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Nature of DNA Damage in Ejaculated Human Spermatozoa and the Possible Involvement of Apoptosis[1]

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ABSTRACT

Numerous studies have shown the presence of DNA strand breaks in human ejaculated spermatozoa. The nature of this nuclear anomaly and its relationship to patient etiology is however poorly understood. The aim of this study was to investigate the relationship between nuclear DNA damage, assessed using the TUNEL assay and a number of key apoptotic markers, including Fas, Bcl-x, and p53, in ejaculated human spermatozoa from men with normal and abnormal semen parameters. We also determined the nature of the DNA damage by examining the percentage of ejaculated spermatozoa exhibiting DNA damage using the comet assay and by challenging sperm chromatin to attack by micrococcal nuclease S7 and DNase I. We show that TUNEL positivity and apoptotic markers do not always exist in unison; however, semen samples that had a low sperm concentration and poor morphology were more likely to show high levels of TUNEL positivity and Fas and p53 expression. In addition, the DNA damage in ejaculated human sperm is represented by both single- and double-stranded DNA breaks, and access to the DNA is restricted by the compacted nature of ejaculated spermatozoa. This DNA protection is poorer in men with abnormal semen parameters. We propose that the presence of DNA damage is not directly linked to an apoptotic process occurring in spermatozoa and arises due to problems in the nuclear remodeling process. Subsequently, the presence of apoptotic proteins in ejaculated spermatozoa may be linked to defects in cytoplasmic remodeling during the later stages of spermatogenesis.

apoptosis, gamete biology, sperm, spermatogenesis

INTRODUCTION

During spermatogenesis, a complex and dynamic process of proliferation and differentiation occurs as spermatogonia are transformed into mature spermatozoa. This unique process involves a series of meioses and mitoses, changes in cytoplasmic architecture, replacement of somatic cell-like histones with transition proteins, and the final addition of protamines, leading to a highly packaged chromatin [1–4]. The tissue remodeling that occurs during spermatogenesis is unique in that it is one of the only mammalian systems that produces a cell type in which the nucleus is transcriptionally inactive and a large part of the cell is stripped off.

Not surprisingly, there is a large body of evidence that some of the ejaculated spermatozoa produced in the human possess a variety of abnormalities at the nuclear, cytoskeletal, and organelle levels and that these anomalies impact fertility [5–7]. The human is of particular interest as a single ejaculate normally contains a heterogeneous population of spermatozoa. In the human, it has been known for many years that the chromatin of the mature sperm nucleus can be abnormally packaged [7]. In addition, abnormal chromatin packaging and nuclear DNA damage appear to be linked [7–11], and there is a strong association between the presence of nuclear DNA damage in the mature spermatozoa of men and poor semen parameters [12–14].

A number of studies have proposed that the presence of spermatozoa with damaged DNA is indicative of apoptosis. The TUNEL technique, a process that identifies DNA breaks by labeling 3′-OH termini using exogenous terminal deoxynucleotidyl transferase, was used by Gorczyca et al. [10] to identify a population of spermatozoa in the ejaculate that were believed to be apoptotic. Numerous other studies using the same technique have followed [11–15]. We have previously shown the presence of one key apoptotic protein, Fas, on mature spermatozoa [16], while others have shown the possible presence of annexin V [17].

The aim of this study was to further investigate the relationship between nuclear DNA damage and a number of key apoptotic markers, including Fas, Bcl-x, and p53, in ejaculated human spermatozoa from men with normal and abnormal semen parameters. We also attempted to determine the nature of the DNA damage in men with normal and abnormal semen parameters by examining the percentage of ejaculated spermatozoa exhibiting DNA damage using the comet assay and by challenging sperm chromatin to attack by micrococcal nuclease S7 (MNase) and DNase I.

MATERIALS AND METHODS

Sample Collection and Preparation

To investigate the presence of apoptotic markers and nuclear damage in ejaculated sperm, we obtained samples for investigation after routine semen analysis from the andrology laboratory of the Assisted Conception Unit, Birmingham Women's Hospital, Birmingham, UK. The use of discarded human spermatozoa was approved by the unit's ethics subcommit-

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tee. Semen parameters were assessed using the criteria for normal concentration and motility according to the World Health Organization (W.H.O.) [18], while morphology was assessed using the strict criteria of Kruger et al. [19], whereby the cutoff for normal morphology is set at 4%. Samples were collected by masturbation after 2–4 days' abstinence and were allowed to liquefy for at least 30 min. The samples were then separated into 45% and 90% fractions by centrifugation on density gradients to remove excess cellular debris. For each sample, the 45% and 90% fractions were aspirated, combined, and washed with PBS plus 0.5% human serum albumin (HSA) (Bio Products Laboratory, Hertfordshire, UK). Washed sperm were then labeled using either monoclonal antibodies or a TUNEL kit.

Antibodies

The monoclonal antibodies used throughout were Ab-2, immunoglobulin (Ig) G_1 (anti-CD95) (Fas); anti-p53 (Calbiochem, Nottingham, UK); anti-Bcl-x, clone 44, IgG_1 (Transduction Laboratories, San Diego, CA); and anti-Bcl-xL, H5, IgG_1 (Santa Cruz Biotechnology, Santa Cruz, CA). These antibodies were detected by incubation of sections with fluorescein isothiocyanate (FITC)-conjugated polyclonal sheep anti-mouse Ig (Sigma Pharmaceuticals, Dorset, UK) or phycoerythrin (PE)-conjugated polyclonal goat anti-mouse Ig (Sigma).

Immunofluorescence Staining for Fas, Bcl-x, and p53

After the initial wash, sperm were resuspended in 200 μl PBS plus 0.5% HSA, primary antibody was added, and the sperm and antibody were incubated for 1 h at 37°C. Antibodies to intracellular antigens were resuspended in 0.1% Triton X-100 in sodium citrate before addition to samples and incubation, whereas Fas labeling was performed without the addition of Triton X-100 so as to specifically label membrane proteins. Samples were washed, resuspended in 100 μl of PBS plus 0.5% HSA containing the FITC-labeled secondary detection antibody, and incubated for a further hour as previously described. In all analyses, spermatozoa labeled with only the secondary antibody were assessed as controls. Additional controls were also performed for the Bcl-x and p53 antibodies in which no Triton X-100 pretreatment was performed, so as to dismiss the observation of nonspecific binding to the extracellular surface of the spermatozoa. Samples were washed and finally fixed in 1.5% paraformaldehyde (BDH, Coventry, UK) in PBS plus 0.5% HSA. Samples were stored at 4°C before being analyzed by flow cytometry (fluorescence-activated cell sorting).

TUNEL Labeling

TUNEL labeling was carried out using a Cell Death Detection kit from Boehringer-Roche (East Sussex, UK) and was performed according to the manufacturer's instructions. Briefly, washed sperm were resuspended in 3.7% paraformaldehyde (BDH) for at least 30 min. Samples were washed in PBS before being resuspended for 2 min in 200 μl of 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate (Sigma). After a further wash, 50 μl of TUNEL reagent was added to each sample (except for the negative control, to which reagent without enzyme was added), and the sample and regents were incubated for 1 h at 37°C. The samples were then washed with PBS and analyzed immediately by flow cytometry.

Double Labeling

Double labeling was performed for 3 pairs of markers: Bcl-x and TUNEL, p53 and TUNEL, and Bcl-x and propidium iodide (PI). Labeling was first carried out as for Bcl-x or p53 only, but using a PE-conjugated secondary antibody rather than an FITC-conjugated secondary antibody. Cells were fixed for 30 min with 3.7% paraformaldehyde before continuing with TUNEL labeling as described previously.

Flow Cytometry

Flow cytometry was performed using a FACscan (Becton Dickinson, Lincoln Park, NY). In all analyses, spermatozoa labeled with only the secondary antibody were assessed as controls. The controls performed showed that the antibodies were not binding to the spermatozoa nonspecifically. Debris was gated out based on light scatter measurements. A minimum of 10 000 spermatozoa per sample was analyzed.

Assessment of the Nature of DNA Damage and the Percentage of Ejaculated Spermatozoa Exhibiting DNA Damage Using the Comet Assay

In the analysis of sperm DNA, we compared samples from patients who had semen parameters above and below those classified as normal by W.H.O. criteria [18]. About 2×10^4 sperm from the semen sample were mixed at 37°C with a low-melting point agarose gel solution (0.8% in PBS). The solution was carefully pipetted onto a glass microscope slide precovered with normal-melting point agarose (1.5% in PBS), a coverslip was then placed on top, and the agarose was allowed to solidify. The coverslip was removed, and a low-melting point agarose gel solution (0.5% in PBS) was used to form a third layer. The slides with coverslips removed were then placed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X, and 10 mM dithiothreitol [DTT] at a pH of 10) for 1 h at 4°C. The slides were then incubated at 37°C in 10 μg/ml of proteinase K in lysis buffer for 1 h.

After the proteinase K (Sigma) solution was drained from the slides, they were placed in a horizontal electrophoresis tank filled with freshly prepared electrophoresis solution (Tris-acetate-EDTA [TAE], pH 11, for the alkaline comet assay; TAE, pH 7.3, for the neutral comet assay) and left undisturbed for 20 min to allow for DNA unwinding.

Electrophoresis was performed at room temperature, at 10 V and 6 mA, for 15 min.

The slides were washed with a neutralizing solution of 0.4 M Tris, pH 7.5 to remove alkali and detergents. After a neutralization step, the slides were each stained with ethidium bromide (20 μg/ml) and mounted with a coverslip. Identification of comets was performed as suggested by Aravindan et al. [20]. In particular, the ethidium bromide-stained agarose gel on the microscopic slide support was gently washed in distilled water and viewed with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) at 120× magnification using the appropriate UV lamp excitation. Within random fields, 200 of the brightly fluorescing sperm from each slide were counted and scored for the presence and absence of comets. A positive score was given to those cells exhibiting a width:length ratio different from 1.

Assessment of the Nature of DNA Damage by Agarose Gels

Human spermatozoa were permeabilized with Triton X-100 (0.1%, Sigma, Buchs, Switzerland) for 30 min at 37°C and then washed twice with IVF medium (Scandinavian IVF, Gotheburg, Sweden). Four experiments were performed using different sperm samples in each replicate. The spermatozoa were incubated at 37°C for 30 min with different concentrations of MNase (Boehringer Mannheim, Germany) in a buffer containing 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, 0.5 mM DTT, 0.25 mM sucrose, 0.05 mM $CaCl_2$, and 3 mM $MgCl_2$ or with DNase I (Boehringer Mannheim) in 3 mM $CaCl_2$ as described previously [21–23]. Enzyme activity was stopped by adding a mixture of EDTA, EGTA, and *N*-laurylsarcosine at final concentrations of 12.5 mM, 12.5 mM, and 62.5 mM, respectively.

The sperm nuclei were treated with digestion buffer (100 mM NaCl; 25 mM EDTA, pH 8; 0.2% SDS; 2 μg; proteinase K; Sigma) for 16 h. at 60°C. The DNA was extracted with phenol:chloroform:isoamylalcohol (25:24:1; Sigma) and precipitated with ethanol (100%, 2.5 volumes) and NaCl (0.5 M) at −70°C for 1 h. The DNA was collected by centrifugation at 15 000 × *g* for 10 min, washed twice with ethanol (70%), and resuspended in buffer containing 10 mM Tris and 1 mM EDTA (TE buffer; Sigma). The amount of DNA was quantified using a spectrophotometer at optical densities of 260 and 280 nm (an optical density at 260 nm of 1 indicates a concentration of 50 μg/ml). The samples were prepared in loading buffer (50% glycerol, 0.1 M EDTA, 0.3 M NaOH, 0.01% bromphenol blue), and 3 μg of DNA per well were resolved in 0.5% or 1.5% agarose gels in TBE buffer (89 mM Tris base, 89 mM borate, 2 mM EDTA; Sigma). The gel was run at 80 V (Bio-Rad submerged gel nucleic acid electrophoresis system, Zurich, Switzerland), stained with ethidium bromide, and photographed (Fuji instant black and white film FP-300B, Fuji photo film, Tokyo, Japan).

Alkaline agarose gels were prepared as previously described [24]. Briefly, agarose gels were prepared and solidified as previously described and equilibrated in alkaline buffer (30 mM NaOH, 2 mM EDTA) for 18 h at room temperature. The gels were run, neutralized in Tris-HCl buffer (500 mM, pH 8) for 18 h at room temperature, and then stained with ethidium bromide.

Statistical Analysis

Analysis of the data was carried out using a Statistical Package for the Social Sciences (SPSS 9.0 for Windows; SPSS Inc., Chicago, IL). The data for each marker (Fas, Bcl-x, TUNEL, and p53) were plotted in a histogram and showed a skewed distribution. To select cutoff values for each marker, the fifth percentile increment closest to the mean was used. Values were compared using the Mann-Whitney test, and correlation tests were performed using the Pearson correlation test with continuous outcomes.

RESULTS

Associations of Fas, Bcl-x, p53, and TUNEL Positivity with Sperm Concentration, Motility, and Morphology

Immunofluorescence staining of mature human spermatozoa showed that expression of Fas, Bcl-x, and p53 varied between samples, as did TUNEL positivity. The overall results for all samples assessed during the course of the study are shown in Table 1. After distribution of the data set for each marker, the fifth percentile closest to the mean of the population values was used as a cutoff value for high and low levels of the expression of each marker. The values used were 10% for Fas and 20% for TUNEL, Bcl-x, and p53. Associations between the expression of these molecules or the presence of DNA breaks and the concentration, motility, and morphology parameters for each sample were then assessed.

When expression of the three apoptotic proteins and TUNEL positivity was evaluated with respect to sperm concentration, it was found that samples with high Fas, p53, and TUNEL positivity were more likely to have a low concentration of spermatozoa, and samples with low Fas, p53, and TUNEL positivity were more likely to have a high concentration of spermatozoa (Fig. 1). The Fas results concur with our previously published data relating Fas expression and semen parameters [16]. This same pattern was also noted on evaluation of Fas, p53, and TUNEL positivity and sperm morphology: samples having high Fas, p53, and TUNEL positivity exhibited a low percentage of normal forms, whereas samples with low Fas, p53, and TUNEL positivity displayed a high percentage of normal forms. Bcl-x expression was not found to be associated with concentration, motility, or morphology of sperm (Fig. 1).

When a correlation analysis was performed for the different markers, we found negative correlations between positivity for Fas (n = 132; $r = -0.21$; $P = 0.01$), TUNEL (n = 65; $r = -0.33$; $P = 0.01$), and p53 (n = 30; $r = -0.33$; $P = 0.05$) and sperm concentration. Similarly, we found negative correlations between positivity for Fas (n = 131; $r = -0.20$; $P = 0.01$), TUNEL (n = 64; $r = -0.39$; $P = 0.004$), and p53 (n = 30; $r = -0.41$; $P = 0.02$) and sperm morphology. There were no correlations between Fas, TUNEL, and p53 positivity and sperm motility.

Finally, as Bcl-x can be either the long (x_L) or short (x_S) form, experiments were carried out using both an anti-Bcl-x monoclonal antibody and a specific anti-Bcl-x_L monoclonal antibody, in an attempt to determine which form was expressed in these samples and whether this affected the lack of a relationship with concentration, motility, and morphology. In 16 sperm samples, the correlation between Bcl-x and Bcl-x_L expression was found to be highly significant ($r = 0.96$; $P < 0.001$), suggesting that Bcl-x_L is one of the forms present; however, the relative proportions of Bcl-x_L and Bcl-x_S remains to be determined.

TABLE 1. Descriptive statistics for Fas, Bcl-x, p53, and TUNEL expression in the samples examined.

Marker	No. of samples stained	% Positive spermatozoa			
		Mean	SEM	Minimum	Maximum
Fas	132	9.7	0.5	1.8	47.3
Bcl-x	68	22.3	2.2	2.9	81.2
TUNEL	68	20.7	1.9	1.0	71.7
p53	30	21.7	5.2	6.7	80.8

Associations Between Fas, Bcl-x, p53, and TUNEL Positivity

Having determined the aforementioned relationships, the expressions of Fas, Bcl-x, and p53 and TUNEL positivity were compared with each other (Table 2). This analysis was initially performed by testing for the Fas, Bcl-x, and TUNEL markers in the same sperm sample. Samples that had high Fas expression (>10%) were more likely to have high Bcl-x expression (>20%) ($r = 0.42$; $P < 0.001$), whereas Fas expression was not significantly associated with TUNEL positivity (Table 2). Although samples with high Bcl-x expression (>20%) were significantly more likely to have high Fas expression, Bcl-x expression was not significantly associated with TUNEL positivity (Table 2). Neither Fas nor Bcl-x expression was significantly different for samples with high versus low TUNEL positivity (Table 2). The only markers that were significantly correlated between different sperm samples were Fas and Bcl-x (n = 68; $r = 0.30$; $P = 0.01$).

When the same sperm samples evaluated for p53 positivity (n = 30) were also evaluated for TUNEL, Fas, and Bcl-x positivity, a significant correlation was observed between p53 and Bcl-x positivity ($r = 0.9$; $P = 0.001$) but not between p53 and TUNEL or p53 and Fas positivity.

Double Labeling for Bcl-x, p53, and TUNEL

A number of samples were double-labeled to determine if any population of spermatozoa in a sample were simultaneously expressing Bcl-x and TUNEL positivity. Of 25 samples assessed, a mean ± SD of 1.9% ± 2.4% of sperm were found to be double-labeled. From the results obtained, it seems that TUNEL-positive sperm do not exclusively express Bcl-x positivity (Table 3). As TUNEL staining detects DNA damage, we also examined expression of p53, as this protein has a major role in DNA repair and apoptosis. As was observed for Bcl-x, TUNEL-positive sperm did not exclusively express p53 positivity (Table 3). To verify the technique, we also labeled seven samples with Bcl-x and PI to ensure that red-green double-labeling was functional. This double-labeling was able to distinguish a high percentage of spermatozoa that were labeled with Bcl-x and PI and with PI alone (data not shown).

Abnormalities in Human Ejaculated Spermatozoa Analyzed by Comet Assay

To further characterize the type of sperm nuclear DNA damage, we determined the type of strand breaks and the susceptibility to DNA digestion in sperm obtained from men with normal and abnormal semen parameters according to W.H.O. parameters [18]. A comparison between values obtained after comet assay analysis carried out in alkaline conditions (which identifies alkali-labile sites and both double- and single-stranded DNA breaks) and neutral

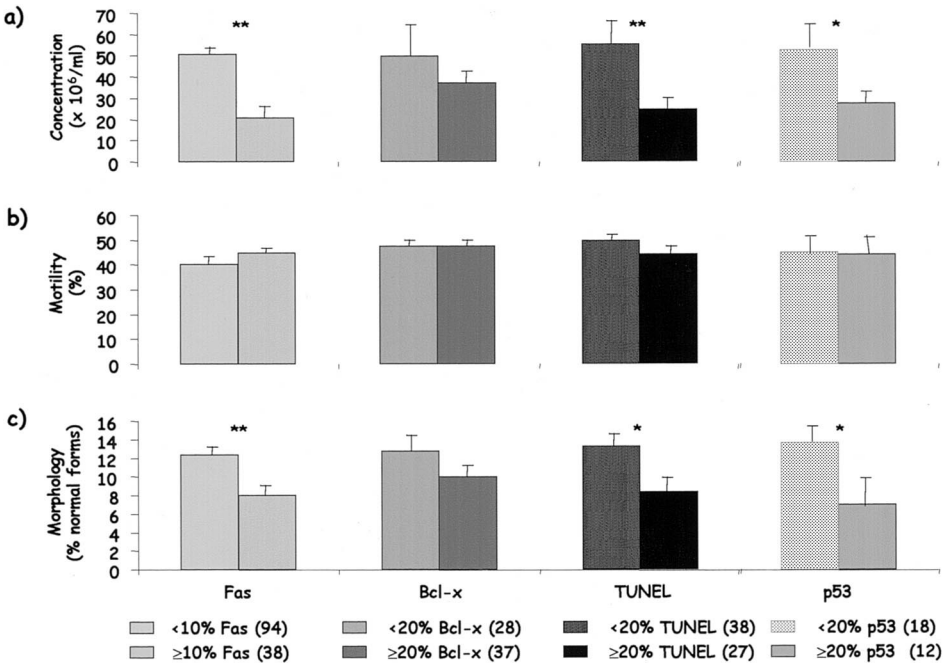

FIG. 1. The relationship between high and low levels of Fas, Bcl-x, and TUNEL and p53 positivity and **a**) concentration, **b**) motility, and **c**) morphology of ejaculated human spermatozoa. Fas, Bcl-x, TUNEL, and p53 positivity are shown as the percentage of positive sperm per sample. The number of samples examined for each group is given in parentheses. * $P < 0.05$, ** $P < 0.01$.

conditions (which identifies double-stranded breaks only) indicated that both double- and single-stranded DNA breaks are present in ejaculated spermatozoa, but there is a significantly greater percentage of both types of breaks in sperm from men with abnormal semen parameters (Fig. 2). Compared with men with normal semen parameters, men with abnormal semen parameters had a significantly greater mean percentage of sperm with strand breaks under both alkaline conditions (11.2% ± 3.7% vs. 23.7% ± 6.9%, $P < 0.05$) and neutral conditions (8.5% ± 2.6% vs. 14.2% ± 5.3%, $P < 0.05$).

Increased Sensitivity of Chromatin Isolated from Abnormal Human Spermatozoa to MNase and DNase I

To compare chromatin structure in sperm nuclei isolated from men with normal and abnormal semen parameters, MNase or DNase I digestion was performed, and the samples were run under alkaline conditions. DNA isolated from both groups of men migrated as a band of 8 kilobase pairs without digestion. Limited access of enzymes to supercondensed chromatin typical of mature sperm was observed in the absence of permeabilization with both DNase I and MNase. When spermatozoa were permeabilized with Triton X-100, the pattern of enzyme-hypersensitive sites detected in chromatin for men with normal semen parameters (Figs. 3a and 4a) was consistently different from that for men with abnormal semen parameters (Figs. 3b and 4b). Furthermore, the enzyme concentrations necessary to digest the chromatin from men with abnormal parameters (9 U) were lower than those required to digest chromatin from men with normal parameters (Figs. 3 and 4).

TABLE 2. Relationship between apoptotic markers when Fas, Bcl-x, and TUNEL expression were assessed in the same sperm samples.

	No. of samples stained	Other apoptotic marker	% Positive spermatozoa (mean ± SEM)	*P*
% Bcl-x positive				
<20	29	Fas	9.1 ± 1.7	
>20	39	Fas	11.9 ± 1.0	0.001
<20	29	TUNEL	17.5 ± 2.2	
>20	39	TUNEL	23.1 ± 2.8	0.3
% Fas positive				
<10	41	Bcl-x	23.2 ± 2.9	
>10	27	Bcl-x	31.0 ± 3.2	0.02
<10	41	TUNEL	17.8 ± 1.9	
>10	27	TUNEL	25.1 ± 3.6	0.13
% TUNEL positive				
<20	40	Bcl-x	27.4 ± 3.1	
>20	28	Bcl-x	24.7 ± 3.1	0.7
<20	40	Fas	10.4 ± 1.3	
>20	28	Fas	11.2 ± 1.2	0.3

DISCUSSION

During spermatogenesis, a complex series of morphologic changes occur in the transition of spermatogonia to mature spermatozoa. In the human, the relationship between apoptosis and the nature of ejaculated spermatozoa with nuclear DNA damage [8, 10] has not been extensively studied. In the present study, we have shown that TUNEL positivity and apoptotic markers do not always exist in unison in spermatozoa. However, semen samples that had a low sperm concentration and poor morphology were more likely to show high levels of TUNEL positivity and Fas

TABLE 3. Double-labeling of ejaculated sperm to determine if Bcl-x or p53 is expressed in the same spermatozoa that are TUNEL positive.[a]

Labeling	% Spermatozoa positive (mean ± SD)
Bcl-x positive	4.4 ± 3.8
TUNEL positive	14.0 ± 9.5
Bcl-x and TUNEL positive	2.0 ± 2.4
p53 positive	1.4 ± 1.0
TUNEL positive	21.9 ± 12.1
p53 and TUNEL positive	1.3 ± 1.3

[a] The results reflect labeling of individual spermatozoa with a single fluorescent marker or both markers. For the Bcl-x and TUNEL double-labeling, 25 samples were examined; for the p53 and TUNEL double-labeling, 16 samples were examined.

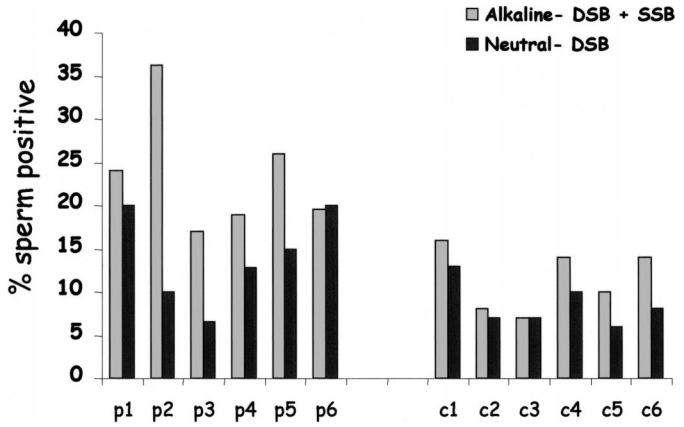

FIG. 2. The percentage of ejaculated spermatozoa positive for DNA strand breaks after analysis by the comet assay in either alkaline or neutral conditions. Columns labeled p1–p6 show semen samples from men with abnormal semen parameters according to W.H.O. criteria [18], whereas columns c1–c6 show semen samples from fertile men with normal semen parameters according to W.H.O. criteria.

and p53 expression. In addition, the DNA damage in ejaculated human sperm consists of both single- and double-stranded DNA breaks. Two theories have been proposed to explain the phenomenon of why ejaculated human spermatozoa possess anomalies in their nuclear DNA. The first theory arises from studies performed in animal models and is linked to the unique manner in which mammalian sperm chromatin is packaged. Endogenous nicks in DNA have been shown to be normally present at specific stages of spermiogenesis in rats and mice [25–27]. In the rat [26, 28] and mouse [27, 29], these endogenous nicks are evident during spermiogenesis but are not observed once chromatin packaging is completed. The presence of nicks is maximal during the transition from round to elongated spermatids in the testis and occurs before the completion of protamination in maturing rat and mouse spermatozoa. It has been proposed that the endogenous nuclease topoisomerase II may play a role in both creating and ligating nicks during spermiogenesis, that these nicks may provide relief of torsional stress, and that they aid chromatin rearrangement during the displacement of histones by the protamines [25, 30]. The second theory of why DNA damage is present in ejaculated human spermatozoa arises from the use of the TUNEL assay as a marker of apoptosis. A number of studies have therefore stated that the presence of TUNEL-positive sperm indicates that these sperm are indeed apoptotic [10, 12, 13, 31].

In this study, we have confirmed that men with abnormal semen parameters have higher levels of Fas expression on their ejaculated spermatozoa [16]. As with Fas expression, both p53 and TUNEL positivity also significantly correlated with sperm concentration and morphology; however, Bcl-x expression did not follow the same pattern. When we assessed whether TUNEL expression was evident in the same samples that had high Fas or Bcl-x expression, we found no strict relationship. When double-labeling was performed, to see if the same population of sperm that were TUNEL positive also showed Bcl-x and p53 positivity, less than 2% of the sperm showed expression of both markers, while 14% and 22% of Bcl-x- and p53-positive sperm showed TUNEL positivity, respectively. The same small percentage of sperm that were p53 positive were also positive for both TUNEL and p53, whereas only about half the percentage of Bcl-x-positive sperm showed positivity for

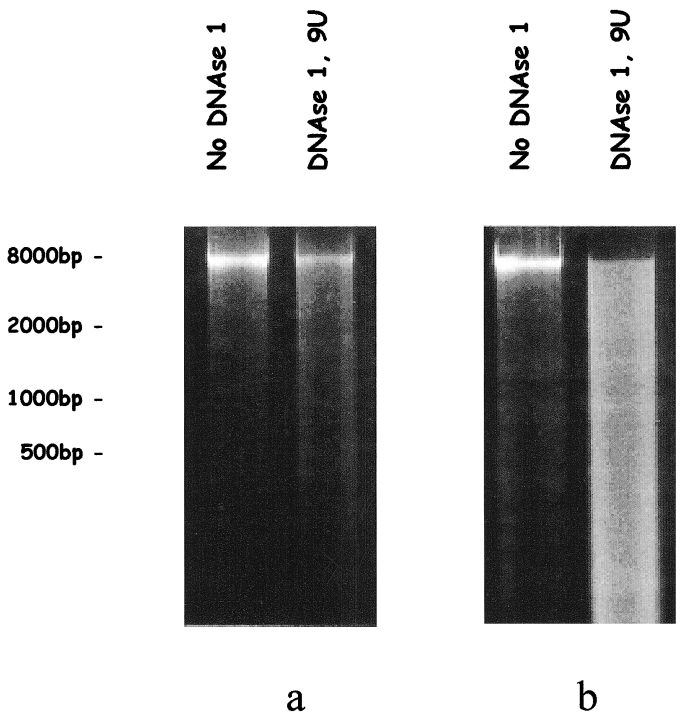

FIG. 3. The digestion of ejaculated human spermatozoa from men with normal (**a**) and abnormal (**b**) semen parameters permeabilized with Triton X-100 and incubated at 37°C for 30 min with different concentrations of DNase I.

both TUNEL and Bcl-x. Both p53 and Bcl-x labeling were significantly lower than TUNEL labeling. It appears from these results that ejaculated sperm exhibiting DNA damage do not necessarily show distinct apoptotic markers. The presence of apoptotic markers and DNA damage may therefore not be strictly related.

First, the persistence of increased levels of expression of

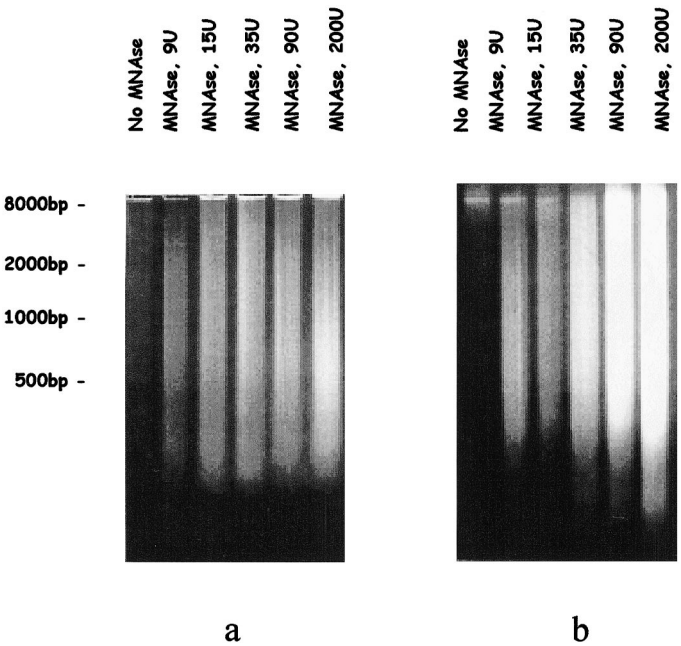

FIG. 4. The digestion of ejaculated human spermatozoa from men with normal (**a**) and abnormal (**b**) semen parameters permeabilized with Triton X-100 and incubated at 37°C for 30 min with different concentrations of MNase.

some apoptotic markers in mature spermatozoa as seen in the present study may also involve a mechanism similar to that reported in the rat [32]. Blanco-Rodriguez and Martinez-Garcia [32] have shown that apoptotic signaling molecules can be restricted to a specific cytoplasmic compartment during rat spermatogenesis and form residual bodies similar to apoptotic bodies. These residual bodies have increased levels of expression of caspase-1, c-jun, p53, and p21. Although human spermatozoa do not normally possess residual bodies, they do sometimes retain cytoplasm. Therefore, a defect in cytoplasmic remodeling may be responsible for some of the mature spermatozoa showing high levels of apoptotic markers. Such a defect would explain the relationship with poor morphology. Cytoplasmic retention has already been shown to vary in human spermatozoa that exhibit different biochemical markers such as creatine kinase [33–35].

Second, the presence of damage may be related to the process of chromatin packaging and the replacement of histones by protamines during spermiogenesis. Recently, a study in a protamine 1 knock-out mouse model has shown that when chromatin assembly is incomplete, the nuclei are less resistant to chemical disruption [36]. This decreased resistance is similar to the findings in sperm from men with abnormal semen parameters in the present study. The comet assay, under both alkaline and neutral conditions, indicated that ejaculated sperm had both single- and double-stranded DNA breaks and that both were more prevalent in men with abnormal semen parameters. When comparing the susceptibility to MNase and DNAse I between men with normal and abnormal semen parameters, we found an increased susceptibility to enzyme digestion in sperm from men in the latter group. This strengthens the argument that chromatin packing is altered in abnormal spermatozoa and that this in turn reflects a higher level of DNA damage. Interestingly, Balhorn et al. [37] and Belokopytova et al. [38] postulated that certain aspects of male infertility may be related to aberrant protamine 1 and 2 ratios in some men. Furthermore, Steger et al. [39] reported that round spermatids from infertile men exhibit decreased protamine 1 and 2 mRNAs.

The mechanisms responsible for producing abnormal spermatozoa in the human ejaculate have been poorly understood. In this study, we have highlighted two mechanisms that play a key role in modulating spermatogenesis. The presence in the ejaculate of spermatozoa showing apoptotic markers indicates one mechanism by which abnormal sperm are produced. This is possibly a population of sperm that have escaped programmed cell death and express various apoptotic markers, a process that we have previously called ''abortive apoptosis'' [16]. This phenomenon may therefore be linked to defects in the remodeling of the cytoplasm that take place during spermatogenesis. The presence of DNA damage may arise from a separate mechanism and is more likely related to problems in nuclear remodeling resulting directly from problems during protamine deposition during spermiogenesis. Abnormalities in both of these processes would lead to populations of abnormal ejaculated spermatozoa, with problems at the nuclear level, cytoplasmic level, or both levels. Studies examining both normal and abnormal spermatogenesis will provide further insights on how these mechanisms are related.

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