CHAPTER 2

Impact of Sperm Morphology on DNA Damage Caused by b-Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Induced Oxidative Stress

Said TM, Agarwal A, Sharma RK, Thomas AJ, Jr., Sikka SC

Fertil Steril, 2005, 83 (1), 95-103

ABSTRACT

Objective: It is not yet clear whether abnormal sperm morphology plays any role in oxidative stress (OS) induced sperm DNA damage. As immature sperm contain high β-nicotinamide adenine dinucleotide phosphate (NADPH) in cytoplasmic droplets, our aim was to investigate the role of NADPH-induced DNA damage of human spermatozoa.

Design: Prospective – controlled study.

Setting: Male infertility clinic, Glickman Urological Institute, Cleveland Clinic Foundation, Cleveland, Ohio.

Patients: Twenty-eight men undergoing infertility screening.

Intervention(s): Chemiluminescence assay and TUNEL assay-coupled flow cytometry after incubating mature and immature sperm separated by density gradient with 5 mM NADPH for 0, 3 and 24 hours.

Main Outcome Measure(s): ROS generation (10^6 counted photons per minute/10^6 sperm) and percentage of spermatozoa with fragmented DNA.

Results: Immature sperm from teratozoospermic semen samples were characterized by significant presence of cytoplasmic residues in the mid-piece when compared to mature normozoospermic samples. Increased ROS production was observed in spermatozoa rich in cytoplasmic residues that showed significant positive correlation with sperm DNA damage in a time dependent manner.

Conclusions: Our data support the role of NAPDH in ROS-mediated sperm DNA damage and suggest that abnormal sperm morphology combined with an elevated ROS production may serve as a useful indicator of potential damage to sperm DNA.

Key words: Immature spermatozoa with cytoplasmic droplets, sperm DNA damage, NADPH, oxidative stress, teratozoospermia.

INTRODUCTION

Mammalian spermatozoa generate a variety of reactive oxygen species (ROS), which when present in limited concentrations are thought to play an important physiological role during sperm capacitation (1-4). However, oxidative stress (OS) occurs if the generation of ROS by human spermatozoa overwhelms their limited antioxidant defenses leading to a wide range of pathologies that may affect the fertilizing ability and the genomic integrity of the spermatozoa (5, 6).

The ability of human spermatozoa to produce ROS inversely correlates with their maturational stage (7, 8). Since sperm maturation involves the remodeling of membrane components and a decrease in the docosahexaenoic acid during the process of spermiogenesis (9), failure of these changes to occur, would result in immature spermatozoa that exhibit cytoplasmic retentions rich in glucose-6-phosphate dehydrogenase (G6PD) enzyme (10-13). G6PD controls the rate of glucose flux and intracellular availability of NADPH through the hexose monophosphate shunt. This in turn is used as a source of electrons by spermatozoa to fuel the generation of ROS via a putative NADPH oxidase located in the sperm plasma membrane (12-14).

The primary product of the immature spermatozoa free radical generating system appears to be the superoxide anion ($O_2^{\bullet-}$), which secondarily dismutates to H_2O_2 through the catalytic action of superoxide dismutase (SOD) (15, 16). The ability to generate $O_2^{\bullet-}$ is linked to the presence of NADPH oxidase-like activity (7, 13, 14, 17, 18). The addition of the substrate NADPH to human spermatozoa has been shown to result in a dose dependent induction of ROS. However, since NADPH is highly membrane impermeable, higher concentrations in the millimolar range (superphysiological) are needed to raise its intracellular concentration to the point where significant ROS induction is triggered (14, 19).

Substantial evidence exists from a number of observational and interventional studies that OS stands as a major causative factor behind increased levels of sperm DNA damage (19-23). Although the characteristic tight packaging of the sperm DNA may offer some protection against oxidative insult (24), spermatozoa are particularly susceptible to OS-induced damage as their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes (25). In addition, deoxyribonucleic acid bases and phosphodiester backbones are susceptible to peroxidation (26), thus affecting sperm genomic integrity that may impair *in vivo* (27, 28) and *in vitro* (29-32) fecundity.

The sperm quality and its fertilizing ability correlate well with its DNA integrity (33-35). In men with teratozoospermia, the control of spermiogenesis is less efficient than that observed under normal conditions, resulting in the release of significantly higher numbers of immature spermatozoa with 'cytoplasmic retention'. This may lead to increased ROS production as

characterized by prevalence of DNA damage (8, 9). Although oxidative DNA damage resulting in strand breaks has been previously reported in human spermatozoa, the pathogenesis behind its occurrence and relationship to teratozoospermic samples is unknown.

The objective of our study was to investigate the impact of abnormal sperm morphology (i.e., sperm with cytoplasmic residues) on ROS production induced by exogenous NADPH and its correlation with sperm DNA damage.

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 (n = 28) undergoing infertility screening. To ensure the presence of sufficient spermatozoa for all our planned evaluations, samples with a sperm concentration < 20 X 10^6/mL and < 2.0 mL volume were excluded from our study.

Semen collection and assessment

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. For morphological evaluations, seminal smears were stained with Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL) and 200 spermatozoa per slide were assessed by a single person (TMS) according to World Health Organization (WHO) guidelines. Presence of cytoplasmic residues in the mid-piece was confirmed if it was greater than one third of sperm head area (36) (Figure 1). All specimens were examined for white blood cell (WBCs) contamination by using myeloperoxidase (Endtz) staining (37). Semen samples containing > 1 X 10^6 WBCs/mL were excluded to avoid potential non-spermatozoa ROS generation.

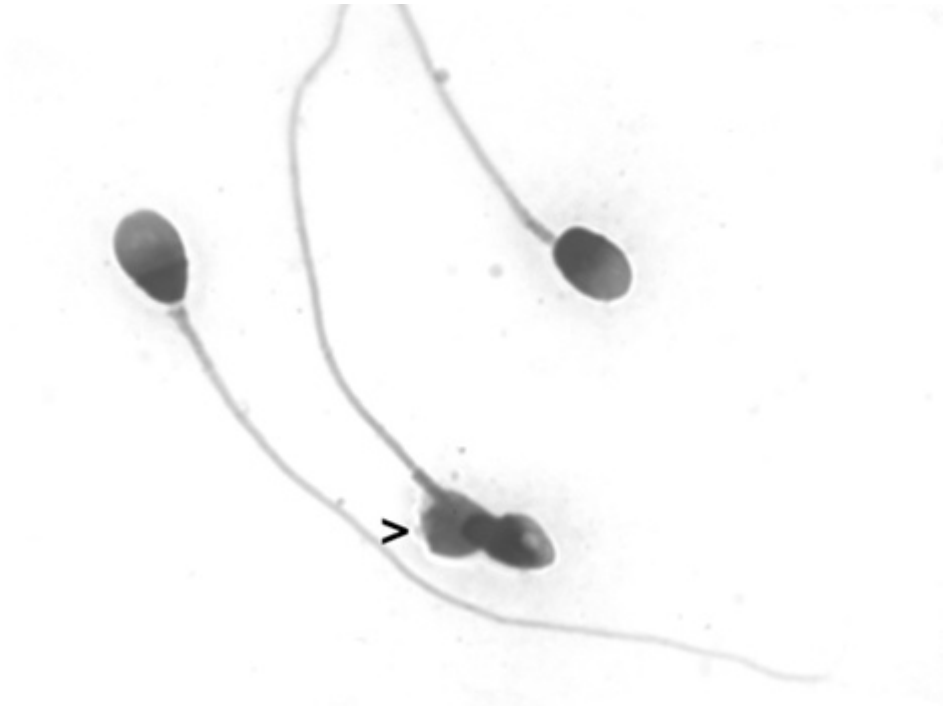

Figure 1. *Spermatozoa stained with Giemsa stain (X 160). Arrow points to cytoplasmic retention in the mid-piece.*

Sample preparation and induction of ROS by exogenous NADPH

Samples were categorized according to the percentage of morphologically normal spermatozoa into 2 separate groups. Samples with ≥ 30% normal forms (n = 13) were considered normozoospermic, while samples with < 30% normal forms; (n = 15) were classified as teratozoospermic (36). In order to separate spermatozoa into predominantly mature and predominantly immature populations, 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 (8). The resulting interface between the 47% and 90% layers (immature spermatozoa) and the 90% pellet (mature spermatozoa) were aspirated, and transferred to separate test tubes.

The pellets from both fractions were re-suspended in Biggers, Whitten-Whittingham media (BWW, Irvine Scientific, Santa Ana, CA) and the assessment of the sperm parameters including morphology was repeated. Both fractions were further subdivided into 3 aliquots and each aliquot was incubated with 5mM NADPH (Sigma, St Louis, MO) for 0, 3 and 24 hours respectively at 37°C and 5% CO_2. Each aliquot had its corresponding control without NADPH.

this dose of NADPH was selected based upon our preliminary dose response study and also recent published reports (14, 19).

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 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 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^{TM}, BD Biosciences, Mississauga, ON) as established earlier (38). Briefly, spermatozoa were washed twice in 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

Normozoospermic and teratozoospermic groups were compared using Mann Whitney test. Within-group differences between samples and controls were assessed using the Wilcoxon matched-pairs test. To evaluate the occurrence of statistically significant change over time in the various fractions, repeated measures ANOVA (Friedman test) was used. Correlation

between variables was assessed using non-parametric Spearman's (*r*). 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.

RESULTS

Sperm morphology

Table 1 demonstrates sperm parameters in various fractions. Significantly lower percentages of morphologically normal spermatozoa were detected in teratozoospermic samples. The difference was evident in neat samples as well as in the mature and immature sperm fractions (P = 0.02; P = 0.001; P = 0.02, respectively). In all the tested samples, immature sperm fractions had higher percentage of spermatozoa characterized with cytoplasmic residues in the mid-piece compared to the neat samples before separation [5.5 (3, 9) vs. 3 (2, 8); P = 0.03] and mature sperm fractions after separation [5.5 (3, 9) vs. 2 (1, 3.75); P = 0.008] (Figure 1). Teratozoospermic samples were characterized by the presence of higher percentage of cytoplasmic residues compared to normozoospermic samples in neat samples, mature and immature sperm fractions (P = 0.008; P = 0.01; P = 0.01, respectively).

ROS measurements

All the samples containing immature spermatozoa, which were incubated with NADPH, had higher ROS values compared to controls (no NADPH) after 3 hours [1.12 (0.75, 2.67) vs. 0.32 (0.19, 0.89), P = 0.008] and 24 hours [3.74 (1.77, 5.96) vs. 1.85 (1.39, 2.72); P = 0.001] respectively. In teratozoospermic samples, the increase in ROS levels occurred immediately (at 0 hour) in the immature fractions regardless of NADPH exposure. The levels of ROS measured after 3 hours of incubation was significantly higher than at 0 hour in samples exposed to NADPH (P < 0.01) as well as in controls (P < 0.05). In normozoospermic samples, immature spermatozoa also showed higher ROS levels after 24 hours compared to 0 hour in samples with (P < 0.001) or without (P = 0.01) NADPH. The only mature spermatozoal fraction to reveal higher ROS levels after 24 hours (P < 0.01) was the one separated from teratozoospermic samples and treated with exogenous NADPH (Figure 2). ROS levels measured in the immature fraction following 3 hours of incubation with NADPH correlated positively with the percentage of DNA damaged spermatozoa (r = 0.47, P = 0.01). After 24 hours the positive correlation was also seen in the immature spermatozoa not exposed to NADPH (r = 0.55, P = 0.002) and the mature fraction incubated with NADPH (r = 0.37, P = 0.04), while no correlation was observed at 0 hour. ROS levels measured in immature fractions also correlated positively with the percentage of spermatozoa exhibiting excessive residual cytoplasm (r = 0.5, P = 0.01).

Variables	Neat semen samples		*P* value*	Mature fraction		*P* value*	Immature fraction		*P* value*
	Normo-zoospermic (n = 13)	Terato-zoospermic (n = 15)		Normo-zoospermic (n = 13)	Terato-zoospermic (n = 15)		Normo-zoospermic (n = 13)	Terato-zoospermic (n = 15)	
Concentration (X 10^6/mL)	65.85 (42.3, 86.5)	53.5 (43.6, 57.75)	N.S	4.46 (2.98, 5.9)	3.45 (2.2, 3.8)	N.S	1.79 (1.4, 2.3)	2.68 (1.78, 5.59)	N.S
Motility (%)	69.35 (56.28, 84.38)	76.5 (64.5, 83.83)	N.S	91 (90.25, 92.15)	85 (78, 86)	N.S	58.4 (40.6, 68.63)	54 (32, 57)	N.S
Morphology (% normal)	30 (25, 32)	21 (18.5, 24.5)	0.02	38 (37, 40)	16 (13.5, 16.5)	0.001	17 (15, 19)	11 (7.5, 11.5)	0.02
Sperm with mid-piece cytoplasmic residues (%)	4 (3.25, 4.75)	8 (6.5, 9.5)	0.008	1 (0.25, 1.38)	4 (3, 4.5)	0.01	5 (4, 5.5)	11 (10.5, 19)	0.01

Table 1. *Sperm parameters of normozoospermic and teratozoospermic neat semen samples as well as post double density gradient centrifugation fractions. Results are expressed as median and interquartile range (25^{th}, 75^{th} percentiles).*P < 0.05 considered significant by Mann Whitney test comparing same fractions in normozoospermic and teratozoospermic groups.*

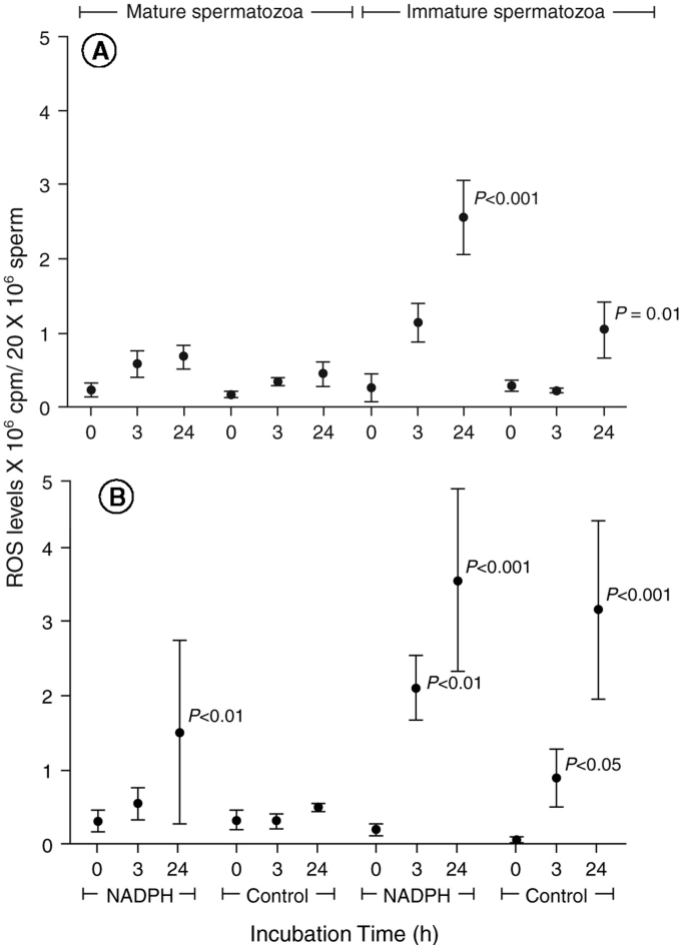

Figure 2. *Analysis of the impact of incubation on the levels of ROS measured in (A) normozoospermic and (B) teratozoospermic samples. All ROS values represent the median and interquartile ranges (25%, 75% percentiles). P < 0.05 considered significant compared to 0 hour value using non-parametric repeated measures ANOVA.*

DNA strand breaks

Mature and immature spermatozoa treated with exogenous NADPH in both groups exhibited higher frequency of DNA strand breaks compared to controls following 24 hours of incubation. On the other hand, higher levels of DNA damage were detected much earlier (at 3 hours) in the immature spermatozoa originating from the teratozoospermic group and treated with NADPH (Table 2). Within NADPH treated samples, DNA damage was more pronounced in immature fractions rather than the mature fractions in the normozoospermic group after 24 hours (P = 0.008) and in the teratozoospermic group after 3 hours (P = 0.08) and 24 hours (P = 0.03).

However, within the controls, DNA damage in immature fractions was more pronounced than mature fractions in the teratozoospermic group after 24 hours ($P = 0.009$).

In general, teratozoospermic samples contained higher percentages of spermatozoa with DNA damage compared to normozoospermic samples. The difference was significant in immature sperm fractions at 0 hour regardless of exogenous NADPH exposure. However, in the mature fractions, it was only detected following 24 hours incubation with NADPH. A high variation was seen in all fractions in the percentage of spermatozoa evaluated for TUNEL.

Teratozoospermic samples show higher levels of DNA strand breaks after 24 hours of incubation with NADPH when compared to levels obtained at 0 and 3 hours in the immature sperm fractions ($P < 0.001$; $P < 0.01$, respectively) and the mature sperm fractions ($P < 0.01$; $P < 0.05$, respectively) (Figure 3). In controls (not exposed to NADPH), the immature sperm fraction also revealed similar findings ($P < 0.01$; $P < 0.05$, respectively), while no significant change in levels of DNA damage was observed in the mature sperm fraction after 24 hours compared to their corresponding values obtained at 0 or 3 hours.

In normozoospermic samples a significant change following 24 hours of incubation was seen only in immature spermatozoa incubated with NADPH ($P < 0.001$; $P < 0.01$, respectively) (Figure 4). The percentage of spermatozoa exhibiting cytoplasmic residues seen in the immature fractions correlated positively with percentage of spermatozoa with DNA strand breaks ($r = 0.55$, $P = 0.002$).

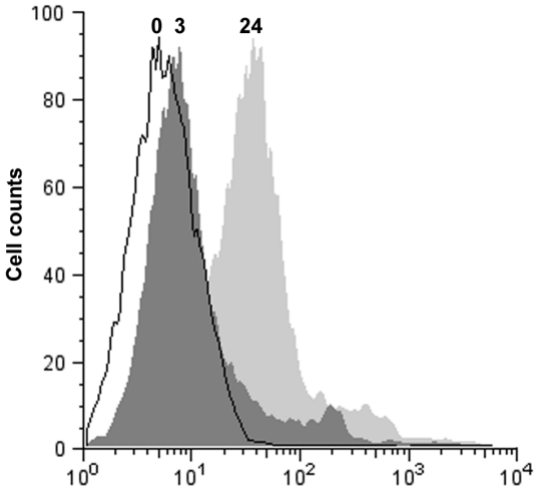

Figure 3. *Frequency distribution histograms of TUNEL positive immature spermatozoa obtained from teratozoospermic semen samples and incubated for 0, 3, and 24 hours with NADPH. The percentage of spermatozoa with DNA strand breaks increases after 24 hour as represented by the white histogram.*

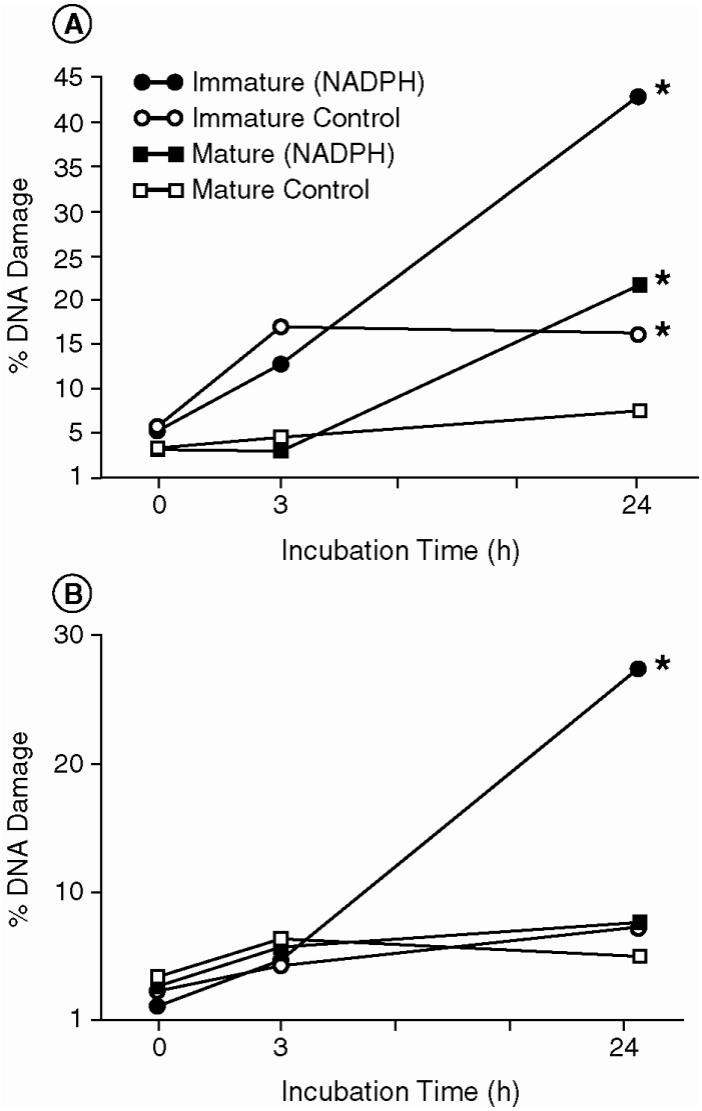

Figure 4. *Effect of NADPH treatment on progression of DNA damage in (A) teratozoospermic and (B) normozoospermic semen samples. Mature and immature sperm from both groups were incubated up to 24 hours in absence (control) and presence of 5mM NADPH (sample) and DNA damage evaluated as described in Methods. Asterisk * denotes comparison between 0 and 24 hour values by non-parametric repeated measures ANOVA, and considered significant at P < 0.05.*

Subjects	Incubation duration (hour)	Mature fraction		*P* value *	Immature fraction		*P* value †
		Sample (NADPH)	Control (No NADPH)		Sample (NADPH)	Control (No NADPH)	
Normozoo-spermic (n = 13)	**0**	3.06 (2.52, 3.95)	3.31 (1.26, 5.26)	N.S	1.12 (1, 3.98)	2.54 (1.54, 4.4)	N.S
	3	5.56 (2.26, 6.74)	6.25 (3.53, 8.58)	N.S	4.46 (2.56, 8.78)	4.11 (1.15, 4.65)	N.S
	24	7.09 (6.79, 14.8)	4.89 (2.13, 8.2)	0.005	27.2 (19.66, 34.11)	7.4 (3.33, 11.42)	0.002
Teratozoo-spermic (n = 15)	**0**	3.1 (1.57, 7.04)	2.88 (2.65, 3.93)	N.S	5.54 (4.02, 7.45)	5.5 (2.49, 7.86)	N.S
	3	2.89 (3.94, 10.64)	4.7 (3.8, 7.23)	N.S	13 (8.51, 16.8)	7 (5.13, 11.81)	0.01
	24	21.23 (19.17, 63.08)	7.23 (6.34, 9.06)	0.0004	42.9 (33.53, 54.24)	16 (10.4, 40)	0.004

Table 2: *Comparison of the percentage of DNA damage in mature and immature spermatozoa of normozoospermic and teratozoospermic semen samples incubated in absence or presence of NADPH (5mM) for various time intervals. Results are expressed as median (25^{th}, 75^{th} percentiles). *P< 0.05 considered significant by Wilcoxon matched pairs test comparing mature fraction samples to controls. †P< 0.05 considered significant by Wilcoxon matched pairs test comparing immature fraction samples to controls.*

DISCUSSION

Teratozoospermic semen samples are characterized by a higher content of morphologically abnormal and immature spermatozoa that retain cytoplasmic residues in the mid-piece. Such immature spermatozoa can be separated by double-density gradient procedures, (8, 9, 39, 40). The enzyme G6PD that is excessively present in sperm residual cytoplasm generates NADPH, which in turn stimulates ROS production (7, 10-12, 14, 19). ROS levels are expected to rise at a faster pace and in greater intensity in such sperm samples in the presence of cytoplasmic residues. We observed that ROS generating potential of NADPH is greatly enhanced in immature spermatozoa (Figure 2), which may be attributed to the abundance of G6PD-rich

cytoplasmic residues. NADPH may also have increased ROS production by increasing the permeability of the sperm plasma membrane.

Although the role of NADPH in causing oxidative insult to the sperm DNA has been reported (13, 19-21), it is not clear why spermatozoa isolated from samples with high prevalence of morphologically abnormal forms have higher incidence of DNA strand breaks as observed in this study. Such immature spermatozoa appear to be more capable of ROS production and more susceptible to oxidative DNA damage. These specific features were not encountered in mature spermatozoa (41-43). Therefore, role of putative NADPH oxidase in human spermatozoa remains controversial.

In our study, elevated ROS levels always precede significant fragmentation of the sperm DNA. These events may be attributed to the presence of increased NADPH oxidase activity presumably present in immature spermatozoa. Using our current model of ROS induction, it was clearly evident that exogenous addition of high amount of NADPH causes early DNA strand breaks in immature spermatozoa. Also, it affects the DNA integrity of mature spermatozoa in samples characterized by teratozoospermia although to a lesser degree in comparison to immature fractions (Figure 4). Thus, the presence of higher DNA damage in immature sperm fractions that exhibit higher rate of cytoplasmic residues may be attributed to high endogenous NADPH oxidase activity. This hypothesis may be further tested in the future by studying the effects of ROS scavengers such as superoxide dismutase and catalase in our ROS-DNA model.

In the present experiment we used exogenous NADPH in a super-physiological concentration (5mM) as a model for intracellular ROS production by spermatozoa. In published reports, the concentration of NADPH used to induce ROS varies widely (500μM – 50mM) (7, 13, 14, 19, 20, 44). The choice of optimum 5mM concentration was based on results of our pilot study, in which samples were exposed to various doses of NADPH. Significant changes were detected in basal $O2\bullet-$ levels and those following incubation with 5 mM NADPH [-26.3 (-52.7, -6.5); $P = 0.001$]. Similarly, levels of $O2\bullet-$ were higher than the basal values when the sperm were incubated with 10 mM NADPH, [-22.4 (-53.5, -8.8); $P = 0.002$]. Therefore, it appears that higher concentrations of NADPH do not significantly increase the amount of ROS produced by the spermatozoa. A limitation in our study was the lack of multiple assessments between 3 and 24 hours. The number of incubations had to be limited by the number of spermatozoa available to conduct all relevant assays. Another limiting factor was the sample size. We did not encounter many teratozoospermic cases (46%).

The duration of incubation in vitro appears to play a major role in triggering sperm DNA damage. Under physiological conditions a protective antioxidant environment exist in the epididymis, which is the main storage site for spermatozoa (45). Spermatozoa stored outside

the epididymis are more likely to possess DNA and chromatin abnormalities (46). The prolonged incubation of isolated mature spermatozoa per se up to 24 hours does not seem to affect their DNA integrity, while the incubation of immature spermatozoa leads to significant deleterious consequences due to oxidative stress.

Excessive ROS production by immature, morphologically abnormal spermatozoa with cytoplasmic residues such as those encountered in teratozoospermic samples may induce oxidative damage of mature spermatozoa during sperm migration from the seminiferous tubules to the epididymis and may be an important cause of male infertility (6). This may also explain why prolonged storage of heterogonous sperm populations in the reproductive tract diminishes their fertilization capacity. This has important clinical implications in the preparation and use of mature spermatozoa in assisted reproductive techniques.

In conclusion, we emphasize that morphologically abnormal and immature spermatozoa appear to be more susceptible to NADPH induced oxidative damage to ejaculated spermatozoa in a time dependent manner. Therefore, if prolonged incubation is mandated during the course of ART, care should be taken to isolate mature spermatozoa as purely as possible to avoid any contamination with ROS producing immature spermatozoa. In addition, a careful assessment of sperm morphology and an evaluation of increased ROS production may indicate extent of potential damage to sperm DNA integrity and serve as an index of poor sperm function.

REFERENCES

1. Oehninger S, Blackmore P, Mahony M, Hodgen G. Effects of hydrogen peroxide on human spermatozoa. J Assist Reprod Genet 1995;12:41-47.
2. de Lamirande E, Harakat A, Gagnon C. Human sperm capacitation induced by biological fluids and progesterone, but not by NADH or NADPH, is associated with the production of superoxide anion. J Androl 1997;19:215-225.
3. Rivlin J, Mendel J, Rubinstein S, Etkovitz N, Breitbart H. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. Biol Reprod 2003;70:518-522.
4. Thundathil J, de Lamirande E, Gagnon C. Nitric oxide regulates the phosphorylation of the threonine-glutamine-tyrosine motif in proteins of human spermatozoa during capacitation. Biol Reprod 2003;68:1291-1298.
5. Sharma R, Pasqualotto F, Nelson D, Thomas AJ, Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 1999;14:2801-2807.
6. Sikka S. Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. J Androl 2004;25:5-18.
7. Fisher H, Aitken R. Comparative analysis of the ability of precursor germ cells and epididymal spermatozoa to generate reactive oxygen metabolites. J Exp Zool 1997;277:390-400.
8. Gil-Guzman E, Ollero M, Lopez M, Sharma R, Alvarez J, Thomas AJ et al. Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod 2001;16:1922-1930.
9. Ollero M, Gil-Guzman E, Lopez M, Sharma R, 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;16:1912-1921.
10. 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;39:268-279.
11. Huszar G, Vigue L. Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. J Androl 1994;15:71-77.
12. Gomez E, Buckingham D, Brindle J, Lanzafame F, Irvine D, Aitken R. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J Androl 1996;17:276-287.
13. Twigg J, Fulton N, Gomez E, Irvine D, Aitken R. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. Hum Reprod 1998;13:1429-1436.
14. Aitken R, Fisher H, 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;47:468-482.
15. Alvarez J, Touchstone J, Blasco L, Storey B. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. J Androl 1987;8:338-348.

16. Aitken R, Ryan A, Curry B, Baker M. Multiple forms of redox activity in populations of human spermatozoa. Mol Hum Reprod 2003;9:645-661.

17. Vernet P, Fulton N, Wallace C, Aitken R. Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. Biol Reprod 2001;65:1102-1113.

18. Lewis B, Aitken R. A redox-regulated tyrosine phosphorylation cascade in rat spermatozoa. J Androl 2001;22:611-622.

19. Aitken R, Gordon E, Harkiss D, Twigg J, Milne P, Jennings Z et al. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 1998;59:1037-1046.

20. Twigg J, Irvine D, Aitken J. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Hum Reprod 1998;13:1864-1871.

21. Barroso G, Morshedi M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum Reprod 2000;15:1338-1344.

22. Kemal Duru N, Morshedi M, Oehninger S. Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertil Steril 2000;74:1200-1207.

23. Agarwal A, Said T. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331-345.

24. Fuentes-Mascorro G, Serrano H, Rosado A. Sperm chromatin. Arch Androl 2000;45:215-225.

25. Saleh R, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. J Androl 2002;23:737-752.

26. Teebor G, Boorstein R, Cadet J. The repairability of oxidative free radical mediated damage to DNA: a review. Int J Radiat Biol 1988;54:131-150.

27. Shen H, Chia S, Ong C. Evaluation of oxidative DNA damage in human sperm and its association with male infertility. J Androl 1999;20:718-723.

28. Zini A, Fischer M, Sharir S, Shayegan B, Phang D, Jarvi K. Prevalence of abnormal sperm DNA denaturation in fertile and infertile men. Urology 2002;60:1069-1072.

29. Sun J, Jurisicova A, Casper R. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod 1997;56:602-607.

30. Tomsu M, Sharma V, Miller D. Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. Hum Reprod 2002;17:1856-1862.

31. Duran E, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. Hum Reprod 2002;17:3122-3128.

32. Larson-Cook K, Brannian J, Hansen K, Kasperson K, Aamold E, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertil Steril 2003;80:895-902.

33. Zini A, Bielecki R, Phang D, Zenzes M. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. Fertil Steril 2001;75:674-677.

34. Tomlinson M, Moffatt O, Manicardi G, 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;16:2160-2165.

35. 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;23:717-723.

36. World Health Organization Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 4th ed. Cambridge University Press, Cambridge, New York, 1999.

37. Endtz A. A rapid staining method for differentiating granulocytes from "germinal cells" in Papanicolaou-stained semen. Acta Cytol. 1974;18:2-7.

38. 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;53:1945-1951.

39. Hall J, Fishel S, Timson J, Dowell K, Klentzeris L. Human sperm morphology evaluation pre- and post-Percoll gradient centrifugation. Hum Reprod 1995;10:342-346.

40. Ollero M, Powers R, Alvarez J. Variation of docosahexaenoic acid content in subsets of human spermatozoa at different stages of maturation: implications for sperm lipoperoxidative damage. Mol Reprod Dev 2000;55:326-334.

41. Armstrong J, Bivalacqua T, Chamulitrat W, Sikka S, Hellstrom W. A comparison of the NADPH oxidase in human sperm and white blood cells. Int J Androl 2002;25:223-229.

42. de Lamirande E, Harakat A, Gagnon C. Human sperm capacitation induced by biological fluids and progesterone, but not by NADH or NADPH, is associated with the production of superoxide anion. J Androl 1998;19:215-25.

43. Richer S, Ford W. A critical investigation of NADPH oxidase activity in human spermatozoa. Mol Hum Reprod 2001;7:237-244.

44. Chen H, Cheung M, Chow P, Cheung A, Liu W, O W. Protection of sperm DNA against oxidative stress in vivo by accessory sex gland secretions in male hamsters. Reproduction 2002;124:491-499.

45. Potts R, Jefferies T, Notarianni L. Antioxidant capacity of the epididymis. Hum Reprod 1999;14:2513-2516.

46. Potts R, Notarianni L, Jefferies T. Extra-epididymal spermatozoa express nuclear abnormalities. Int J Androl 1999;22:282-288.