Blackwell Publishing

Asian J Androl 2005; 7 (2): 121–126
DOI: 10.1111/j.1745-7262.2005.00022.x

AJA

·Original Article·

Novel association between sperm deformity index and oxidative stress-induced DNA damage in infertile male patients

Tamer M. Said[1], Nabil Aziz[2], Rakesh K. Sharma[1], Iwan Lewis-Jones[2], Anthony J. Thomas Jr[1], Ashok Agarwal[1]

[1]Center for Advanced Research in Human Reproduction, Infertility and Sexual Function, Glickman Urological Institute and Department of Obstetrics-Gynecology, The Cleveland Clinic Foundation, Cleveland, Ohio 44195, USA
[2]Reproduction Medicine Unit, Liverpool Women's Hospital, Liverpool L87SSS, UK

Abstract

Aim: To investigate the impact of abnormal sperm morphology using the sperm deformity index (SDI) on reactive oxygen species (ROS) production and its correlation with sperm DNA damage. **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 mmol/L β-nicotinamide adenine dinucleotide phosphate (NADPH) for up to 24 h 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 terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assays, respectively. **Results:** SDI values (median [interquartiles]) were higher in patients than 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 [0.30, 1.87] *vs.* 0.39 [0.10, 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, 5.10], P = 0.008). Higher DNA damage was also seen following 24 h of incubation in patients compared to donors. SDI correlated with the percentage increase in sperm DNA damage following incubation for 24 h in samples treated with NADPH (r = 0.7, P = 0.008) and controls (r = 0.58, P = 0.04). **Conclusion:** SDI may be a useful tool in identifying potential infertile males with abnormal prevalence of oxidative stress (OS)-induced DNA damage. NADPH plays a role in ROS-mediated sperm DNA damage, which appears to be more evident in infertile patients with semen samples containing a high incidence of morphologically abnormal spermatozoa. *(Asian J Androl 2005 Jun; 7: 121–126)*

Keywords: β-nicotinamide adenine dinucleotide phosphate; oxidative stress; sperm deformity index; sperm DNA damage

1 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 because of the lack of a universally acceptable method. One drawback of attempts to classify sperm into morphological subgroups as proposed by WHO 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 [1]. However, it may not serve as the best discriminator between normal and func-

Correspondence to: Prof. Ashok Agarwal, Center for Advanced Research in Human Reproduction, Infertility and Sexual Function, Glickman Urological Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195, USA.
Tel: +1-216-444-9485, Fax: +1-216-445-6049
E-mail: agarwaa@ccf.org
Received 2004-07-15 Accepted 2004-12-10

tionally impaired samples due to the lack of a cut-off point for normal values. In a report by Menkveld *et al.* [2], the average percentage of normal forms in the fertile population was 6.5 %, while in subfertile it was 3.0 %. On the other hand, successful oocyte fertilization and pregnancies have been reported in couples with 0 % normal sperm morphology [3].

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 [4]. 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 [5]. The fertilizing potential of the semen sample may be compromised at sperm deformity index >1.6 despite the presence of normal forms [4].

In defective spermiogenesis, there is failure of the remodeling of sperm membrane components, which results in morphologically abnormal spermatozoa that exhibit cytoplasmic residues. 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 [6, 7].

A significant positive correlation was observed between sperm ROS production and the proportion of sperm with abnormal morphology characterized by high SDI scores [8]. High levels of ROS lead to oxidative stress (OS), which is one of the leading causes of sperm DNA damage [9]. Despite the protective tight packaging of the sperm DNA, deoxyribonucleic acid bases and phosphodiester backbones are susceptible to peroxidation [10]. 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 [11].

The prevalence of spermatozoa with fragmented DNA is considered among the most common causes for male infertility that may pass undetected [12]. The correlation between sperm morphology and DNA integrity remains controversial. 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.

2 Materials and methods

2.1 Subject selection

The present 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 < 20×10^6/mL and < 2.0 mL volume were excluded from our study to ensure the presence of sufficient spermatozoa for all our planned evaluations.

2.2 Semen collection and evaluation

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

2.3 Assessment of sperm morphology

For morphological evaluations, seminal smears were stained with Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, USA). Slides were coded (Andrology Laboratories, Cleveland Clinic Foundation) and evaluated by the investigator (N. Aziz, Liverpool Women's Hospital, Liverpool, UK). A total of 100 spermatozoa were scored per slide using bright field illumination and an oil immersion objective with a total magnification of ×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 [13]. 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. Therefore, the ratio of the number of deformed sperm to the num-

ber of deformities in each sperm should not affect the final results of the SDI.

2.4 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, USA) and centrifuged at 500 × *g* for 20 min. The resulting 90 % pellet (mature spermatozoa) was aspirated, re-suspended in Biggers, Whitten-Whittingham media (BWW, Irvine Scientific, Santa Ana, USA) and the assessment of the sperm parameters including morphology was repeated. The mature sperm suspension was further subdivided into two aliquots and each aliquot was incubated with 5 mmol/L NADPH (Sigma, St Louis, USA) for 0 and 24 h respectively at 37 °C and 5 % CO_2. Each aliquot had its corresponding control without NADPH.

2.5 Measurement of ROS

ROS levels in all fractions were measured in 400 μL aliquots containing > 2 million sperm/mL using 4 μL of 25 mmol/L lucigenin (bis-N-methylacridnium nitrate, Sigma, St Louis, USA) at final concentration of 0.25 mmol/L. Negative controls were prepared by adding equal volume of lucigenin to 400 μL 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 $\times 10^6$ counted photons per min (cpm) per 20 million sperm.

2.6 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, USA) as established earlier [14]. Data acquisition was performed within 3 h on a flow cytometer equipped with 488 nm argon laser as a light source (Becton Dickinson FACScan, San Jose, USA). A minimum of 10 000 spermatozoa were examined for each assay at a flow rate of < 100 cells/second. Fluorescein isothiocyanate (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, USA).

2.7 Statistical analysis

Patient and donor groups were compared using the 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 (25th and 75th percentile). All hypothesis testing was 2-tailed, with a significance level of 0.05.

3 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 a 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- and 0-h 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 a 24-h incubation, regardless of NADPH exposure.

Similarly, the increase in DNA damage levels following incubation was calculated as the difference between 24 h and 0 h 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 h 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

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 (25th and 75th percentiles); $^{b}P < 0.05$ considered significant comparing patient to donor groups using the Mann–Whitney test.

Parameter	Patients (n = 7)	Donors (n = 6)	P-value
Sperm count ($\times 10^6$)	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[b]
SDI	2 (1.8, 2.1)	1.53 (1.52, 1.58)	0.008[b]

not exposed to NADPH (5.1 [3.87, 7.74] *vs.* 1.79 [2.87, 3.36], $P = 0.03$) (Figure 1).

Samples with an SDI score > 1.6 had higher increase in DNA damaged sperm compared to those with an 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 h 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.

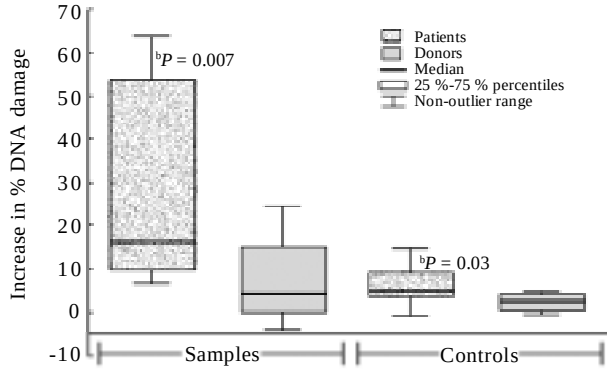

Figure 1. Increase in sperm DNA damage in samples (treated with NADPH) and controls (without NADPH) following incubation for 24 h in patients undergoing infertility screening and donors. Values represent median and interquartile (25 %, 75 % percentiles). $^{b}P < 0.05$ considered significant comparing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

4 Discussion

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 a reason for infertility. In addition, it reflects that the SDI may be capable of distinguishing semen samples with potentially impaired fertility.

Samples with an SDI higher than 1.6 were previously described to have decreased fertilizing potential [4]. This observation consistent with our current results, in which almost all patients undergoing infertility screening (6/7) had an 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 [15]. Based on results of our pilot study, we have selected to use exogenous NADPH in a concentration of 5 mmol/L as a model for increased ROS production by spermatozoa. Using this model, we were able to detect an 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 to 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 consistent with a previously published report that documents the presence of impaired DNA integrity in semen samples with abnormal sperm parameters in absence of leukocytospermia [16].

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 an increased SDI and cytoplasmic residues, which may result in increased ROS production [17]. 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 [18, 19]. 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 as well as the concern of transmission of genetic diseases through these techniques. The transmission of defective paternal DNA may increase the incidence of genomic imprinting errors leading to increased incidence of birth defects [20].

Unfortunately, the heterogeneity of sperm populations usually complicates proper DNA quality assessment. The choice of which assay to be used for the evaluation of the sperm chromatin status depends on many factors such as the expense, the available laboratory facilities, and the presence of experienced technicians. The correlation between the morphological pattern of spermatozoa and its DNA integrity in ejaculate may be an alternate strategy. Since the increase in DNA damage was more marked in samples with an SDI > 1.6, our preliminary findings suggest that samples with high SDI scores may be more likely to present with prevalent DNA fragmented sperm. However, our study has limitations due to small sample size and our findings 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.

References

1 Grow DR, Oehninger S, Seltman HJ, Toner JP, Swanson RJ, Kruger TF, *et al*. Sperm morphology as diagnosed by strict criteria: probing the impact of teratozoospermia on fertilization rate and pregnancy outcome in a large *in vitro* fertilization population. Fertil Steril 1994; 62: 559–67.

2 Menkveld R, Wong WY, Lombard CJ, Wetzels AM, Thomas CM, Merkus HM, *et al*. Semen parameters, including WHO and strict criteria morphology, in a fertile and subfertile population: an effort towards standardization of *in-vivo* thresholds. Hum Reprod 2001; 16: 1165–71.

3 Seibel MM, Zilberstein M. The shape of sperm morphology. Hum Reprod 1995; 10: 247–8.

4 Aziz N, Buchan I, Taylor C, Kingsland CR, Lewis-Jones I. The sperm deformity index: a reliable predictor of the outcome of oocyte fertilization *in vitro*. Fertil Steril 1996; 66: 1000–8.

5 Panidis D, Matalliotakis I, Papathanasiou K, Roussos C, Koumantakis E. The sperm deformity and the sperm multiple anomalies indexes in patients who underwent unilateral orchectomy and preventive radiotherapy. Eur J Obstet Gynecol Reprod Biol 1998; 80: 247–50.

6 Aitken J, Krausz C, Buckingham D. Relationships between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation, and the presence of leukocytes and precursor germ cells in human sperm suspensions. Mol Reprod Dev 1994; 39: 268–79.

7 Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, *et al*. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. Mol Reprod Dev 1997; 47: 468–82.

8 Aziz N, Saleh RA, Sharma RK, Lewis-Jones I, Esfandiari N, Thomas AJ Jr., *et al*. Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil Steril 2004; 81: 349–54.

9 Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, *et al*. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 1998; 59: 1037–46.

10 Teebor GW, Boorstein RJ, Cadet J. The repairability of oxidative free radical mediated damage to DNA: a review. Int J Radiat Biol 1988; 54: 131–50.

11 Saleh R, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. J Androl 2002; 23: 737–52.

12 Sharma RK, Said T, Agarwal A. Sperm DNA damage and its clinical relevance in assessing reproductive outcome. Asian J

Androl 2004; 6: 139-48.
13 Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Veeck LL, *et al*. New method of evaluating sperm morphology with predictive value for human *in vitro* fertilization. Urology 1987; 30: 248–51.
14 Gorczyca W, Gong J, Darzynkiewicz Z. Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res 1993; 53: 1945–51.
15 Fisher HM, Aitken RJ. Comparative analysis of the ability of precursor germ cells and epididymal spermatozoa to generate reactive oxygen metabolites. J Exp Zool 1997; 277: 390–400.
16 Erenpreiss J, Hlevicka S, Zalkalns J, Erenpreisa J. Effect of leukocytospermia on sperm DNA integrity: a negative effect in abnormal semen samples. J Androl 2002; 23: 717–23.
17 Ollero M, Gil-Guzman E, Lopez MC, Sharma RK, Agarwal A, Larson K, *et al*. Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. Hum Reprod 2001; 16: 1912–21.
18 Muratori M, Piomboni P, Baldi E, Filimberti E, Pecchioli P, Moretti E, *et al*. Functional and ultrastructural features of DNA-fragmented human sperm. J Androl 2000; 21: 903–12.
19 Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M, Sakkas D. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. Hum Reprod 2001; 16: 2160–5.
20 Marques CJ, Carvalho F, Sousa M, Barros A. Genomic imprinting in disruptive spermatogenesis. Lancet 2004; 363: 1700–2.