Journal of Andrology, Vol. 26, No. 6, November/December 2005

DNA Integrity Is Compromised in Protamine-Deficient Human Sperm

VINCENT W. AOKI,*†‡ SERGEY I. MOSKOVTSEV,§ JENNIFER WILLIS,§ LIHUA LIU,*‡
J. BRENDAN M. MULLEN,§ AND DOUGLAS T. CARRELL*†‡¶

*From the *Andrology and IVF Laboratories and the Departments of †Surgery and ‡Physiology, University of Utah School of Medicine, Salt Lake City, Utah; the §Andrology Laboratory, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada; and the ¶Department of Obstetrics and Gynecology, University of Utah School of Medicine, Salt Lake City, Utah.*

ABSTRACT: The objective of this study was to examine the relationship between DNA integrity and protamines in human sperm. One hundred forty-nine male infertility patients were included in an Institutional Review Board–approved study. Sperm were evaluated for DNA fragmentation using the DNA Integrity Assay, a test equivalent to the sperm chromatin structure assay (SCSA). Additionally, nuclear proteins were extracted and the protamine-1/protamine-2 ratio (P1/P2), protamine-1 (P1), protamine-2 (P2), and total protamine concentrations were evaluated. We identified 37 patients with abnormally low P1/P2 ratios, 99 patients with normal P1/P2 ratios, and 13 patients with abnormally high P1/P2 ratios. DNA fragmentation was significantly elevated in patients with low P1/P2 ratios (37.1 ± 6.02) vs those with normal and high P1/P2 ratios (26.7 ± 1.9 and 23.8 ± 3.2, respectively; $P < .05$) and was inversely correlated with the P1/P2 ratio (R_s −0.18, $P < .05$), P1 concentration (R_s −0.29, $P < .001$), P2 concentration (R_s −0.24, $P < .005$), and total protamine concentration (R_s −0.28, $P < .001$). Furthermore, χ^2 analysis revealed a significant increase in the incidence of marked DNA fragmentation in patients with diminished levels of either P1 or P2. The present study is the first to report that human sperm protamine content is significantly related to DNA fragmentation. In particular, sperm P1 and P2 concentrations inversely correlate with DNA fragmentation, indicating a protective role of the protamines against sperm DNA damage. In light of recent studies highlighting the negative effect of sperm DNA damage on ART outcomes, these findings indicate a possible clinical significance for human sperm protamine levels.

Key words: Chromatin, DFI, DNA damage.
J Androl 2005;26:741–748

During spermiogenesis, sperm chromatin undergoes substantial compaction. Sperm chromatin packaging occurs in a 2-step process (Oliva and Dixon, 1991). In the first step, the transition nuclear proteins (TP1 and TP2) replace the somatic cell histones. In the second step, during the elongating spermatid stage, the sperm protamine proteins replace the transition proteins. The result is a highly compact sperm chromatin, which fosters DNA stability and transcriptional quiescence.

In humans there are 2 forms of sperm protamine: protamine-1 (P1) and protamine-2 (P2), which occur in a strictly regulated 1-to-1 ratio (Corzett et al, 2002). Sperm protamine deficiency has been implicated in male infertility (Chevaillier et al, 1987; Balhorn et al, 1988; Belokopytova et al, 1993; de Yebra et al, 1993, 1998; Carrell and Liu, 2001; Aoki and Carrell, 2003; Aoki et al, 2005). In particular, aberrant P1/P2 ratios significantly relate to fertility status. The studies conducted by Yebra et al (1998) and Carrell and Liu (2001) describe a population of infertile males with undetectable sperm P2. Recently, P1 deficiency has also been identified in a population of subfertile males (Aoki et al, 2005).

It has been postulated that protamine deficiency is related to DNA damage in human sperm. A number of recent studies have focused on the relationship between sperm DNA damage and male infertility (Evenson et al, 2002; Tomsu et al, 2002; Virant-Klun et al, 2002; Seli et al, 2004). Although the biological significance of sperm DNA damage remains unclear, it appears to be detrimental to fertility in humans and has been linked to lower embryo quality (Tomsu et al, 2002; Virant-Klun et al, 2002), blastulation rates (Seli et al, 2004), and in vitro fertilization (IVF) pregnancy rates (Evenson et al, 2002; Bungum et al, 2004; Henkel et al, 2004; Virro et al, 2004). Mice that are haplo-insufficient for either P1 or P2 are sterile and have increased levels of sperm cell apoptosis, DNA damage, and embryonic arrest (Cho et al, 2001, 2003). However, relatively little is known about sperm DNA integrity in protamine-deficient human males.

The objective of this study was to examine the relationship between DNA integrity and protamines in human

Correspondence to: Douglas T. Carrell, PhD, University of Utah IVF and Andrology Laboratories, 675 Arapeen Drive, Suite 205, Salt Lake City, UT 84108 (e-mail: douglas.carrell@hsc.utah.edu).

Received for publication March 21, 2005; accepted for publication May 31, 2005.

DOI: 10.2164/jandrol.05063

sperm. Specifically, the DNA fragmentation index (DFI) was used as a measure of DNA integrity. Protamine levels (P1/P2 ratio, P1, P2, and total protamine concentrations) are compared to DFI levels in the sperm of male infertility patients.

Materials and Methods

Unless otherwise noted, all chemicals were obtained from Sigma Chemical Company (St Louis, Mo). Reagents for gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, Calif). Acridine orange was purchased from Polysciences Inc (Warrington, Pa).

Institutional Review Board approval was obtained before initiation of this study. Semen was collected and evaluated from 149 randomly selected male patients presenting for infertility assessment. A single semen sample was used for all assays, including the DNA Integrity Assay and protamine protein extraction and quantification. Samples with a sperm concentration of less than 3×10^6/mL were excluded because they offered insufficient material. After semen analysis and within 1 hour of the time of ejaculation, aliquots of raw semen were frozen at −80°C for later analysis.

DNA Integrity Assay

The DNA Integrity Assay was used to measure the DNA fragmentation index (DFI) and was performed as previously described (Evenson et al, 2002; Fischer et al, 2003). At the time of analysis, semen samples were thawed on ice and diluted with TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4) to $1–2 \times 10^6$ cells/mL. Two hundred–microliter aliquots of diluted sample were mixed with 400 μL of a low-pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl for 30 seconds; this was followed by staining with 1.2 mL of 6 μg/mL chromatographically purified acridine orange (AO) in a phosphate citrate buffer (pH 6.0).

Three minutes after the staining procedure started, the cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif) equipped with an air-cooled argon laser. Measurements were collected in duplicate on 5000 cells per sample, and 2 aliquots were analyzed for each semen specimen. Under these conditions, AO intercalated with double-stranded DNA emits green fluorescence, and AO associated with single-stranded DNA emits red fluorescence. To avoid instrument drift, reference samples were used to set the red and green photomultiplier tube voltages. A new reference sample was run every 6 to 10 samples. FCS Express Version 2 (De Novo Software, Thornhill, Canada) was used for off-line analysis of the flow cytometric data.

DNA denaturation was expressed as the DFI, which represents the ratio of red to red plus green fluorescence intensity (Figure 1). This is similar to the SCSA definition of DFI as red (F > 630 nm)/red + green (F515–530 band pass). Based on a previously published categorization system (Evenson et al, 2002), 3 levels of DNA fragmentation were reported: low (<15% DFI), moderate (15%–30% DFI), and high (>30% DFI). These levels correspond to excellent, good, and fair-to-poor fertility potential, respectively.

Purification of Nuclear Proteins

Sperm nuclear proteins were extracted from the semen aliquots of all 149 patients. The P1/P2 ratio, P1, P2, and total protamine concentrations were subsequently quantified in all 149 patients (Aoki et al, 2005). Prior to extraction, sperm concentrations and white blood cell counts were determined using World Health Organization (WHO) criteria. All samples were run in duplicate, and the average P1/P2 ratio, P1, and P2 concentrations from the 2 runs were reported.

Semen aliquots with a known number of sperm ($5.0–20 \times 10^6$ cells) were centrifuged at $500 \times g$ for 5 minutes at 4°C. The pellet was washed in 1 mM phenylmethylsulfonylfluoride (PMSF) in distilled water, centrifuged at $500 \times g$ for 5 minutes at 4°C, and the pellet was resuspended in 100 μL of 100 mM Tris buffer containing 20 mM EDTA and 1 mM PMSF (pH 8.0). One hundred microliters of 6 M guanidine and 575 mM dithiothreitol were added and mixed, followed by addition of 200 μL 522 mM sodium iodoacetate.

The suspension was maintained at room temperature for 30 minutes while being protected from light. To this suspension, 1.0 mL of 100% ethanol at 4°C was added and maintained for 1 minute before centrifugation at $12\,000 \times g$ for 10 minutes at 4°C. The ethanol wash was repeated and the pellet was resuspended in 0.5 M HCl (0.8 mL), incubated for 15 minutes at 37°C, and centrifuged at $10\,000 \times g$ for 10 minutes.

The supernatant was retained and the nuclear proteins were precipitated by the addition of 100% trichloroacetic acid (TCA) to a final concentration of 20% TCA. The solution was incubated at 4°C for 5 minutes and centrifuged at $12\,000 \times g$ for 10 minutes. The pellet was washed twice in 1% 2-mercaptoethanol in acetone (500 μL). The final pellet was dried and stored at −20°C until gel electrophoresis analysis.

Preparation of the Human Protamine Standard

A human protamine standard was prepared as previously described (Mengual et al, 2003). Twenty semen samples were pooled to extract and quantitate a highly purified sperm protamine sample. Briefly, sperm were washed twice with 0.5 M HCl before protamine extraction to remove other acid-extracted proteins. After acid treatment, the protamines were extracted as described above. The protein extract contained highly purified protamine, as verified by gel electrophoresis and Western blot. The final protamine concentration was determined using the RC DC protein assay kit (Bio-Rad). The protamine extract was run using acid-acrylamide gel electrophoresis to determine the ratio of P1 to P2.

The final concentrations of P1 and P2 were calculated from the percent composition of each of the protamines in the total protamine standard. Subsequently, 1.52, 0.76, 0.38, and 0.19 μg of human sperm protamine standard were loaded in each gel and a standard regression curve was made to calculate the amount of protamine in each of the patient samples. The r^2 value of the regression curve was 0.98 or better for each gel run in this study.

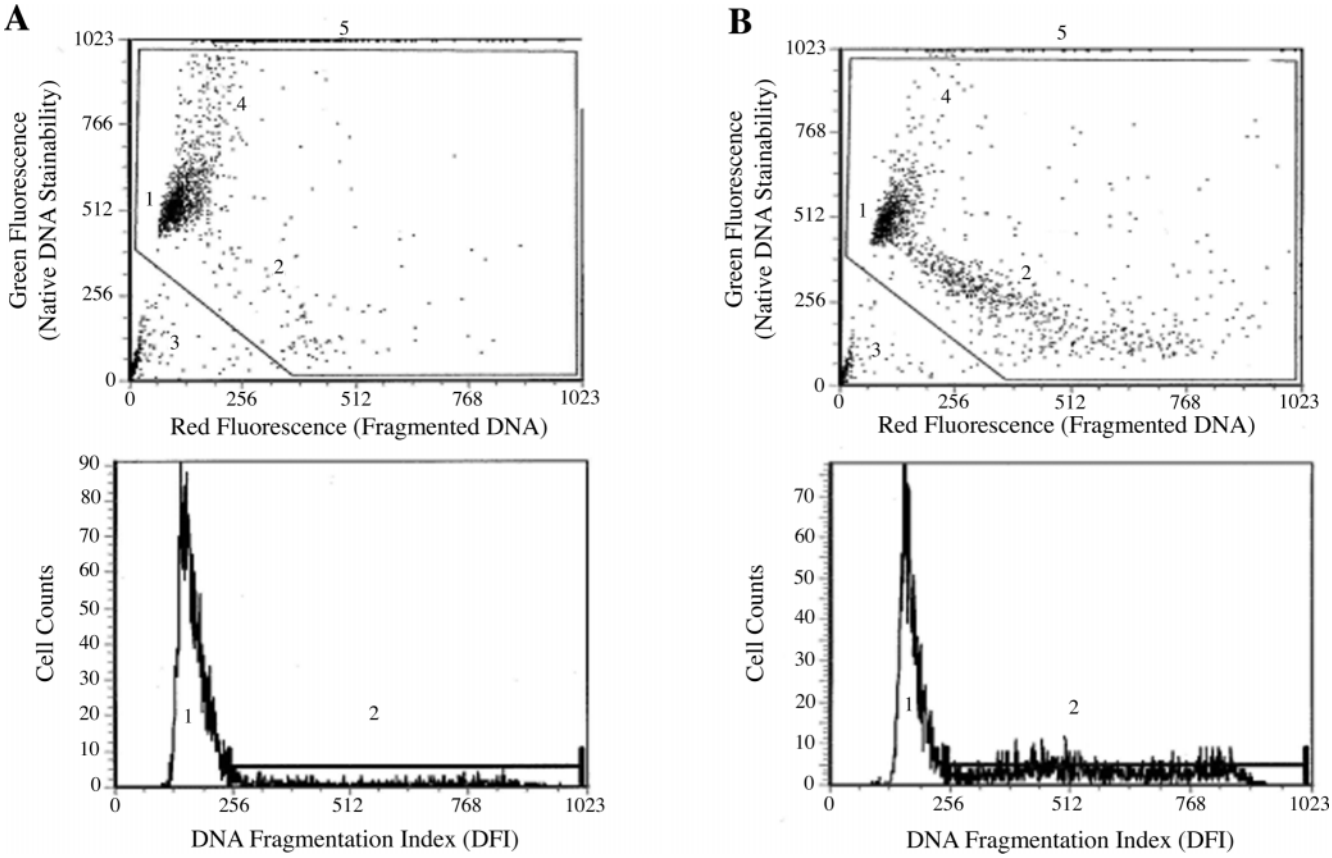

Figure 1. DNA Integrity Assay fluorescence cytograms for samples with **(A)** low and **(B)** high DNA fragmentation index (DFI) values. The top traces for both **A** and **B** show fragmented DNA staining vs native DNA staining. The numbered regions represent (1) cells with normal DNA, (2) cells with fragmented DNA, (3) seminal debris, (4) ''high-green'' stained cells that are not fully condensed, and (5) immature cells, bacteria, and cellular debris. The bottom panels for both **A** and **B** show a frequency histogram for recorded cellular staining intensity. The highlighted regions represent (1) cells with normal DNA and (2) cells with fragmented DNA. The DFI was calculated based on the amount of red fluorescence divided by the red plus green fluorescence. These particular cytograms show **(A)** low (11.4%) and **(B)** high DFI (43.3%).

P1/P2 Quantification

Acetic-acid urea gel electrophoresis was performed as previously described (Aoki et al, 2005). Briefly, the separating gel contained 20% acrylamide, 0.1% bisacrylamide, 0.9 N acetic acid, and 2.5 M urea. The stacking gel was comprised of 7.5% acrylamide, 0.2% bisacrylamide, 2.5 M urea, and 0.375 M potassium acetate (pH 4.0). Gels were stained with Coomassie blue using standard techniques and scanned using an Umax-SE scanner with the SilverFast scanning software package (Umax Technologies, Dallas, Tex).

Band intensities corresponding to P1 and P2 were quantified using National Institutes of Health Image-J software. P1 and P2 quantity were calculated against the standard curve generated from the human protamine standard (above). Protein quantity is reported as ng protein/10^6 sperm. Identity of protamine bands were established using Western blot analysis, as reported in a previous study (Carrell and Liu, 2001).

Table 1. *Reproducibility of protamine measurements validated via multiple extractions of aliquots from identical samples*

N	13
Mean P1/P2 ratio	0.84 ± 0.01 (CV = 1.19%)
Mean P1 concentration (ng/10^6 sperm)	443.2 ± 5.1 (CV = 1.15%)
Mean P2 concentration (ng/10^6 sperm)	525.2 ± 6.1 (CV = 1.16%)

Protamine Quantification Quality Control

We used 2 measures of quality control to ensure our protamine quantification protocol was valid and reproducible. First, aliquots of 20 × 10^6 sperm were made from a common semen sample taken from a pool of 20 semen samples. One of these aliquots was run with each round of extractions (n = 13). The resulting mean P1/P2 ratio (0.84 ± 0.01), P1 concentration (443.2 ± 5.1 ng/10^6 sperm), and P2 concentration (525.2 ± 6.1 ng/10^6 sperm) showed little sample-to-sample variation (coefficient of variation [CV]; 1.19%, 1.15%, and 1.16%, respectively) and ensured reproducible results within individual samples (Table 1).

Second, to evaluate variations in the P1/P2 ratio, [P1], and [P2] between ejaculates from the same individual, in another study we analyzed the semen from 2 different ejaculates (obtained 6 months apart) in 42 individuals (Aoki et al, 2005). Results indicate no significant differences between ejaculates with

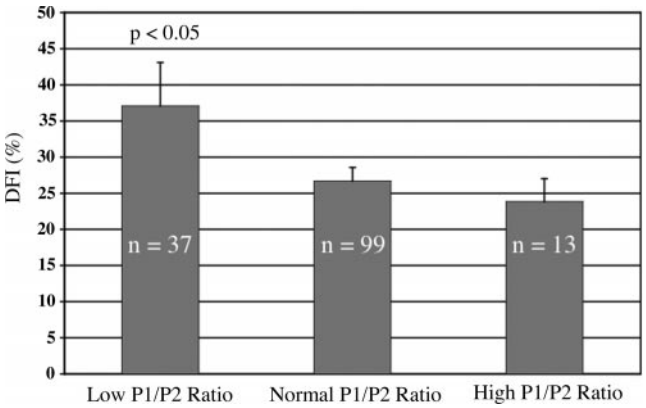

Figure 2. Mean DNA fragmentation index (DFI) levels among patients with low, normal, and high P1/P2 ratios. The Kruskal-Wallis test showed DNA fragmentation was significantly elevated in patients with low P1/P2 ratios (37.1 ± 6.02, n = 37) vs those with normal and high P1/P2 ratios (26.7 ± 1.9, n = 99; 23.8 ± 3.2, n = 13, respectively, $P < .05$).

respect to the P1/P2 ratio (1.03 ± 0.04 vs 1.11 ± 0.08), P1 concentration (560.4 ± 42.2 vs 571.9 ± 49.6 ng/10^6 sperm), or P2 concentration (535.5 ± 30.9 vs 527.1 ± 37.2 ng/10^6 sperm), as assessed by a paired Student's *t* test. The CV in the estimation of all sperm cell concentrations was within acceptable standards (3.9%).

Statistical Evaluation

Study subjects were stratified into 3 groups based on the P1/P2 ratio: normal P1/P2 patients, low P1/P2 patients, and high P1/P2 patients. Cut-off values for the low (<0.8) and high (>1.2) P1/P2 ratio groups were established in a previous study (Aoki et al, in press). In that study, the P1/P2 ratios of 95% of fertile men were found to lie within those ranges (0.8–1.2). The Kruskal-Wallis test was used to evaluate differences in DNA damage and semen analysis outcome measures (where available) between P1/P2 ratio groups.

Patients were also identified who displayed a significant underexpression of the protamine proteins (both P1 and P2). The normal ranges of P1 and P2 concentrations in fertile men were established in a previous study (Aoki et al, 2005). The critical values defining the concentrations at which P1 and P2 were significantly underexpressed are used in this study (483 ng P1/10^6 sperm and 474 ng P2/10^6 sperm, respectively). Chi-square analysis was used to detect differences in the incidence of significantly elevated DNA damage among patients underexpressing P1 or P2.

Spearman's correlation coefficient was used to evaluate the correlation between sperm protamine levels (P1/P2 ratios, P1, P2, and total protamine concentrations) and DNA damage (DFI).

Results

Relationship of Semen Quality to the P1/P2 Ratio

Gel electrophoresis scanning densitometry revealed that in the group of 149 infertility patients, we identified 37 with abnormally low P1/P2 ratios, 99 with normal P1/P2 ratios, and 13 with abnormally high P1/P2 ratios (Figure 2).

White blood cells (range, 0.0–0.4 × 10^6/mL) were present in 7.5% of the semen samples. None of the specimens qualified as leukocytospermic according to the WHO classification. Evaluation of semen quality parameters with respect to P1/P2 ratio groups revealed that sperm cell concentrations were significantly reduced in the low (34.7 ± 4.0, n = 37) and high (41.4 ± 15.3, n = 13) P1/P2 ratio groups compared with the normal group (69.7 ± 5.2, n = 97, $P < .001$). Sperm motility was significantly decreased in the low (17.6 ± 2.5, n = 17) and high (16.3 ± 3.9, n = 13) P1/P2 groups compared to the normal group (39.5 ± 1.7, n = 66, $P < .0001$). Normal sperm head morphology was significantly reduced in the low (14.1 ± 1.9, n = 17) and high (6.0 ± 3.5, n = 13) ratio groups compared to the normal group (25.5 ± 1.3, n = 66, $P < .05$; Table 2).

Relationship of Semen Quality to DFI

Evaluation of semen quality parameters with respect to DFI categories revealed that sperm cell concentrations were significantly reduced in the moderate (54.0 ± 5.8, n = 48) and high (45.1 ± 7.6, n = 53) DFI groups vs the low DFI group (70.9 ± 7.4, n = 48, $P < .05$; Table 2). Progressive motility significantly declined with increasing DFI group. The high DFI group (20.2 ± 2.7, n

Table 2. *Semen quality parameters within DNA fragmentation index (DFI) and P1/P2 ratio categories*

	Normal DFI	Moderate DFI	Abnormal DFI	*P* Value
Sperm concentration (10^6/mL)	70.9 ± 7.4	54.0 ± 5.8	45.1 ± 7.6	<.05*
Progressive motility (%)	42.9 ± 2.6	29.6 ± 2.5	20.2 ± 2.7	<.001*
Normal head shapes (%)	28.1 ± 2.0	19.1 ± 2.3	17.3 ± 1.6	<.005*
	Low P1/P2	Normal P1/P2	High P1/P2	*P* Value
Sperm concentration (10^6/mL)	34.7 ± 4.0	69.7 ± 5.2	41.4 ± 15.3	<.001†
Progressive motility (%)	17.6 ± 2.5	39.5 ± 1.7	16.3 ± 3.9	<.0001†
Normal head shapes (%)	14.1 ± 1.9	25.5 ± 1.3	6.0 ± 3.5	<.005‡

* Significant difference between column 1 and columns 2 and 3.
† Significant difference between column 2 and columns 1 and 3.
‡ Significant difference among all columns.

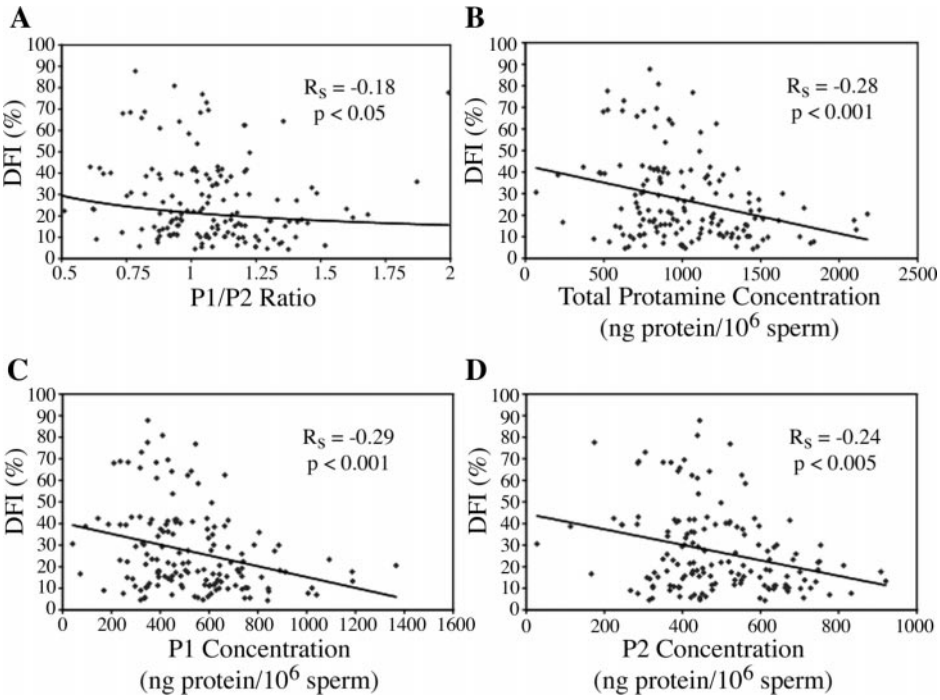

Figure 3. Correlations between DNA fragmentation index (DFI) and **(A)** the P1/P2 ratio, **(B)** total protamine concentration, **(C)** P1 concentration, and **(D)** P2 concentration. Spearman's correlation coefficient revealed significant inverse correlations between DFI and **(A)** the P1/P2 ratio (R_s, −0.18, $P < .05$), **(B)** total protamine concentration (R_s, −0.28, $P < .001$), **(C)** P1 concentration (R_s, −0.29, $P < .001$), and **(D)** P2 concentration (R_s, −0.24, $P < .005$).

= 34) had significantly lower progressive motility than the moderate DFI group (29.6 ± 2.5, n = 33). In turn, the moderate DFI group had significantly lower progressive motility than the low DFI group (42.9 ± 2.6, n = 34, $P < .001$; Table 2). Normal head morphology was also significantly reduced in the moderate (19.1 ± 2.3, n = 33) and high (17.3 ± 1.6, n = 34) DFI groups vs the low DFI group (28.1 ± 2.0, n = 34, $P < .005$; Table 2).

Relationship of Sperm Protamine Content to DFI

DNA fragmentation was significantly elevated in patients with low P1/P2 ratios (37.1 ± 6.02) vs those with normal and high P1/P2 ratios (26.7 ± 1.9, 23.8 ± 3.2, respectively, $P < .05$; Figure 2).

A negative relationship was observed between the explanatory variables (P1/P2 ratio, P1, P2, and total protamine concentrations) and the DFI outcome variable (Figure 3). Spearman's correlation coefficient revealed a significant inverse correlation between DFI and the P1/P2 ratio (R_s, −0.18, $P < .05$). A greater significant inverse correlation was observed between DFI and P1 concentration (R_s, −0.29, $P < .001$), P2 concentration (R_s, −0.24, $P < .005$), and total protamine concentration (R_s, −0.28, $P < .001$).

A group of 70 patients were identified to show significant P1 underexpression. Chi-square analysis revealed a significant increase in the frequency of patients with abnormal DFI within this group compared to the 79 patients that expressed P1 normally ($P < .005$; Table 3). Additionally, 79 patients were identified with significant P2 underexpression. A significant increase in the frequency

Table 3. *Frequency of P1 and P2 underexpression within DNA fragmentation index (DFI) categories*

	Normal DFI (%)	Moderate DFI (%)	Abnormal DFI (%)	*P*
P1 normally expressed	42 (33/79)	37 (29/79)	21 (17/79)	<.005
P1 underexpressed	21 (15/70)	27 (19/70)	52 (36/70)	
P2 normally expressed	41 (29/70)	36 (25/70)	23 (16/70)	<.01
P2 underexpressed	24 (19/79)	29 (23/79)	47 (37/79)	
P1 & P2 normally expressed*	41 (20/49)	37 (18/49)	22 (11/49)	<.01
P1 & P2 underexpressed*	22 (8/37)	22 (8/37)	56 (21/37)	

* Patients included were only those with a normal P1/P2 ratio.

of abnormal DFI patients was observed in the group underexpressing P2 vs in patients expressing P2 normally ($P < .01$; Table 3). Therefore, there was a significant increase in DNA fragmentation observed in patients with diminished levels of either P1 or P2. Meanwhile, in patients with a normal P1/P2 ratio, there was a significant increase in DNA fragmentation observed in patients underexpressing both P1 and P2 vs in those normally expressing P1 and P2 ($P < .01$; Table 3).

Discussion

In this study, we provide the first description of the relationship between DNA integrity and direct quantitative measurements of human sperm protamine quantity. Previous studies have attempted to correlate DNA damage with indirect measurements of protamine levels using the chromomycin A_3 (CMA_3) fluorochrome (Bianchi et al, 1993; Manicardi et al, 1995). CMA_3 is a fluorochrome that competes with protamines for DNA binding and that is indirectly related to the degree of sperm protaminization (Bianchi et al, 1993). Results of these studies indicate that protamine deficiency is related to increasing levels of DNA damage (Bianchi et al, 1993; Manicardi et al, 1995).

In the present study, direct quantitative measurements of sperm protamine concentrations revealed that P1, P2, and total protamine quantity inversely correlated with DNA fragmentation. Furthermore, patients who underexpressed either P1 or P2 displayed elevated DNA damage much more frequently than patients who expressed the protamines normally. This increase in DNA damage was also observed in patients with a normal P1/P2 ratio who underexpressed both P1 and P2. These data are consistent with those obtained in studies using CMA_3 staining that indicate that protamine deficiency is significantly related to increases in DNA damage. In addition, our data highlight the fact that both P1 and P2 contribute to this relationship.

The P1/P2 ratio was also correlated with DNA fragmentation. Numerous studies have implicated aberrant P1/P2 ratios in human male infertility (Balhorn et al, 1988; de Yebra et al, 1993, 1998; Khara et al, 1997; Carrell and Liu, 2001; Mengual et al, 2003; Aoki et al, 2005). The semen quality data presented here are consistent with those from these studies and show significantly reduced sperm counts, motility, and head morphology. In particular, patients with abnormally low P1/P2 ratios (<0.8) are reported to have severely affected sperm quality (Aoki et al, 2005), consistent with the present observation that DNA fragmentation is elevated in low–P1/P2 ratio patients. Although there is no fertile control group in the present study, a number of reports have established that protamine abnormalities and DNA damage are rare in men of known fertility (Evenson et al, 1999; Evenson and Jost, 2000; Carrell and Liu, 2001; Aoki et al, 2005).

Numerous studies have related sperm DNA integrity to semen quality. Many of these studies indicate that sperm motility, concentration, viability, and morphology correlate with DNA fragmentation (Evenson et al, 1991; Sun et al, 1997; Irvine et al, 2000; Muratori et al, 2000; Tomlinson et al, 2001; Zini et al, 2001; Sharma et al, 2004). Our data are consistent with those of these studies and show progressively diminished counts, motility, and morphology across the low, moderate, and high DFI categories. However, others have reported no meaningful correlation between DNA integrity and standard semen parameters (Evenson and Jost, 2000; Larson et al, 2000; Evenson et al, 2002).

The results of this study indicate that normally expressed sperm protamines may serve a protective function against DNA damage. Elegant studies in mice have established that protamine haplo-insufficiency is a direct causative factor involved in sperm DNA damage induction (Cho et al, 2003). Although a number of reports have established a link between sperm protamine content and chromatin structure, in humans the current data fall short of proving this hypothesis.

The protamines are critical for proper sperm chromatin packaging (Balhorn et al, 2000). Therefore, it is not surprising that sperm protamine concentrations correlate with DNA integrity. The protamine proteins are rich in cysteine content, which facilitates both inter- and intraprotamine cross-links via disulphide bond formation (Fuentes-Mascorro et al, 2000). Several studies have demonstrated that sperm chromatin stability is dependent on the quantity of these disulphide cross-links (Dadoune, 1995; Fuentes-Mascorro et al, 2000). Experimental evidence indicates that a reduction in disulphide bonding may alter sperm chromatin structure and reduce nuclear integrity (Kosower et al, 1992; Love and Kenney, 1999). However, the data remain conflicting on this issue, as one study demonstrated no meaningful correlation between sperm protamine thiol status and DNA denaturation in stallions (Evenson et al, 2000).

Additionally, the use of protamine-deficient human sperm for intracytoplasmic sperm injection results in sperm premature chromatin decondensation, indicating a less stable sperm chromatin in these patients (Nasr-Esfahani et al, 2004). Taken together, these studies indicate that protamine-deficient sperm adopt a less-stable chromatin structure, which may possibly arise as a consequence of decreased inter- and intraprotamine interactions, rendering them more susceptible to DNA damage. Future human studies are needed to test this hypothesis and to evaluate the relationship between sperm protamine levels, thiol content, and DNA damage.

In addition to absolute protamine quantity, the P1/P2

ratio also appears to be critical for chromatin stability. Although the P1/P2 ratio varies between genera, within a species it is highly conserved (Corzett et al, 2002). In humans, aberrations in the 1-to-1 ratio of P1 to P2 correlate significantly with male infertility, and the results of this study indicate that patients with abnormally low P1/P2 ratios have significantly elevated DNA fragmentation (Chevaillier et al, 1987; Balhorn et al, 1988; Belokopytova et al, 1993; de Yebra et al, 1993, 1998; Carrell and Liu, 2001; Aoki and Carrell, 2003; Aoki et al, 2005). However, patients with abnormally high P1/P2 ratios presented with DNA fragmentation levels comparable to those patients with normal P1/P2 ratios. These data are surprising considering the correlations between abnormal semen quality and high P1/P2 ratios. DNA fragmentation differences in these high–P1/P2 ratio patients may not have been elucidated because of the low number of patients identified. Further studies are needed that evaluate DNA fragmentation in a larger group of high–P1/P2 ratio patients.

The effects of aberrant protamine ratios on human sperm chromatin structure have not yet been elucidated. Clues may lie in studies using sophisticated atomic force microscopy and proton-induced X-ray emission spectroscopy, which provide insights into the details of protamine-DNA binding interactions (Hud et al, 1994; Bench et al, 1996; Balhorn et al, 2000). First, P1 and P2 are not randomly distributed along the DNA; instead, they tend to form characteristic clusters within species (Balhorn et al, 2000; Corzett et al, 2002). Thus, aberrations in the species-specific P1/P2 ratio may disrupt this characteristic protamine-DNA binding, possibly influencing secondary chromatin structure.

Second, we now have a description of the length of DNA that must be covered by each P1 and P2 molecule to facilitate their relative binding. Based on amino acid sequencing, P2 requires a longer stretch of DNA (approximately 15 bp) than P1 (approximately 11 bp) for proper chromatin incorporation (Bench et al, 1996). This may provide evidence for abnormal chromatin packaging when P2 is more abundant that P1, as is the case in patients with significantly reduced P1/P2 ratios. Given this scenario, physical size constraints dictate that there would be an overall reduction in the amount of protamine bound to the DNA, thereby reducing the overall number of disulphide cross-links.

Alternatively, chromatin structure aberrations and perturbations in the P1/P2 ratio may themselves be unrelated and may simply reflect generalized problems during spermiogenesis. Further studies are critical for our understanding of these proposed chromatin structure changes in patients with aberrant P1/P2 ratios.

Additional studies are necessary to fully understand how these various protamine aberrations influence abnormal DNA integrity. We suggest human studies to evaluate the protective role protamines play against sperm DNA damage. Additionally, it would be useful to evaluate the chromatin structure in patients with aberrant P1/P2 ratios to detect abnormal protamine-DNA interactions and perturbations of normal disulfide cross-linking. In the present study, only a limited number of severely oligospermic patients were included as a result of cell concentration requirements for the DNA integrity test. Therefore, a larger population of patients with severe oligospermia should be evaluated for protamine content and DNA integrity.

To conclude, we provide the first study investigating the relationship between DNA damage and direct quantitative measures of protamine levels in human sperm. Protamine concentrations (P1, P2, and total protamine) inversely correlate with the DNA fragmentation index. Additionally, patients with low P1/P2 ratios have markedly increased DNA damage. In light of recent reports highlighting reduced assisted reproductive technique (ART) fecundity in cases using DNA-damaged sperm, these data indicate that the sperm protamines may be of clinical significance in ARTs.

References

Aoki VW, Carrell DT. Human protamines and the developing spermatid: their structure, function, expression and relationship with male infertility. *Asian J Androl.* 2003;5:315–324.

Aoki VW, Liu L, Carrell DT. Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. *Hum Reprod.* 2005;20:1298–1306.

Balhorn R, Brewer L, Corzett M. DNA condensation by protamine and arginine-rich peptides: analysis of toroid stability using single DNA molecules. *Mol Reprod Dev.* 2000;56:230–234.

Balhorn R, Reed S, Tanphaichitr N. Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. *Experientia.* 1988;44:52–55.

Belokopytova IA, Kostyleva EI, Tomilin AN, Vorob'ev VI. Human male infertility may be due to a decrease of the protamine p2 content in sperm chromatin. *Mol Reprod Dev.* 1993;34:53–57.

Bench GS, Friz AM, Corzett MH, Morse DH, Balhorn R. DNA and total protamine masses in individual sperm from fertile mammalian subjects. *Cytometry.* 1996;23:263–271.

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. *Biol Reprod.* 1993;49:1083–1088.

Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod.* 2004;19:1401–1408.

Carrell DT, Liu L. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. *J Androl.* 2001;22:604–610.

Chevaillier P, Mauro N, Feneux D, Jouannet P, David G. Anomalous protein complement of sperm nuclei in some infertile men. *Lancet.* 1987;2:806–807.

Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xu Z, Schultz RM, Hecht NB, Eddy EM. Protamine 2 deficiency leads to sperm

DNA damage and embryo death in mice. *Biol Reprod.* 2003;69:211–217.

Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM. Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nat Genet.* 2001;28:82–86.

Corzett M, Mazrimas J, Balhorn R. Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. *Mol Reprod Dev.* 2002; 61:519–527.

Dadoune JP. The nuclear status of human sperm cells. *Micron.* 1995;26: 323–345.

de Yebra L, Ballesca JL, Vanrell JA, Bassas L, Oliva R. Complete selective absence of protamine p2 in humans. *J Biol Chem.* 1993;268: 10553–10557.

de Yebra L, Ballesca JL, Vanrell JA, Corzett M, Balhorn R, Oliva R. Detection of p2 precursors in the sperm cells of infertile patients who have reduced protamine p2 levels. *Fertil Steril.* 1998;69:755–759.

Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment. *Methods Cell Sci.* 2000;22:169–189.

Evenson D, Jost L, Varner D. Stallion sperm nuclear protamine -sh status and susceptibility to DNA denaturation are not strongly correlated. *J Reprod Fert Suppl.* 2000;56:401–406.

Evenson DP, Jost LK, Baer RK, Turner TW, Schrader SM. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod Toxicol.* 1991;5:115–125.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod.* 1999;14:1039–1049.

Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl.* 2002;23:25–43.

Fischer MA, Willis J, Zini A. Human sperm DNA integrity: correlation with sperm cytoplasmic droplets. *Urology.* 2003;61:207–211.

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

Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB, Kruger TF. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril.* 2004;81: 965–972.

Hud NV, Milanovich FP, Balhorn R. Evidence of novel secondary structure in DNA-bound protamine is revealed by raman spectroscopy. *Biochemistry.* 1994;33:7528–7535.

Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ. DNA integrity in human spermatozoa: relationships with semen quality. *J Androl.* 2000;21:33–44.

Khara KK, Vlad M, Griffiths M, Kennedy CR. Human protamines and male infertility. *J Assist Reprod Genet.* 1997;14:282–290.

Kosower NS, Katayose H, Yanagimachi R. Thiol-disulfide status and acridine orange fluorescence of mammalian sperm nuclei. *J Androl.* 1992;13:342–348.

Larson KL, DeJonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod.* 2000;15: 1717–1722.

Love CC, Kenney RM. Scrotal heat stress induces altered sperm chromatin structure associated with a decrease in protamine disulfide bonding in the stallion. *Biol Reprod.* 1999;60:615–620.

Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, Sakkas D. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin a3 accessibility. *Biol Reprod.* 1995;52:864–867.

Mengual L, Ballesca JL, Ascaso C, Oliva R. Marked differences in protamine content and p1/p2 ratios in sperm cells from Percoll fractions between patients and controls. *J Androl.* 2003;24:438–447.

Muratori M, Piomboni P, Baldi E, Filimberti E, Pecchioli P, Moretti E, Gambera L, Baccetti B, Biagiotti R, Forti G, Maggi M. Functional and ultrastructural features of DNA-fragmented human sperm. *J Androl.* 2000;21:903–912.

Nasr-Esfahani MH, Razavi S, Mozdarani H, Mardani M, Azvagi H. Relationship between protamine deficiency with fertilization rate and incidence of sperm premature chromosomal condensation post-ICSI. *Andrologia.* 2004;36:95–100.

Oliva R, Dixon GH. Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Prog Nucleic Acid Res Mol Biol.* 1991; 40:25–94.

Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril.* 2004;82:378–383.

Sharma RK, Said T, Agarwal A. Sperm DNA damage and its clinical relevance in assessing reproductive outcome. *Asian J Androl.* 2004; 6:139–148.

Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol Reprod.* 1997;56:602–607.

Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M, Sakkas D. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. *Hum Reprod.* 2001;16:2160–2165.

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.

Virant-Klun I, Tomazevic T, Meden-Vrtovec H. Sperm single-stranded DNA, detected by acridine orange staining, reduces fertilization and quality of ICSI-derived embryos. *J Assist Reprod Genet.* 2002;19: 319–328.

Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril.* 2004;81:1289–1295.

Zini A, Kamal K, Phang D, Willis J, Jarvi K. Biological variability of sperm DNA denaturation in infertile men. *Urology.* 2001;58:258–261.