

Impact of sperm morphology on DNA damage caused by oxidative stress induced by β -nicotinamide adenine dinucleotide phosphate

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Objective: To investigate the role of DNA damage induced by β -nicotinamide adenine dinucleotide phosphate (NADPH) in human spermatozoa.

Design: Prospective controlled study.

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

Patient(s): Twenty-eight men undergoing infertility screening.

Intervention(s): Chemiluminescence assay and terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling (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): Reactive oxygen species (ROS) generation (10^6 counted photons per minute/ 10^6 sperm) and percentage of spermatozoa with fragmented DNA.

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

Conclusion(s): Immature sperm contain high nicotinamide adenine dinucleotide phosphate (NADPH) in cytoplasmic droplets, but it has not yet been clear whether abnormal sperm morphology plays any role in sperm DNA damage induced by oxidative stress. Our data support the role of NADPH in ROS-mediated sperm DNA damage and suggest that abnormal sperm morphology combined with elevated ROS production may serve as a useful indicator of potential damage to sperm DNA. (Fertil Steril® 2005;83:95–103. ©2005 by American Society for Reproductive Medicine.)

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

Mammalian spermatozoa generate a variety of reactive oxygen species (ROS), which, when present in limited concentrations, are thought to play an important physiologic role during sperm capacitation (1–4). However, oxidative stress 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). Because sperm maturation involves the remodeling of mem-

brane 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). This enzyme, G6PD, controls the rate of glucose flux and intracellular availability of β -nicotinamide adenine dinucleotide phosphate (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 system of generating free radicals appears to be the superoxide anion ($O_2^{\cdot-}$), which secondarily dismutates to H_2O_2 through the catalytic action of superoxide dismutase (SOD) (15, 16). The ability to generate $O_2^{\cdot-}$ 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

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been shown to result in a dose-dependent induction of ROS. However, because NADPH is highly membrane impermeable, higher concentrations in the millimolar range (supraphysiologic) are needed to raise its intracellular concentration to the point where substantial ROS induction is triggered (14, 19).

Substantial evidence exists from a number of observational and interventional studies that oxidative stress 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 damage induced by oxidative stress 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

Patient 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 $<20 \times 10^6/\text{mL}$ 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, $5 \mu\text{L}$ of each specimen was loaded on a 20-micron Microcell chamber (Conception Technologies, San Diego, CA) and analyzed for sperm concentration and motility. For morphologic evaluations, seminal smears were stained with Giemsa stain (Diff-Quik; Baxter Scientific Products, McGaw

FIGURE 1

Spermatozoa stained with Giemsa stain (magnification $\times 160$). Arrow points to cytoplasmic retention in the mid-piece.



Said. ROS-induced sperm DNA damage. *Fertil Steril* 2005.

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) (Fig. 1). All specimens were examined for white blood cell (WBC) contamination by using myeloperoxidase (Endtz) staining (37). Semen samples containing $>1 \times 10^6$ WBCs/mL were excluded to avoid potential ROS generation by anything but spermatozoa.

Sample Preparation and Induction of ROS by Exogenous NADPH

Samples were categorized according to the percentage of morphologically normal spermatozoa into two separate groups. Samples with $\geq 30\%$ normal forms ($n = 13$) were considered normozoospermic, and samples with $<30\%$ normal forms; ($n = 15$) were classified as teratozoospermic (36). 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 $500 \times g$ 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 resuspended in Biggers, Whitten-Whittingham media (Irvine Scientific, Santa Ana, CA), and the assessment of the sperm parameters including morphology was repeated. Both fractions were further subdivided into three aliquots, and each aliquot was incubated with 5 mM of NADPH (Sigma, St Louis, MO) for 0, 3 and 24 hours respectively at 37°C and $5\% \text{CO}_2$. Each aliquot had its corresponding control without NADPH. This

dose of NADPH was selected based on our preliminary dose-response study and on published reports (14, 19).

Measurement of ROS

The ROS levels in all fractions were measured in 400- μ L aliquots containing >2 million sperm/mL using 4 μ L of 25-mM Lucigenin (bis-N-methylacridinium nitrate; Sigma) at a final concentration of 0.25 mM. Negative controls were prepared by adding an equal volume of Lucigenin to 400 μ L of phosphate-buffered saline (PBS). The ROS levels were determined by chemiluminescence assay using a luminometer (model LKB 953; Berthold Technologies, Bad-Wilbad, Germany) for 15 minutes, and were expressed as $\times 10^6$ 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 (38). Briefly, spermatozoa were washed twice in PBS, resuspended in 1% paraformaldehyde at a concentration of $1-2 \times 10^6$ sperm/mL and placed on ice for 30 to 60 minutes. These spermatozoa were again washed and resuspended in 70% ice-cold ethanol by centrifugation at $300 \times g$ for 5 minutes as per the kit instructions. The ethanol supernatant was removed and the cell pellets were washed twice in wash buffer and resuspended in 50 μ L of the staining solution for 60 minutes 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 a 488-nm argon laser as a light source (FACScan; Becton Dickinson, San Jose, CA). A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/second. The 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 the 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 analysis of variance (ANOVA), the Friedman test, was used. Correlation between variables was assessed using nonparametric Spearman's (r). Summary sta-

tistics are presented as median and interquartiles (25th and 75th percentile). All hypothesis testing was two-tailed, and $P < .05$ was considered statistically significant.

RESULTS

Sperm Morphology

Table 1 demonstrates sperm parameters in various fractions. Statistically significantly lower percentages of morphologically normal spermatozoa were detected in the teratozoospermic samples. The difference was evident in neat samples as well as in the mature and immature sperm fractions ($P = .02$, $P = .001$, $P = .02$, respectively). In all the tested samples, immature sperm fractions had higher percentage of spermatozoa characterized with cytoplasmic residues in the mid-piece compared with the neat samples before separation [5.5 (3, 9) vs. 3 (2, 8); $P = .03$] and mature sperm fractions after separation [5.5 (3, 9) vs. 2 (1, 3.75); $P = .008$] (see Fig. 1). Teratozoospermic samples were characterized by the presence of a higher percentage of cytoplasmic residues compared with normozoospermic samples in neat samples and in mature and immature sperm fractions ($P = .008$, $P = .01$, $P = .01$, respectively).

ROS Measurements

All the samples containing immature spermatozoa, which were incubated with NADPH, had higher ROS values compared with controls (no NADPH) after 3 hours (1.12 [0.75, 2.67] vs. 0.32 [0.19, 0.89], $P = .008$) and 24 hours (3.74 [1.77, 5.96] vs. 1.85 [1.39, 2.72]; $P = .001$), 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 were statistically significantly higher than at 0 hour in samples exposed to NADPH ($P < .01$) as well as in controls ($P < .05$). In normozoospermic samples, immature spermatozoa also showed higher ROS levels after 24 hours compared with 0 hour in samples with ($P < .001$) or without ($P = .01$) NADPH. The only mature spermatozoal fraction to reveal higher ROS levels after 24 hours ($P < .01$) was the one separated from teratozoospermic samples and treated with exogenous NADPH (Fig. 2).

The ROS levels measured in the immature fraction following 3 hours of incubation with NADPH correlated positively with the percentage of spermatozoa with DNA damage ($r = 0.47$, $P = .01$). After 24 hours, the positive correlation was also seen in the immature spermatozoa not exposed to NADPH ($r = 0.55$, $P = .002$) and the mature fraction incubated with NADPH ($r = 0.37$, $P = .04$), but no correlation was observed at 0 hour. The ROS levels measured in immature fractions also correlated positively with the percentage of spermatozoa exhibiting excessive residual cytoplasm ($r = 0.5$, $P = .01$).

TABLE 1

Sperm parameters of normozoospermic and teratozoospermic neat semen samples as well as after double density gradient centrifugation fractions.

Variables	Neat semen samples			Mature fraction			Immature fraction		
	Normozoo- spermic (n = 13)	Teratozoo- spermic (n = 15)	<i>P</i> value ^a	Normozoo- spermic (n = 13)	Teratozoo- spermic (n = 15)	<i>P</i> value ^a	Normozoo- spermic (n = 13)	Teratozoo- spermic (n = 15)	<i>P</i> value ^a
Concentration (× 10 ⁶ /mL)	65.85 (42.3, 86.5)	53.5 (43.6, 57.75)	NS	4.46 (2.98, 5.9)	3.45 (2.2, 3.8)	NS	1.79 (1.4, 2.3)	2.68 (1.78, 5.59)	NS
Motility (%)	69.35 (56.28, 84.38)	76.5 (64.5, 83.83)	NS	91 (90.25, 92.15)	85 (78, 86)	NS	58.4 (40.6, 68.63)	54 (32, 57)	NS
Morphology (% normal)	30 (25, 32)	21 (18.5, 24.5)	.02	38 (37, 40)	16 (13.5, 16.5)	.001	17 (15, 19)	11 (7.5, 11.5)	.02
Sperm with mid-piece cytoplasmic residues (%)	4 (3.25, 4.75)	8 (6.5, 9.5)	.008	1 (0.25, 1.38)	4 (3, 4.5)	.01	5 (4, 5.5)	11 (10.5, 19)	.01

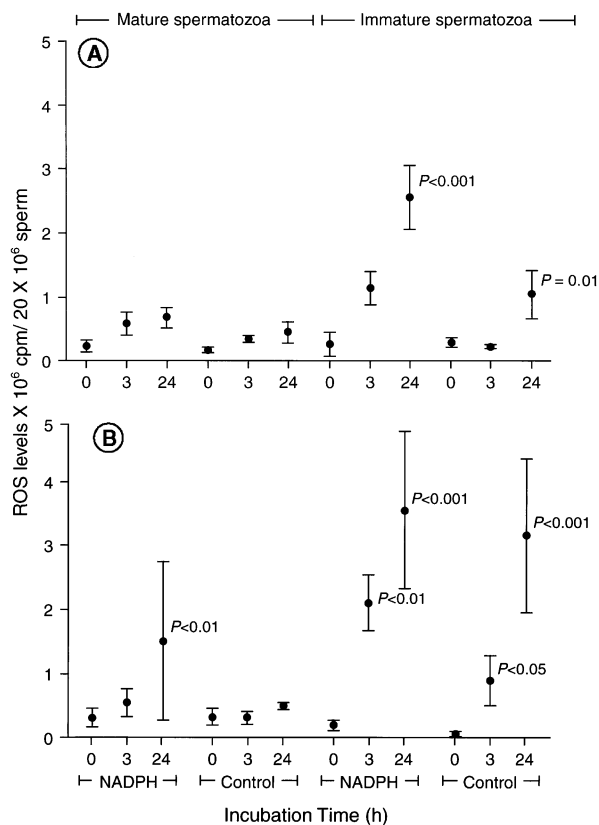
Note: Results are expressed as median and interquartile range (25th, 75th percentiles). NS = Not statistically significant.

^a*P* < .05 considered statistically significant by Mann-Whitney test comparing same fractions in normozoospermic and teratozoospermic groups.

Said. ROS-induced sperm DNA damage. Fertil Steril 2005.

FIGURE 2

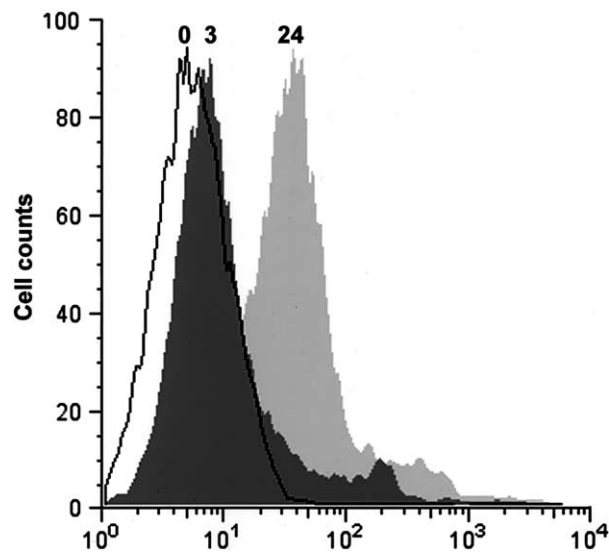
Analysis of the impact of incubation on the levels of ROS measured in (A) normozoospermic and (B) teratozoospermic samples. All ROS values represent the median and interquartile ranges (25%, 75% percentiles). $P < .05$ was considered statistically significant compared with 0 hour value using nonparametric repeated measures ANOVA.



Said. ROS-induced sperm DNA damage. *Fertil Steril* 2005.

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 increased after 24 hours, as represented by the light grey histogram.



Said. ROS-induced sperm DNA damage. *Fertil Steril* 2005.

In general, teratozoospermic samples contained higher percentages of spermatozoa with DNA damage compared with normozoospermic samples. The difference was statistically 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 of 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 with levels obtained at 0 and 3 hours in the immature sperm fractions ($P < .001$ and $P < .01$, respectively) and the mature sperm fractions ($P < .01$ and $P < .05$, respectively) (Fig. 3). In controls (not exposed to NADPH), the immature sperm fraction also revealed similar findings ($P < .01$ and $P < .05$, respectively), but no statistically significant change in levels of DNA damage was observed in the mature sperm fraction after 24 hours compared with their corresponding values obtained at 0 or 3 hours.

In normozoospermic samples a statistically significant change following 24 hours of incubation was seen only in immature spermatozoa incubated with NADPH ($P < .001$ and $P < .01$, respectively) (Fig. 4). The percentage of spermatozoa exhibiting cytoplasmic residues seen in the imma-

DNA Strand Breaks

Mature and immature spermatozoa treated with exogenous NADPH in both groups exhibited a higher frequency of DNA strand breaks compared with 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 mature fractions in the normozoospermic group after 24 hours ($P = .008$) and in the teratozoospermic group after 3 hours ($P = .08$) and 24 hours ($P = .03$). However, within the controls, DNA damage in immature fractions was more pronounced than mature fractions in the teratozoospermic group after 24 hours ($P = .009$).

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 (5 mM) for various time intervals.

Patients	Incubation duration (hour)	Mature fraction			Immature fraction		
		Sample (NADPH)	Control (No NADPH)	<i>P</i> value ^a	Sample (NADPH)	Control (No NADPH)	<i>P</i> value ^b
Normozoospermic (n = 13)	0	3.06 (2.52, 3.95)	3.31 (1.26, 5.26)	NS	1.12 (1.0, 3.98)	2.54 (1.54, 4.4)	NS
	3	5.56 (2.26, 6.74)	6.25 (3.53, 8.58)	NS	4.46 (2.56, 8.78)	4.11 (1.15, 4.65)	NS
	24	7.09 (6.79, 14.8)	4.89 (2.13, 8.2)	.005	27.2 (19.66, 34.11)	7.4 (3.33, 11.42)	.002
Teratozoospermic (n = 15)	0	3.1 (1.57, 7.04)	2.88 (2.65, 3.93)	NS	5.54 (4.02, 7.45)	5.5 (2.49, 7.86)	NS
	3	2.89 (3.94, 10.64)	4.7 (3.8, 7.23)	NS	13 (8.51, 16.8)	7 (5.13, 11.81)	.01
	24	21.23 (19.17, 63.08)	7.23 (6.34, 9.06)	.0004	42.9 (33.53, 54.24)	16 (10.4, 40)	.004

Note: Results are expressed as median (25th, 75th percentiles). NS = Not statistically significant.

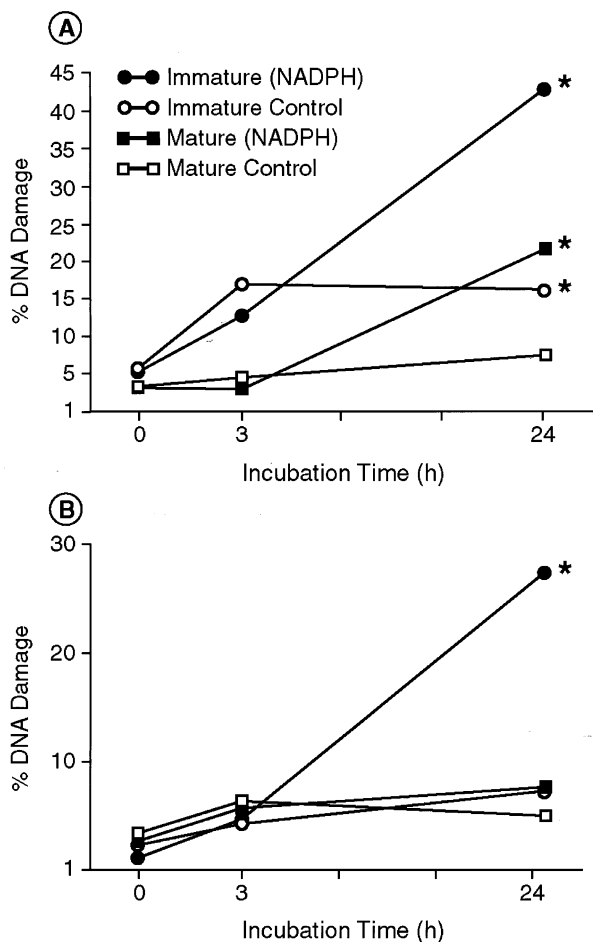
^a*P* < .05 considered statistically significant by Wilcoxon matched pairs test comparing mature fraction samples with controls.

^b*P* < .05 considered statistically significant by Wilcoxon matched pairs test comparing immature fraction samples with controls.

Said. ROS-induced sperm DNA damage. *Fertil Steril* 2005.

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 the absence (control) and presence of 5 mM of NADPH (sample), and the DNA damage was evaluated. *Comparison between 0-hour and 24-hour values by nonparametric repeated measures ANOVA; $P < .05$ was considered statistically significant.



Said. ROS-induced sperm DNA damage. *Fertil Steril* 2005.

ture fractions correlated positively with percentage of spermatozoa with DNA strand breaks ($r = .55$, $P = .002$).

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, which is excessively present in sperm residual

cytoplasm, generates NADPH, which in turn stimulates ROS production (7, 10–12, 14, 19). The 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 (see Fig. 2), which may be attributed to the abundance of G6PD-rich cytoplasmic residues. The ROS production may also have been increased by NADPH via 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 a high prevalence of morphologically abnormal forms have a 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 substantial fragmentation of the sperm DNA. These events may be attributed to the presence of the 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 with immature fractions (see Fig. 4). Thus, the presence of higher DNA damage in immature sperm fractions that exhibit a 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 supraphysiologic concentration (5 mM) as a model for intracellular ROS production by spermatozoa. In published reports, the concentration of NADPH used to induce ROS varies widely (500 μ M to 50 mM) (7, 13, 14, 19, 20, 44). The choice of optimum 5-mM concentration was based on results of our pilot study, in which samples were exposed to various doses of NADPH. Statistically significant changes were detected in basal $O_2^{\cdot -}$ levels and in those samples following incubation with 5 mM of NADPH (-26.3 [-52.7 , -6.5]; $P = .001$).

Similarly, levels of $O_2^{\cdot -}$ were higher than the basal values when the sperm were incubated with 10 mM of NADPH, (-22.4 [-53.5 , -8.8]; $P = .002$). Therefore, it appears that higher concentrations of NADPH do not increase the amount of ROS produced by the spermatozoa in a statistically significant manner. 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 physiologic conditions, a protective antioxidant environment exists 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 for up to 24 hours does not seem to affect their DNA integrity, but the incubation of immature spermatozoa leads to 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 heterogeneous 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 assisted reproduction, 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.

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