 24 hours following incubation was significantly higher in patients compared with donors in aliquots exposed to NADPH [16.56 (11.29, 40) vs. 4.4 (3.92, 5.25), *P* = 0.007] and in controls aliquots not exposed to NADPH [5.1 (3.87, 7.74) vs. 1.79 (2.87, 3.36), *P* = 0.03] (Figure 1).

Samples with SDI score >1.6 had higher increase in DNA damaged sperm compared to those with SDI score <1.6 [9.76 (4.19, 16.16) vs. 3.98 (3.02, 5.09), *P* = 0.04]. SDI scores correlated with the percentage increase in sperm DNA damage following incubation for 24 hours in samples exposed to NADPH (*r* = 0.7, *P* = 0.008) as well as controls not exposed to NADPH (*r* = 0.58, *P* = 0.04). Other sperm parameters assessed pre- and post-double density centrifugation (sperm count, motility, percentage sperm with normal morphology and percentage sperm with cytoplasmic droplet) showed no correlation with the sperm DNA damage.

Parameter	Patients (n = 7)	Donors (n = 6)	P-value*
Sperm count ($X10^6$)	27.2 (12.6, 27.6)	29.88 (22.54, 37.22)	0.45
Motility (%)	85 (54.5, 85.5)	92.5 (91.75, 93.25)	0.23
Strict morphology (%)	5 (1.5, 9)	8.5 (3, 12.5)	0.44
Cytoplasmic droplets (%)	4 (3, 4.5)	1 (0.25, 1.38)	0.004
SDI	2 (1.8, 2.1)	1.53 (1.52, 1.58)	0.008

Table 1. *Summary of sperm characteristics in mature spermatozoa isolated by double density gradient centrifugation. SDI = sperm deformity index. Results are expressed as median and interquartile values ($25^{th}$and 75^{th} percentiles); *P<0.05 considered significant comparing patient to donor groups using Mann-Whitney test.*

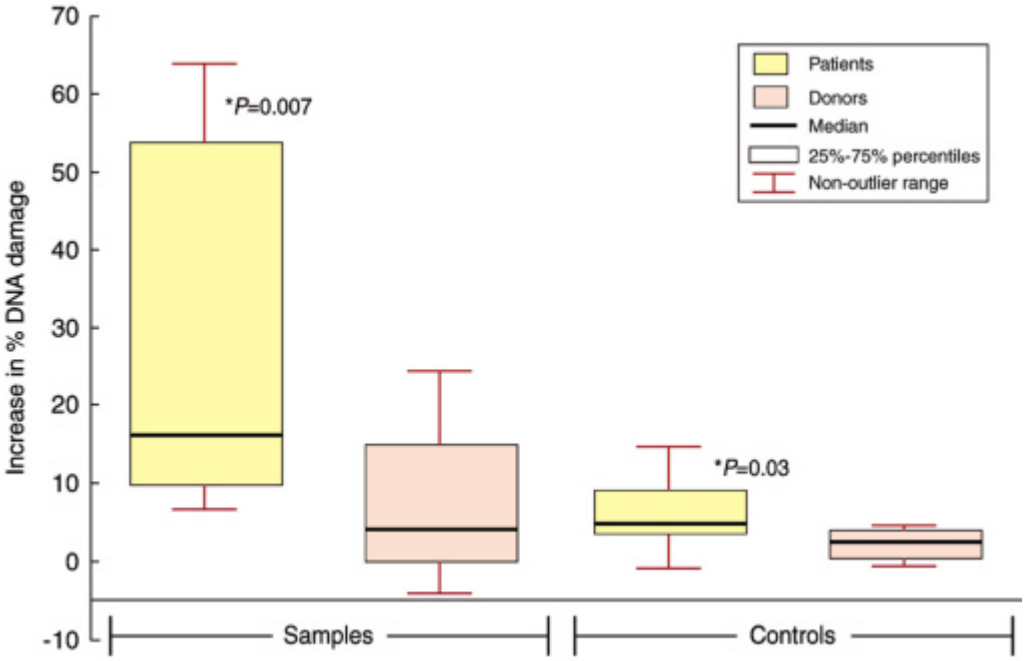

Figure 1. *Increase in sperm DNA damage in samples (treated with NADPH) and controls (without NADPH) following incubation for 24 hours in patients undergoing infertility screening and donors. Values represent median and interquartile (25%, 75% percentiles). * P<0.05 considered significant comparing patient to donor groups using Mann Whitney test.*

DISCUSSION

The absolute values of SDI scores reflect the interplay between the proportion of sperm with normal morphology and the proportion with one or more deformities as seen in a fixed stained semen smear. We have detected higher SDI scores in a heterogeneous group of males undergoing infertility screening compared to donors. On the other hand, we found that the percentages of sperm with normal morphology applying the strict Tygerberg's criteria were comparable in both groups. Therefore, this slight aberration from normal may be the reason for infertility. In addition, it reflects that the SDI may be capable of distinguishing semen samples with potentially impaired fertility.

Samples with SDI higher than 1.6 were previously described to have decreased fertilizing potential [5]. This observation is in agreement with our current results, in which almost all patients undergoing infertility screening (6/7) had SDI>1.6 despite the presence of equivocal sperm concentration and motility.

Exposure of spermatozoa to exogenous NADPH has been shown to result in a dose dependent increase in ROS. However, high concentrations of NADPH are required to increase its

intracellular concentration for significant ROS induction since the substrate is membrane impermeable [12-14, 16, 28]. Based on results of our pilot study, we have selected to use exogenous NADPH in a concentration of 5 mM as a model for increased ROS production by spermatozoa. Using this model, we were able to detect increase in ROS levels with a simultaneous increase in sperm DNA fragmentation following exogenous addition of NADPH.

Patients undergoing infertility screening had a significantly higher increase in sperm DNA damage compared to healthy donors. Significantly higher SDI scores and sperm with cytoplasmic residues were also noted in these patients. Therefore, we hypothesize that morphologically impaired spermatozoa that retain cytoplasmic residues may be more susceptible for DNA damage. High levels of ROS appear to mediate such damage. Increased ROS production may be attributed to NADPH, which is mediated by G6PD abundant in cytoplasmic residues. Our results are in agreement with a previously published report that documents the presence of impaired DNA integrity in semen samples with abnormal sperm parameters in absence of leukocytospermia [29].

The presence of increased DNA damage following prolonged incubation in the absence of exogenous NADPH in patients undergoing infertility screening further supports our hypothesis that morphologically impaired spermatozoa are susceptible for DNA damage. These samples had increased SDI and cytoplasmic residues, which may have lead to increased ROS production. Spermatozoa incubated in plain culture media have revealed spontaneous capacity to generate ROS and DNA fragmentation [30, 31]. In men with teratozoospermia, spermiogenesis is impaired resulting in deformed spermatozoa with residual cytoplasm, which may lead to increased ROS production and DNA damage [8, 32].

In our previous study, we demonstrated that sperm ROS production was positively correlated with the percentage of sperm with amorphous heads, acrosomal damage, cytoplasmic droplets, midpiece, and tail defects as well as the SDI scores [15]. Our present results also establish for the first time a potential correlation between the SDI scores and sperm DNA damage. However, our results showed no correlation between sperm DNA integrity and percentage normal sperm morphology, sperm concentration and sperm motility as reported previously [33, 34]. The difference in the assays used for evaluation in addition to the difference in the study population and the relatively larger number included in these studies may explain the discrepancy.

In the last decade, the focus on the sperm genomic integrity has been further intensified by the frustrating low success rates of assisted reproductive techniques (ART) as well as the concern of transmission of genetic diseases through these techniques [35]. Children who are born after intracytoplasmic sperm injection (ICSI) have an increased risk of a major congenital malformation compared with those born after spontaneous conception [36]. The transmission of

defective paternal DNA may increase the incidence of genomic imprinting errors leading to increased incidence of birth defects [37, 38].

Unfortunately, the heterogeneity of sperm populations usually complicates proper quality assessment and the ability to predict the fertilization potential. There are multiple assays that may be used for the evaluation of the sperm chromatin status. The choice of which assay to be performed depends on many factors such as the expense, the available laboratory facilities, and the presence of experienced technicians. The establishment of a cut-off point between normal levels in the average fertile population and the minimal levels of sperm DNA integrity required for achieving pregnancy still remains to be investigated. Such an average range or value is still lacking for most of these assays except for the sperm chromatin structure assay (SCSA) [39].

The correlation between the morphological pattern of spermatozoa and its DNA integrity in ejaculate may be an alternate strategy. However, this only allows the probability analysis of the fertility potential of a particular semen sample and relies on strict reproducible techniques of assessing sperm morphology. Since the increase in DNA damage was more marked in samples with SDI>1.6, our preliminary findings suggest that samples with high SDI scores may be more prone to present with prevalent DNA fragmented sperm. However, our study may be limited by its sample size and our findings still require further validation.

In conclusion, our preliminary results suggest that SDI may be a useful tool to detect the prevalence of sperm DNA damage and to identify potential infertile men. Infertile patients with semen samples containing high proportion of sperm morphological abnormalities specifically cytoplasmic droplets may be more susceptible to develop ROS-mediated sperm DNA damage.

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