

Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility

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BACKGROUND: The aim of this study was to examine the role of apoptosis and reactive oxygen species (ROS) in inducing DNA damage in ejaculated spermatozoa. **METHODS:** We examined ejaculated spermatozoa from 31 patients examined for infertility and 19 healthy donors for apoptosis, production of ROS and DNA damage using annexin V binding, chemiluminescence assay and sperm chromatin structure assay. **RESULTS:** The percentage of spermatozoa that underwent apoptosis in the whole ejaculate and mature fraction was higher in the patients than in the donors ($P < 0.001$ and $P = 0.009$, respectively). Levels of ROS in the whole ejaculate and immature fraction were higher in the patients than in the donors ($P = 0.002$ and $P = 0.009$). Apoptosis was significantly correlated with ROS within patients in the whole ejaculate [r (95% confidence interval) = 0.53 (0.19–0.86)] and in the mature [0.71 (0.39–1.00)] and immature spermatozoa [0.75 (0.45–1.00)]. Only apoptosis and the DNA fragmentation index (DFI) were significantly correlated within patients in the whole ejaculate [0.57 (0.18–0.97)]. **CONCLUSIONS:** DNA damage may be induced by oxidative assault. Apoptosis may not contribute significantly to the DNA damage.

Key words: apoptosis/DNA damage/reactive oxygen species/spermatozoa

Introduction

Intact human sperm DNA is an essential prerequisite for successful fertilization and embryo development. Spermatogenesis is a complex process, which involves a series of meioses and mitosis, changes in cytoplasmic architecture, replacement of somatic cell histones with transition proteins, and the final addition of protamines (Goldberg *et al.*, 1977; Poccia, 1986; Nonchev and Tsansev, 1990; Balhorn *et al.*, 1999). Ejaculated spermatozoa comprise a heterogeneous population of cellular components such as immature germ cells, round cells such as spermatids, leukocytes, and immature morphologically abnormal and normal spermatozoa.

Alterations in the spermatogenic events result in the release of immature, abnormal spermatozoa in the ejaculate. Immature spermatozoa display a high content of DNA damage, alterations in protamination, chromatin packaging and excessive reactive oxygen species (ROS) production (Balhorn *et al.*, 1988; Gorczyca *et al.*, 1993a,b; Sailer *et al.*, 1995; Bianchi *et al.*, 1996; de Yebra *et al.*, 1998; Manicardi *et al.*, 1998; Evenson *et al.*, 2000). Spermatozoa from infertile men have been shown to contain various nuclear alterations, including

abnormal chromatin structure, microdeletions, chromosomal rearrangements, aneuploidy and DNA strand breaks (Gerardo *et al.*, 2000).

Although the extent of DNA damage is closely related to sperm function and male infertility (Aitken, 1999; Sakkas *et al.*, 1999), the origin of such damage is still largely controversial. Apoptosis is a mode of cellular death based on a genetic mechanism that induces a series of cellular morphological and biochemical alterations leading the cell to suicide (Kerr *et al.*, 1972; Nagata, 1997). There are a number of indications that apoptosis occurs during spermatogenesis in humans (Gorczyca *et al.*, 1993a,b; Manicardi *et al.*, 1995; Baccetti *et al.*, 1996; Hadziselimovic *et al.*, 1997; Sun *et al.*, 1997; Lopes *et al.*, 1998; Manicardi *et al.*, 1998; Sakkas *et al.*, 1999; Barroso *et al.*, 2000; Irvine *et al.*, 2000). Recently, attention has focused on the role of apoptosis in ejaculated sperm (Koopman *et al.*, 1994; Glander and Schaller, 1999; Barroso *et al.*, 2000; Oosterhuis *et al.*, 2000; Duru *et al.*, 2001a,b; Schuffner *et al.*, 2001, 2002; Shen *et al.*, 2002). Whether defective apoptosis accounts for a significant proportion of DNA damage seen in the spermatozoa of infertile men is still an open question. The

observation that mature ejaculated spermatozoa are positive for the TUNEL assay has led to the theory that apoptosis is occurring (Gorczyca *et al.*, 1993a,b; Baccetti *et al.*, 1996; Sun *et al.*, 1997; Lopes *et al.*, 1998; Manicardi *et al.*, 1998; Tesarik *et al.*, 1998).

Another mechanism that has been studied extensively is oxidative stress, which is caused by the overproduction of ROS (Sharma and Agarwal, 1996; Aitken and Krausz, 2001; Sikka, 2001; Agarwal and Saleh, 2002; Saleh and Agarwal, 2002; Saleh *et al.*, 2002a,b; Agarwal *et al.*, 2003). ROS cause lipid peroxidation of sperm plasma membranes, resulting in alteration of sperm function and fertilizing capacity (Duru *et al.*, 2000). They are also known to affect the sperm genome, causing high frequencies of single- and double-strand DNA breaks (Twigg *et al.*, 1998). Both superoxide (O_2^-) and the hydroxyl radical ($OH\cdot$) are known to be mutagenic and cause chromosome deletions, dicentric and sister chromatid exchanges (Twigg *et al.*, 1998; Aitken and Krausz, 2001). These abnormalities in chromatin packaging and nuclear DNA damage appear to be linked, and there is a strong association between the presence of nuclear DNA damage in the mature spermatozoa of men and poor semen parameters (Lopes *et al.*, 1998; Irvine *et al.*, 2000).

Studies have shown DNA fragmentation in ejaculated spermatozoa (Aitken *et al.*, 1998; Irvine *et al.*, 2000; Zini *et al.*, 2001; Saleh *et al.*, 2002a). DNA damage has been linked to poor pregnancy outcome (Sun *et al.*, 1997; Lopes *et al.*, 1998; Evenson *et al.*, 1999, 2000; Host *et al.*, 2000a,b; Larson *et al.*, 2000; Scholl and Stein, 2001; Duran *et al.*, 2002; Benchaib *et al.*, 2003; Saleh *et al.*, 2003). Three hypotheses have been postulated to explain the source of DNA damage in sperm. First, it is believed that DNA damage is caused by improper packaging and ligation during sperm maturation (McPherson and Longo, 1992, 1993a,b; Gorczyca *et al.*, 1993a,b; Sailer *et al.*, 1995). Secondly, oxidative stress causes DNA damage (Agarwal and Saleh, 2002; Saleh *et al.*, 2002a,b; Agarwal *et al.*, 2003), and the increased levels of specific forms of oxidative damage such as 8-hydroxydeoxyguanosine in sperm DNA supports such a theory (Lopes *et al.*, 1998; Aitken, 1999; Shen and Ong, 2000). Thirdly, observed DNA fragmentation is caused by apoptosis (Sakkas *et al.*, 1999, 2002).

The objectives of our study were (i) to assess the role of apoptosis in the pathogenesis of DNA damage in ejaculated spermatozoa from patients examined for infertility; and (ii) to assess the correlation of apoptosis with conventional semen parameters (sperm concentration, motility and morphology) and ROS levels in sperm from patients examined for infertility.

Materials and methods

Sample collection and semen analysis

This study was approved by the Institutional Review Board of the Cleveland Clinic Foundation. The study included a randomly selected group of patients ($n = 31$) attending the male infertility clinic with a history of infertility of at least 1 year's duration. Semen samples were obtained by masturbation into a wide-mouthed sterile specimen jar after 2–3 days of sexual abstinence. Controls consisted of samples obtained from 19 donors of unproven fertility. If a patient had

$<20 \times 10^6$ /ml sperm concentration, $<50\%$ motility or $<30\%$ normal forms as assessed by the World Health Organization (WHO) guidelines (World Health Organization, 1999), they were considered to have abnormal semen parameters. A cut-off value of 1×10^6 counted photons per minute (c.p.m.) per 20×10^6 sperm/ml was used to differentiate between ROS-negative and -positive patients (Kobayashi *et al.*, 2001). Following liquefaction, semen specimens were evaluated for semen volume, appearance, pH and viscosity.

Measurement of sperm morphology

Smears of raw semen were prepared for sperm morphology assessment. The smears were fixed and stained using the Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw, IL). Immediately after staining, the smears were rinsed in distilled water and air-dried. Smears were scored for sperm morphology using WHO guidelines (World Health Organization, 1999), and Tygerberg strict criteria (Mortimer and Menkveld, 2001).

Isolation of mature sperm population

Aliquots of 0.5–1 ml of the liquefied semen were loaded onto a 47 and 90% discontinuous ISolate gradient (Irvine Scientific, Santa Ana, CA) and centrifuged at 500 g for 20 min at room temperature. The presence of mature and immature spermatozoa in the two fractions was verified by preparing smears for sperm morphological abnormalities and the presence of excessive residual cytoplasm as described in our earlier publication (Gil-Guzman *et al.*, 2001). The resulting interfaces between the 47% pellet (fraction 1, immature spermatozoa) and the 90% pellet (fraction 2, containing mature spermatozoa) were aspirated, and these were transferred to separate test tubes. The pellet from fraction 2 was suspended in 1 ml of Biggers–Whitten–Whittingham medium (BWW) and centrifuged at 500 g for 7 min. The pellet was then re-suspended in 1 ml of BWW, and an aliquot was examined for sperm concentration, percentage motility, total sperm count, sperm morphology and ROS production.

Measurement of reactive oxygen species

Production of ROS was measured in washed semen by the chemiluminescence assay method using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St Louis, MO) as a probe (Kobayashi *et al.*, 2001). Aliquots of liquefied semen were centrifuged at 300 g for 7 min. Seminal plasma was separated and frozen at -80°C for later measurement of the total antioxidant capacity (TAC) level. The sperm pellet was washed twice with phosphate-buffered saline (PBS) pH 7.4, and resuspended in the same medium at a concentration of 20×10^6 sperm/ml. A 10 μl aliquot of luminol prepared as 5 mmol/l stock in dimethylsulfoxide (DMSO) was added to 400 μl of the washed sperm suspension; 10 ml of 5 mol/l luminol added to 400 μl of PBS served as a negative control. Levels of ROS were determined by measuring chemiluminescence with a luminometer (LKB 953, Wallac Inc., Gaithersburg, MD) in the integrated mode for 15 min. Results were expressed in $\times 10^6$ c.p.m. per 20×10^6 sperm per ml.

Total non-enzymatic antioxidant capacity was measured in the seminal plasma with an enhanced chemiluminescence assay (Saleh *et al.*, 2002b). Liquefied semen samples were centrifuged at 250 g for 7 min, and seminal plasma was separated and stored at -80°C . Frozen samples of seminal plasma were thawed at room temperature, diluted 1:20 with de-ionized water (dH_2O) and filtered through a 0.20 μl filter (Allegiance Healthcare Corporation, McGaw Park, IL). Signal reagent was prepared by adding 30 μl of H_2O_2 (8.8 molar/l), 10 μl of para-iodophenol stock solution (41.72 $\mu\text{mol/l}$) and 110 μl of luminol stock solution (3.1 mol/l) to 10 ml of Tris buffer (0.1 mol/l, pH 8.0). Horseradish peroxidase (HRP) working solution was prepared from

HRP stock solution by making a dilution of 1:1 of dH₂O to give a chemiluminescence output of 3×10^7 c.p.m. Trolox (6-hydroxyl-2,5,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was prepared as a standard solution (25, 50 and 75 $\mu\text{mol/l}$) for TAC calibration.

With the luminometer in the kinetic mode, 100 μl of signal reagent and 100 μl of HRP working solution were added to 700 μl of dH₂O and mixed. The mixture was equilibrated to the desired level of chemiluminescence output (between 2.8 and 3.2×10^7 c.p.m.) for 100 s. Three concentrations (25, 50 and 75 $\mu\text{mol/l}$) of 100 mmol/l of standard Trolox solution were immediately added to the mixture, and chemiluminescence was measured. Suppression of luminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence were recorded. The same steps were repeated with replacement of Trolox solutions with 100 μl aliquots of the prepared seminal plasma.

TAC calculation

Seminal TAC levels were calculated using the following equation:

$$Y = (Mx \pm C) \times d.$$

In this equation, Y = antioxidant concentration in μM ; M = slope of the curve; x = recovery time in seconds (the shorter the recovery time, the lower the amount of antioxidant present in the sample); C = intercept and not the daily variability (this will be zero if the intercept passes through zero, positive if the intercept passes above zero, or negative if it passes below zero); and d = dilution factor and expressed as molar Trolox equivalents.

Assessment of apoptosis by annexin-V staining

Externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane is an early step in the apoptotic process. Annexin V is a calcium-dependent phospholipid-binding protein with a very high affinity for PS. Annexin-V binding was examined in aliquot I and in fractions 1 and 2 (Vermes *et al.*, 1995). Once the annexin-V label had been applied, spermatozoa were assessed using epifluorescent microscopy. To differentiate apoptotic from necrotic spermatozoa, we included propidium iodide (PI) stain.

Exclusion of PI coupled with binding of annexin-V indicates membrane changes that are characteristic of early apoptosis. Complete disruption of the plasma membrane as seen in necrosis allows for both PS and PI to be expressed. Samples were classified as normal (negative annexin and PI), apoptotic (positive annexin-V and negative PI) or necrotic (positive PI). From both sperm fractions, sperm cells were resuspended in PBS (Sigma) and incubated with annexin-V reagent in HEPES buffer containing PI. The sample was analysed using epifluorescent microscopy. An excitation wavelength in the range of 450–500 nm and a detection wavelength in the range of 515–565 nm were used. A total of 200 spermatozoa were randomly assessed per slide in five fields and identified as normal, apoptotic or necrotic.

Measurement of mature sperm nuclear DNA damage by SCSA

DNA damage was measured in separate aliquots after a simple wash and density gradient centrifugation (fractions 1 and 2) by sperm chromatin structure assay (SCSA; Evenson *et al.*, 1999, 2002). A total of 5000 acridine orange-stained sperm were measured at a rate of ~250 cells/s for the amount of green (515–530 nm = native DNA) and red (> 630 nm = denatured DNA) fluorescence/cell. Computer analysis determined the DNA fragmentation index (DFI = red fluorescence/total [red and green fluorescence]). Computer gating defined the X DFI (mean of DFI ranging from 0 to 1024 channels), the

SD DFI (standard deviation of DFI), the %DFI (percentage of sperm with DNA fragmentation) and the %HDS (percentage of sperm with high DNA stainability).

Statistical methods

We used repeated measures analysis of variance with a compound symmetry correlation structure to test all two- and three-way interactions between factors, i.e. donor/patient group, mature/immature spermatozoa and normal/abnormal semen parameters. If significant interactions were found, we assessed one factor within levels of the other.

We compared donors versus patients examined for infertility and donors with normal semen parameters versus patients on continuous variables using the Wilcoxon rank-sum test or Student's *t*-test, as appropriate. Pairwise comparisons of donors with normal semen parameters, ROS-negative patients and ROS-positive patients were made on continuous variables using Dunn's or Tukey's multiple comparison tests. Within donor and patient groups, we compared mature versus immature spermatozoa on continuous variables using the Wilcoxon signed-rank test or paired *t*-test. We assessed the relationship of apoptosis to ROS and %DFI by computing Spearman correlation coefficients (r) and corresponding 95% confidence intervals (CIs) within donor and patient groups. Correlations of the groups were compared using a z -test for two independent correlation coefficients (Wilcox, 1987).

ROS-TAC scores were generated through principal component analysis and standardized so that the mean ROS-TAC score for the whole ejaculate was 50 and the SD was 10 for the donors with normal semen parameters (Sharma *et al.*, 1999). The results are reported as either median (25th and 75th percentile) or mean (\pm SD). Two-tailed tests were performed, and the significance level for each test was 0.05. A Bonferroni correction was applied to the significance criteria for multiple comparisons such that $P < 0.01$ (0.05/5 comparisons) was considered significant. Analysis was done with SAS 8.2 (SAS Institute Inc., Cary, NC), and graphics were produced with S-PLUS 6.0 (Insightful Corp.).

Results

There were significant two-way interactions between the donor/patient group and mature/immature spermatozoa for many variables. Therefore, for each variable, we compared donor and patient groups within the whole ejaculate, mature and immature spermatozoa separately. Additionally, the donors with normal semen parameters were compared with the patients examined for infertility as a group and by ROS-positive/negative status.

Semen parameters (Table I)

Of the 19 donors, 58% (11 of 19) had normal semen parameters. The donors had significantly higher sperm concentration and percentage motility than the patients. The 11 donors with normal semen parameters had: (i) significantly higher sperm concentration, percentage motility and normal forms as assessed by the WHO guidelines than the patients; (ii) higher percentage motility and normal forms as assessed by the WHO guidelines and Kruger's strict criteria than the ROS-negative patients; and (iii) higher sperm concentration and percentage motility than the ROS-positive patients. The ROS-negative patients had significantly higher sperm concentration than the ROS-positive patients.

Table I. Comparison of semen parameters (SP) between donors and patients examined for infertility

Variable	Donors (D)		Patients (P)		D versus P P-value ^b	Donors with normal SP (ND)		ROS-negative patients (R ⁻)		ROS-positive patients (R ⁺)		P-value			
	n	Statistics ^a	n	Statistics		n	Statistics	n	Statistics	n	Statistics	ND ^b versus P	ND ^d versus R ⁻	ND ^d versus R ⁺	R ^{-d} versus R ⁺
Sperm concentration (×10 ⁶ /ml)	19	83 (62, 130)	31	41 (18, 73)	0.003	11	100 (69, 135)	17	67 (41, 97)	12	24 (18, 44)	0.004	0.13	<0.001	0.004
Motility (%)	19	67 ± 18	31	49 ± 17	<0.001 ^c	11	78 ± 9	17	51 ± 17	12	46 ± 19	<0.001 ^c	<0.001 ^c	<0.001 ^c	0.38 ^e
Morphology															
WHO	19	35 (27, 39)	29	22 (17, 36)	0.022	11	37 (35, 44)	17	22 (15, 27)	11	30 (20, 43)	0.008	0.004	0.09	0.28
Kruger	19	10 (8, 14)	29	6 (4, 12)	0.010	11	12 (10, 16)	17	5 (4, 11)	11	7 (3, 18)	0.011	0.006	0.10	0.35

^aMedian (25th and 75th percentile) or mean ± SD.

^bWilcoxon rank-sum test, unless noted.

^cStudent's *t*-test.

^dDunn's test, unless noted.

^eTukey's test.

P-values <0.01 (0.05/5 comparisons) are significant.

Table II. Comparison of apoptosis and necrosis between donors and patients examined for infertility

Variable	Donors (D)		Patients (P)		D versus P P-value ^b	Donors with normal SP (ND)		ROS-negative patients (R ⁻)		ROS-positive patients (R ⁺)		P-value			
	n	Statistics ^a	n	Statistics		n	Statistics	n	Statistics	n	Statistics	ND ^b versus P	ND ^d versus R ⁻	ND ^d versus R ⁺	R ^{-d} versus R ⁺
Apoptosis (%)															
Whole	19	5.9 ± 2.3	29	12.7 ± 5.2	<0.001 ^c	11	6.1 ± 2.5	17	10.6 ± 4.4	12	15.7 ± 4.9	<0.001 ^c	0.008 ^e	<0.001 ^c	0.003 ^e
Mature	19	11.5 (7.0, 13.5)	24	20.0 (11.3, 26.0)	0.009	11	11.0 (4.5, 13.0)	15	12.5 (9.5, 20.5)	7	29.0 (25.0, 30.5)	0.010	0.19	<0.001	0.005
Immature	19	8.5 (4.5, 10.5)	24	10.5 (6.0, 13.8)	0.087	11	8.5 (2.5, 9.5)	15	6.5 (4.5, 11.0)	7	15.0 (13.0, 18.0)	0.065	0.71	<0.001	<0.001
P-value ^f		<0.001 ^g		<0.001			0.002 ^g		<0.001 ^g		<0.001				
Necrosis (%)															
Whole	19	35 ± 12	29	53 ± 13	<0.001 ^c	11	33 ± 9	17	52 ± 14	12	55 ± 11	<0.001 ^c	<0.001	<0.001	0.51
Mature	19	18 (14, 27)	24	23 (20, 31)	0.065	11	18 (17, 29)	15	22 (19, 29)	7	30 (19, 37)	0.14	0.37	0.09	0.32
Immature	19	58 (41, 65)	24	62 (55, 73)	0.047	11	61 (53, 65)	15	61 (55, 73)	7	62 (57, 69)	0.20	0.21	0.27	0.92
P-value ^f		<0.001		<0.001			<0.001		<0.001		0.016 ^g				

^aMedian (25th and 75th percentile) or mean ± SD.

^bWilcoxon rank-sum test, unless noted.

^cStudent's *t*-test.

^dDunn's test, unless noted.

^eTukey's test.

^fMature versus immature: paired *t*-test, unless noted.

^gWilcoxon signed-rank test.

P-values <0.01 (0.05/5 comparisons) are significant.

Apoptosis and necrosis (Table II)

The patients had a significantly higher percentage of apoptosis in the whole ejaculate than the donors and the donors with normal semen parameters. The ROS-positive patients had a higher percentage of apoptosis in the whole ejaculate than the donors with normal semen parameters and ROS-negative patients. Moreover, the ROS-negative patients had a higher percentage of apoptosis in the whole ejaculate than the donors with normal semen parameters. Within every donor/patient group or subgroup, the percentage of spermatozoa that underwent apoptosis was higher in the mature spermatozoa than in the immature spermatozoa.

Patients had a significantly higher percentage of necrosis in the whole ejaculate than the donors as a whole and the donors with normal semen parameters. The ROS-positive and

ROS-negative patients had a higher percentage of necrosis than donors with normal semen parameters in the whole ejaculate. Within every donor/patient group or subgroup except for the ROS-positive patients, the immature spermatozoa had a higher percentage of necrosis than mature spermatozoa.

ROS and TAC (Table III)

The patients had significantly higher levels of ROS in the whole ejaculate and immature spermatozoa than the donors. As expected, the ROS-positive patients had higher ROS levels in the whole ejaculate and mature and immature spermatozoa than the donors with normal semen parameters and the ROS-negative patients. The immature spermatozoa had higher levels of ROS than the mature spermatozoa within donors, patients and ROS-negative patients. Levels of TAC were not significantly different between any of the groups that were compared.

Table III. Comparison of ROS, TAC and ROS-TAC scores between donors and patients examined for infertility

Variable	Donors (D)		Patients (P)		D versus P	Donors with normal SP (ND)		ROS-negative patients (R ⁻)		ROS-positive patients (R ⁺)		P-value			
	n	Statistics ^a	n	Statistics		P-value ^b	n	Statistics	n	Statistics	n	Statistics	ND ^b versus P	ND ^d versus R ⁻	ND ^d versus R ⁺
ROS															
Whole	19	0.1 (0.0, 0.2)	29	0.5 (0.3, 7.5)	0.002	11	0.1 (0.0, 0.2)	17	0.3 (0.1, 0.4)	12	11.1 (4.4, 94.7)	0.010	0.44	<0.001	<0.001
Mature	19	0.0 (0.0, 0.0)	23	0.0 (0.0, 0.2)	0.055	11	0.0 (0.0, 0.0)	15	0.0 (0.0, 0.0)	7	0.5 (0.1, 8.2)	0.021	0.29	<0.001	0.002
Immature	19	0.0 (0.0, 0.1)	23	0.2 (0.1, 9.1)	0.009	11	0.0 (0.0, 0.1)	15	0.1 (0.0, 0.2)	7	14.0 (9.1, 130.9)	0.012	0.23	<0.001	<0.001
P-value ^f		0.008 ^g		<0.001 ^g			0.08 ^g		0.002 ^g		0.016 ^g				
TAC	18	639 (432, 766)	26	433 (339, 591)	0.086	10	691 (298, 810)	16	458 (379, 655)	9	364 (339, 449)	0.23	0.49	0.12	0.30
ROS-TAC															
Whole	18	49.7 ± 8.2	25	37.1 ± 13.1	<0.001 ^c	10	50.0 ± 10.0	16	45.2 ± 6.1	9	22.7 ± 8.8	0.005 ^c	0.15 ^e	<0.001 ^c	<0.001 ^c
Mature	18	55.4 ± 8.0	19	49.9 ± 7.3	0.039 ^c	10	56.9 ± 10.1	14	52.8 ± 5.2	4	42.3 ± 7.6	0.073 ^c	0.20 ^e	0.003 ^c	0.022 ^c
Immature	18	51.4 ± 7.7	19	41.8 ± 10.8	0.004 ^c	10	53.3 ± 7.6	14	46.6 ± 6.4	4	25.7 ± 8.6	0.003 ^c	0.030 ^e	<0.001 ^c	<0.001 ^c
P-value ^f		0.024		<0.001			0.15		0.006		0.007				

^aMedian (25th and 75th percentile) or mean ± SD.

^bWilcoxon rank-sum test, unless noted.

^cStudent's *t*-test.

^dDunn's test, unless noted.

^eTukey's test.

^fMature versus immature: paired *t*-test, unless noted.

^gWilcoxon signed-rank test.

P-values <0.01 (0.05/5 comparisons) are significant.

Table IV. Comparison of DNA damage between donors and patients examined for infertility

Variable	Donors (D)		Patients (P)		D versus P	Donors with normal SP (ND)		ROS-negative patients (R ⁻)		ROS-positive patients (R ⁺)		P-value			
	n	Statistics ^a	n	Statistics		P-value ^b	n	Statistics	n	Statistics	n	Statistics	ND ^b versus P	ND ^c versus R ⁻	ND ^c versus R ⁺
% DFI															
Whole	14	19 (16, 26)	21	24 (20, 31)	0.092	6	15 (12, 19)	14	23 (20, 26)	7	35 (25, 60)	0.023	0.07	0.003	0.10
Mature	13	11 (6, 28)	18	27 (14, 33)	0.099	6	9 (6, 27)	12	23 (12, 30)	4	32 (18, 51)	0.15	0.33	0.10	0.31
Immature	13	20 (18, 31)	18	25 (17, 29)	0.65	6	19 (14, 21)	12	21 (17, 27)	4	42 (20, 59)	0.13	0.37	0.06	0.20
P-value ^d		0.020		0.90			0.55		0.71		0.61				
\bar{X} DFI															
Whole	14	245 (228, 290)	21	278 (259, 329)	0.057	6	229 (214, 245)	14	270 (253, 283)	7	349 (298, 431)	0.010	0.037	<0.001	0.07
Mature	13	219 (183, 281)	18	293 (220, 329)	0.050	6	212 (183, 267)	12	276 (213, 297)	4	323 (265, 441)	0.061	0.17	0.025	0.19
Immature	13	260 (235, 325)	18	286 (245, 299)	0.32	6	257 (220, 260)	12	273 (242, 289)	4	388 (281, 444)	0.054	0.17	0.025	0.19
P-value ^d		0.003		0.83			0.17		0.91		0.85				
SD DFI															
Whole	14	171 (153, 205)	21	199 (170, 228)	0.14	6	146 (137, 172)	14	188 (165, 215)	7	253 (228, 278)	0.018	0.08	<0.001	0.034
Mature	13	133 (100, 190)	18	220 (154, 240)	0.044	6	124 (97, 181)	12	195 (130, 231)	4	235 (183, 288)	0.036	0.11	0.018	0.21
Immature	13	189 (178, 235)	18	213 (163, 226)	0.57	6	178 (145, 189)	12	193 (157, 221)	4	250 (204, 268)	0.070	0.24	0.022	0.13
P-value ^d		<0.001		0.80 ^e			0.023		0.52		0.98				
%HDS															
Whole	14	3.7 (2.9, 5.7)	21	6.2 (4.0, 10.6)	0.040	6	3.6 (2.9, 5.7)	14	4.3 (3.3, 10.0)	7	9.9 (7.7, 29.3)	0.13	0.32	0.029	0.12
Mature	13	1.3 (1.1, 2.8)	18	2.4 (1.3, 4.8)	0.19	6	1.7 (1.0, 2.8)	12	2.3 (1.3, 3.4)	4	3.9 (2.0, 10.8)	0.27	0.45	0.16	0.36
Immature	13	3.6 (2.9, 5.3)	18	4.4 (3.6, 11.1)	0.25	6	4.0 (3.0, 5.3)	12	4.2 (3.7, 7.9)	4	12.1 (6.3, 19.8)	0.42	0.55	0.18	0.33
P-value ^d		<0.001		<0.001 ^e			<0.001		<0.001 ^e		0.053				

^aMedian (25th and 75th percentile).

^bWilcoxon rank-sum test.

^cDunn's test.

^dMature versus immature: paired *t*-test, unless noted.

^eWilcoxon signed-rank test.

P-values <0.01 (0.05/5 comparisons) are significant.

The donors as a whole and donors with normal semen parameters had significantly higher ROS-TAC scores in the whole ejaculate and immature spermatozoa than the patients.

Donors with normal semen parameters and ROS-negative patients had higher ROS-TAC scores in the whole ejaculate and immature spermatozoa than the ROS-positive patients.

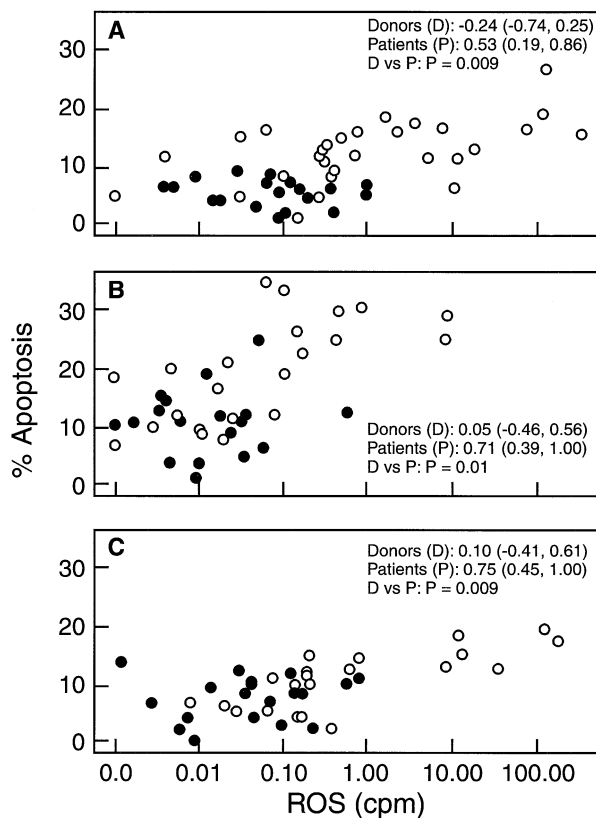


Figure 1. Correlation between percentage apoptosis and ROS in donors and patients examined for infertility. The figure shows the Spearman correlation coefficient (95% confidence interval). Filled circles = donors; empty circles = patients. (A) Whole ejaculate; (B) mature spermatozoa; and (C) immature spermatozoa.

Within patients and the ROS-positive and -negative patient subgroups, ROS-TAC scores were higher in the mature spermatozoa than in the immature spermatozoa

DNA damage (Table IV)

The ROS-positive patients had significantly higher %DFI in the whole ejaculate than the donors with normal semen parameters. There were no statistically significant differences in the %DFI between the donors and patients or between the mature and immature spermatozoa within each subgroup. The ROS-positive patients had a significantly higher mean and SD DFI in the whole ejaculate than the donors with normal semen parameters. Within donors, the immature spermatozoa had higher mean and SD DFI than the mature spermatozoa. There were no significant differences in %HDS between any of the donor and patient groups or subgroups compared. Within each donor and patient group or subgroup except for the ROS-positive patients, the immature spermatozoa had significantly higher %HDS than the mature spermatozoa.

Relationship between apoptosis and ROS

Figure 1 illustrates the relationship between apoptosis and ROS within the donor and patient groups. Apoptosis and ROS were not significantly correlated within the donors. Apoptosis was

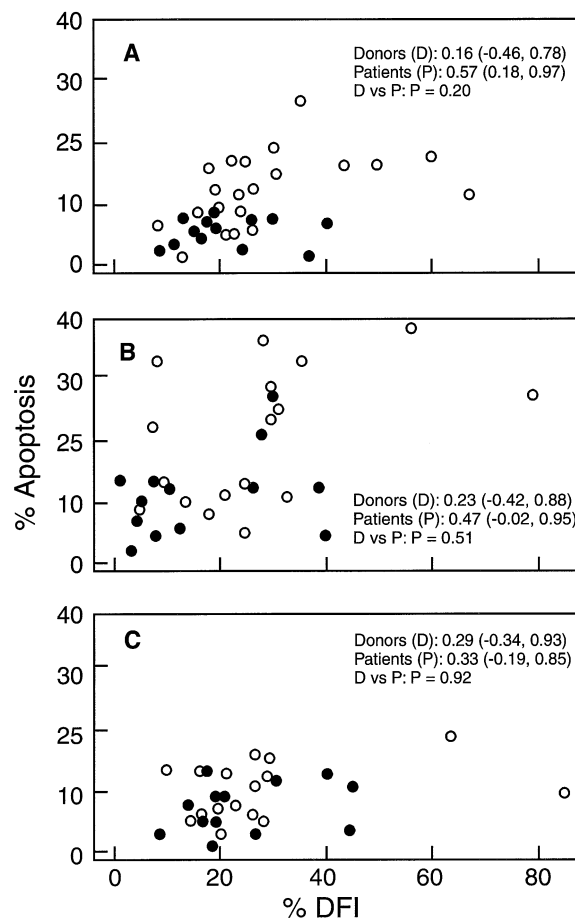


Figure 2. Correlation between percentage apoptosis and %DFI in donors and patients examined for infertility. The figure shows the Spearman correlation coefficient (95% confidence interval). Filled circles = donors; empty circles = patients. (A) Whole ejaculate; (B) mature spermatozoa; and (C) immature spermatozoa.

significantly correlated with ROS within patients in the whole ejaculate [r (95% CI) = 0.53 (0.19–0.86)] and in the mature [r (95% CI) = 0.71 (0.39–1.00)] and immature spermatozoa [r (95% CI) = 0.75 (0.45–1.00)]. Patients had higher absolute value correlation coefficients than donors in the whole ejaculate, mature and immature spermatozoa.

Relationship between apoptosis and DFI

Figure 2 illustrates the relationship between apoptosis and %DFI within the donor and patient groups. Apoptosis and %DFI were not significantly correlated within the donors in the whole ejaculate or in the mature or immature spermatozoa. Apoptosis and %DFI were significantly correlated within the patients, but only in the whole ejaculate [r (95% CI) = 0.57 (0.18–0.97)]. There was no statistical difference between the donor and patient correlation coefficients in the whole ejaculate and mature or immature spermatozoa.

Discussion

Different theories have been proposed to explain the origin of DNA damage in mature spermatozoa from infertile men,

including apoptosis and ROS. Our goal was to elucidate the involvement of these markers in inducing DNA damage in ejaculated spermatozoa. We also wanted to study if these two markers could explain the overall DNA damage or if there are other factors involved in the final DNA damage.

To examine these issues, ROS-positive and -negative samples from donors with both normal and abnormal semen parameters were fractionated into mature and immature subsets and then examined for the extent of apoptosis and DNA damage. The primary function of density gradient separation is to separate the population of sperm with greatest fertility potential. In our earlier study (Gil-Guzman *et al.*, 2001), using a three-layer density gradient (47, 70 and 90%), we identified the immature and mature spermatozoa in the different subsets separated. These were identified by staining both for morphological abnormalities and for the presence of spermatozoa with excessive residual cytoplasm.

We demonstrated that immature spermatozoa produce significantly higher levels of ROS and DNA damage (Gil-Guzman *et al.*, 2001; Ollero *et al.*, 2001). In the present study, mature spermatozoa (sperm fractions from the lower 90% layer of the Percoll gradient) showed lower generation of ROS and markedly superior sperm motion characteristics. Forty-one percent of the patients in our study (12 of 29) had high ROS levels. The higher levels of ROS among the patients examined for infertility imply that ROS is a significant contributor of male infertility.

Functionally abnormal spermatozoa have been considered to be the main source of ROS. However, other cells, particularly leukocytes, can also generate ROS. Activated leukocytes are capable of producing 100-fold higher amounts of ROS than non-activated leukocytes (Plante *et al.*, 1994). Oxidative injury to spermatozoa is a major cause of sperm dysfunction (Oehninger *et al.*, 1995; Sharma and Agarwal, 1996; Ollero *et al.*, 2001; Agarwal and Saleh, 2002; Alvarez *et al.*, 2002; Saleh *et al.*, 2002a,b; Agarwal *et al.*, 2003).

We did not observe a significant reduction in the total antioxidant capacity associated with increased levels of ROS. The pathological levels of ROS detected in the semen of infertile men are more likely to be caused by increased ROS production than by reduced antioxidant capacity of seminal plasma (Zini *et al.*, 1993). Both ROS and TAC markers by themselves are not good discriminators of oxidative stress. Low ROS-TAC scores in our study indicate high seminal oxidative stress. This score may serve as an important measure in identifying men with a clinical diagnosis of infertility who are likely to initiate a pregnancy over time (Sharma *et al.*, 1999; Pasqualotto *et al.*, 2000).

The mechanism of DNA damage in a mature spermatozoon that is transcriptionally inactive is unclear. It is critical to understand how DNA damage occurs. Anomalies in the DNA of ejaculated human spermatozoa can occur in two ways. The first theory is the unique manner in which sperm chromatin is packaged, while the second attributes the nuclear DNA damage in mature spermatozoa to apoptosis (Sakkas *et al.*, 2002). At the nuclear level, histones are replaced by protamines in a process called protamination. To achieve this purpose, nicks must be created endogenously to relieve the torsional stress of

the DNA double helix. The endogenous nuclease, topoisomerase II (topo II), might play a role in both creating and ligating nicks during spermiogenesis (McPherson and Longo, 1992, 1993,b; Sakkas *et al.*, 1995). The presence of endogenous nicks in ejaculated spermatozoa indicates incomplete maturation during spermiogenesis. Once protamination is complete, the nicks completely disappear (Ward and Coffey, 1991). Disruption of the critical process of chromatin packaging may result in persistence of endogenous nicks that would, in turn, be reflected as DNA damage.

The second theory proposes that the presence of endogenous nicks in ejaculated human spermatozoa is characteristic of programmed cell death as seen in apoptosis of somatic cells (Gorczyca *et al.*, 1993a,b). Activation of the endogenous endonuclease, which causes extensive DNA breakage, may therefore represent a ubiquitous mechanism of cell inactivation (death), or functional elimination of possibly defective germ cells from the reproductive pool.

The evidence points to an abortive apoptosis taking place in many males that exhibit sperm parameters that are below normal. Apoptosis in mature sperm is initiated during spermatogenesis in which some cells, earmarked for elimination, may escape the removal mechanism and contribute to poor sperm quality (Sakkas *et al.*, 2002; Shen *et al.*, 2002). In certain males, abortive apoptosis may fail in the total clearance of spermatozoa earmarked for elimination by apoptosis. One of the factors implicated in apoptosis is the cell surface protein, Fas. Results indicate that subfertile men have increased presence of Fas-positive spermatozoa (Sakkas *et al.*, 2002). In these subfertile men, spermatozoa that have been earmarked to undergo apoptosis escape this process, suggesting that the correct clearance of spermatozoa via apoptosis is not occurring.

Apoptotic spermatozoa showed variable degrees of annexin-V positivity, depending on the stage of the apoptotic process. This staining pattern depends on the degree of external expression of the phospholipid PS. As cells progress through the stages of apoptosis, higher amounts of PS are expressed on the cell surface. In general, the external expression of PS takes place in an early stage of apoptosis (Vermees *et al.*, 1995). Simultaneous staining with PI for necrotic spermatozoa allows for differentiation.

Annexin V staining is a physiological marker of early apoptosis; however, it is also associated with bicarbonate-mediated phospholipid scrambling during capacitation (Gadella and Harrison, 2002; de Vries *et al.*, 2003). Therefore, it is important to complement annexin-V staining with other markers of DNA damage such as TUNEL (strand breaks) or DNA fragmentation by SCSA, both of which are highly correlated (Sailer *et al.*, 1995). We therefore also examined the extent of DNA damage in these specimens utilizing SCSA as a measure of DNA damage. In our study, apoptosis occurred in spermatozoa from donors with normal semen parameters and also in spermatozoa from patients examined for infertility at increased levels, reflecting the significant role of this process in male infertility.

A number of studies using TUNEL, Fas binding on mature spermatozoa and annexin-V staining have proposed that the

presence of spermatozoa with damaged DNA is indicative of apoptosis (Manicardi *et al.*, 1995, 1998; Sun *et al.*, 1997; Lopes *et al.*, 1998; Sakkas *et al.*, 1999; Barroso *et al.*, 2000; Irvine *et al.*, 2000). Mature spermatozoa had lower levels of DNA damage than immature spermatozoa in donors with normal semen parameters. However, in patients examined for infertility, mature spermatozoa had increased levels of DNA damage. Variable amounts of DNA fragmentation are seen in the high and low motility fractions of ejaculates from infertile men and normozoospermic donors (Sun *et al.*, 1997; Aitken *et al.*, 1998; Lopes *et al.*, 1998; Barroso *et al.*, 2000; Donnelly *et al.*, 2000; Oosterhuis *et al.*, 2000). The differences in the proportion of sperm with DNA fragmentation among all these studies may be due to the sperm samples analysed (donors versus different patient populations), the various sperm separation methods, and the different methods used to detect DNA fragmentation.

This is important as mature spermatozoa with DNA damage may exhibit lower functional potential and this may explain the patients' subfertility status. Furthermore, it raises the concern of using such spermatozoa during ICSI. We found that the level of DNA damage was higher in patients examined for infertility in our study.

ROS and their role in male infertility have been researched extensively. Many studies have shown the adverse effects of ROS on the different cellular compartments of spermatozoa including DNA. ROS can result in single- and double-strand DNA breaks. Impaired chromatin packaging, apoptosis and ROS alone can induce DNA damage in human spermatozoa. Thus, a cause-effect relationship exists between apoptosis and DNA damage. This explains the positive correlation between apoptosis and DNA damage observed in our study.

However, comparing levels of apoptosis with those of DNA damage in the same sample has indicated that apoptosis might be responsible only for a fraction of DNA damage in human spermatozoa. Additionally, we have observed a strong correlation between ROS and the level of apoptosis. This may reflect a causal relationship in the way that ROS can induce apoptosis. Our results are supported by a recent publication by Sakkas *et al.* (2002) in which they reported that TUNEL positivity and apoptotic markers do not always exist in unison in spermatozoa. In this study, semen samples that had low sperm concentration and poor morphology were more likely to show high levels of TUNEL positivity and Fas and p53 expression. However, no strict correlation was evident between Fas and p53 expression and the population of sperm that were positive for TUNEL. The authors proposed that DNA damage is not directly linked to an apoptotic process occurring in spermatozoa, and may arise due to problems in the nuclear remodelling process during the later stages of spermatogenesis (Sakkas *et al.*, 2002).

In our study, we have demonstrated a strong correlation between apoptosis and sperm DNA damage in the whole ejaculate of our patient group. However, the total amount of the DNA damage observed in our study cannot be explained by apoptosis alone. Furthermore, not all apoptotic spermatozoa observed in our study possessed DNA damage. A proportion of them should be in an early stage of apoptosis. Thus, apoptosis can explain only a fraction of such DNA damage.

The role for caspases and apoptosis in ejaculated sperm is still in question. Caspase, c-jun, p53 and p21 are present in a restricted site for apoptosis (cytoplasmic droplets) in spermatids and immature spermatozoa (Weil *et al.*, 1998; Blanco-Rodriguez and Martinez-Garcia, 1999). Inactive and active form of caspase markers have been detected in human sperm cells (Weng *et al.*, 2002; de Vries *et al.*, 2003) in both low and high motility fractions of donors and patients. A significant positive correlation has been shown between *in situ*-active caspase 3 in the sperm midpiece and DNA fragmentation in the low motility fractions of patients. This suggests that caspase-dependent apoptotic mechanisms could originate in the cytoplasmic droplet or within mitochondria, and function in the nucleus (Weng *et al.*, 2002).

Mature sperm do not have efficient operative mechanisms for protein synthesis. Both active and inactive forms of caspases (caspase 3) are absent in mature sperm cells (de Vries *et al.*, 2003). They do not show bicarbonate/PKA-dependent signs of apoptosis such as fractionation of DNA or mitochondrial inner membrane depolarization, but do show rapid aminophospholipid exposure (de Vries *et al.*, 2003). There may be a temporal disassociation between caspase activation and the expression of cellular changes suggestive of apoptosis in mature spermatozoa.

On the other hand, Paasch *et al.* (2003) recently reported that active caspases were present in subpopulations of mature sperm and to a greater extent in sperm from infertile patients. However, caspases are not completely removed during undisturbed spermatogenesis and may therefore contribute to inhibition of normal sperm function. This finding offers new insight into the current concepts in remodelling defects during spermatogenesis.

Sperm cells with immature appearance and/or cytoplasmic droplets fail to expose PS and also show no phosphotyrosine labelling (de Vries *et al.*, 2003). Alternatively, triggering of PS externalization and DNA fragmentation could be due to activation of other caspases or cellular pathways. It also leaves open the possibility that sperm apoptosis may be caspase independent, at least to some extent. Our data also support this observation since we did not find a significant correlation of apoptosis with DNA damage, suggesting a nuclear remodelling phenomenon rather than apoptosis as a cause of ROS-induced DNA damage.

From all of these observations, we conclude that ROS generation contributes to DNA damage in spermatozoa. Mature spermatozoa had lower levels of DNA damage compared with immature spermatozoa in our group of normal men who serve as volunteers for our research studies. In patients examined for infertility, however, there may be an increased number of spermatozoa with DNA damage, particularly in the mature fraction, explaining their subfertility. The contributing role of each one of these mechanisms will vary among individuals and within a given individual from time to time depending on the circumstances that affect spermatogenesis. The patients in our study consisted of men presenting with a history of infertility at our infertility clinic. These men could not be characterized as being infertile (or not), due to a lack of

information on either male or female factors, and therefore may have included men with apparently normal semen.

The observation that ejaculated human spermatozoa possess DNA damage raises numerous problems. How these spermatozoa arise in the ejaculate of some men and what consequences they have if they succeed in their genetic mission are unclear. Further investigations are required to assess the potential capacity of ejaculated human spermatozoa to undergo apoptotic cell death. It is unclear why the apoptotic process is not fully completed in these sperm.

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