Functional and Ultrastructural Features of DNA-Fragmented Human Sperm

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ABSTRACT: The functional significance of deoxyribonucleic acid (DNA) fragmentation in ejaculated human sperm is unclear. In this study the extent of DNA strand breakage in swim-up selected spermatozoa was evaluated by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL)-coupled flow cytometry and correlated with several functional and morphological sperm parameters. The extent of DNA fragmentation (mean = 11.07% ± 8.00%, range = 0.79%–42.64%, n = 140) was positively related to abnormal morphology and associated with defects of the sperm tail. A negative correlation was found between DNA breakage and progressive motility. When a stepwise multiple linear regression model was used to analyze the relationship between DNA fragmentation and the aforementioned parameters, only motility results were included in the model. The presence of sper-

matozoa showing submicroscopic characteristics resembling those of somatic apoptosis has been reported in human ejaculate. To verify whether sperm DNA fragmentation was associated with the presence of such apoptotic-like cells, we performed electron microscopy and TUNEL-coupled flow cytometry in a limited number of sperm samples (n = 24). Although we did not observe any significant relationship between DNA breakage and the characteristics that are suggestive of apoptosis, an association was found with several ultrastructural features, indicating an impaired motility. Hence, we conclude that in ejaculated sperm, DNA fragmentation does not correspond to the apoptosis-like phenomenon and that it is associated with defects of motility.

Key words: Apoptosis, DNA fragmentation.

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In the last 2 decades, progress in the field of assisted reproduction and micromanipulation has been surprising and the number of infertile couples making use of these techniques has dramatically increased. Poor sperm quality was initially considered a problem for in vitro fertilization (IVF) programs (Acosta et al, 1988). However, with more recent procedures such as intracytoplasmic sperm injection (ICSI), pregnancy rates have become less dependent on sperm quality (Cohen et al, 1992; Van Steirteghem et al, 1993).

One of the main concerns during ICSI is to select sperm of good enough quality (and in particular without damaged deoxyribonucleic acid [DNA]) to be injected into an oocyte. In fact, the injection-induced mechanical bypass of the natural oocyte barriers with an abnormal sperm may result in fertilization failure (Lopes et al, 1998a) or even in possible damage to the fetus (Cummins and Jequier, 1995). In addition, some reports demonstrate

an association between DNA-damaged spermatozoa and elevated levels of childhood cancer in newborns (Fraga et al, 1996; Ji et al, 1997; Sorahan et al, 1997). Hence, there is an increasing awareness of the importance of studying DNA integrity of male gametes. Routine semen analysis primarily evaluates several sperm characteristics such as count, motility, forward progression, morphology, and agglutination but it does not elicit information on DNA integrity. Major DNA damage is the presence of single- or double-strand breaks. DNA fragmentation has been observed in human ejaculated spermatozoa from both fertile and infertile men (Hughes et al, 1996). Among existing methods to investigate DNA breakage, flow cytometry is a rapid and objective technique that provides measures of individual cells. In this study we investigated the reliability of the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assay coupled with flow cytometric detection to quantitatively study DNA breakage in human sperm. In addition, we studied whether or not expression of DNA breakage in germ cells correlated with several morphological and functional characteristics of both swim-up selected and unselected ejaculated sperm. In par-

ticular, we evaluated whether the extent of DNA breakage in selected sperm was correlated to motility, morphology, and viability in the same population and to seminal parameters from routine semen evaluation.

In somatic cells, DNA fragmentation derived from internucleosomal cleavage is considered one of the hallmarks of programmed cell death (ie, apoptosis) and is classically detected by laddering of the nucleosomes. However, in mature sperm chromatin, the nearly complete loss of histones (substituted by protamines) and nucleosomal arrangement does not allow for this classic analysis. In addition, TUNEL labeling of DNA breakage can be considered as evidence of apoptotic cleavage in somatic cells (Gorczyca et al, 1993a; Gold et al, 1994; Nakamura et al, 1995), but not in mature germ cells (Manicardi et al, 1998). Thus, although the presence of TUNEL-positive cells has been demonstrated in ejaculated sperm, it is not clear whether DNA breakage in ejaculated germ cells indicates the presence of apoptosis and whether DNA breakage occurs in single- or double-stranded DNA (Manicardi et al, 1998). Hence, throughout the present paper, we refer to sperm TUNEL positivity by using generic terms such as DNA fragmentation or DNA breakage. However, submicroscopic features similar to the typical ultrastructure of apoptosis occurring in somatic cells have been described in ejaculated human sperm (Baccetti et al, 1996, 1997). Therefore, we investigated whether DNA breakage in TUNEL-labeled sperm and the apoptosis-like phenomenon (as evaluated by electron microscopy) are correlated.

Materials and Methods

Sample Collection and Sperm Preparation

A total of 140 semen samples were collected from men examined in the Andrology Clinic of the University of Florence. Semen samples were collected after at least 48 hours of abstinence. After 30 minutes of liquefaction, routine semen analysis was performed using standard techniques (World Health Organization [WHO], 1992). For swim-up selection, 1 mL of human tubal fluid (Celbio, Milan, Italy) medium with 1% human serum albumin (Celbio) was layered on top of an equal volume of semen sample. After 1 hour of incubation at 37°C, 800 µL of medium were collected. Selected cells were counted and sperm morphology and motility were assessed by optical microscopy according to WHO criteria. Sperm morphology was scored by determining the percentage of normal forms, head, neck/midpiece, tail defects, and cytoplasmic droplets. Sperm motility was scored by determining the percentage of progressive motile, nonprogressive motile, and immotile spermatozoa.

TUNEL Assay

DNA fragmentation in swim-up selected spermatozoa was assessed by TUNEL assay. Swim-up selected spermatozoa (2–10

$\times$ 10^6 cells) were centrifuged at 500 $\times$ g for 10 minutes and fixed in paraformaldehyde (200 µL, 4% in phosphate-buffered saline [PBS], pH 7.4) for 30 minutes at room temperature. After 2 washes with 200 µL of PBS with 1% bovine serum albumin (BSA), spermatozoa were permeabilized with 0.1% Triton X 100 in 0.1% sodium citrate (100 µL, for 2 minutes in ice). Sperm samples were then washed as previously described and split into 2 aliquots. Both aliquots were incubated in 50 µL of the label solution with (as a test sample) or without (as a negative control) the terminal deoxynucleotidyl transferase (TdT) enzyme (supplied by In Situ Cell Death Detection Kit, fluorescein, Roche Molecular Biochemicals, Milan, Italy). The labeling reaction was carried out for 1 hour in the dark at 37°C. After labeling, 2 subsequent washes were able to remove unspecific fluorescence up to the basal level (autofluorescence). Finally, sperm were resuspended in PBS. In some samples, positive controls were also prepared as described before but with an additional treatment with DNAse I (Pharmacia Biotech Italia, Milan, Italy), 2 IU for 20 minutes at 37°C, before the labeling reaction.

Flow Cytometry and Fluorescence Microscopy

Green fluorescence was detected at 515–555 nm using an FL-1 detector of a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif) equipped with a 15-mW argon-ion laser for excitation. For each sample, 10 000 events were recorded at a flow rate of 200–300 cells/sec. Debris were gated out by establishing a region around the population of interest, on the basis of light scatter characteristics of swim-up selected sperm. These characteristics were assessed in several swim-up selected sperm samples before starting the study. Swim-up selected sperm were fixed and permeabilized as described earlier; then sperm DNA was stained with red fluorescent propidium iodide (PI; 50 µg/mL in PBS). Stained samples were acquired and PI fluorescence (FL-2 detector, 563–607 nm wavelength band) was detected. Thus, a region that included red fluorescent events could be established on the forward scatter vs. side scatter dot plot. Light scatter as well as green fluorescence detection was obtained at a fixed instrumental setting for all times of the study. Data were processed with CELL FIT, MANL model analysis software (Becton Dickinson). The percentage of labeled sperm was determined by setting a region that included all the events in the frequency histogram of the negative control. Hence, all the events out of this region were considered positive. In order to verify the reliability of this procedure of data processing, we examined 13 samples by using both fluorescence microscopy and flow cytometry. Spermatozoa were processed with TUNEL as described earlier, then spread onto slides, dried at room temperature, and scored by counting labeled cells in at least 3 fields (about 300 cells) per slide.

Hypo-Osmotic Swelling Test

Sperm that recovered after the swim-up procedure were centrifuged (200 $\times$ g for 10 minutes) and resuspended in hypo-osmotic swelling medium (WHO, 1992) for 30 minutes at 37°C. After centrifugation, sperm were resuspended in 50 µL of ice-cold methanol and layered onto slides. Percentage of curly tail sperm was determined by observing 100 cells/slide.

Transmission Electron Microscopy

After swim-up selection, an aliquot of each sperm sample was fixed for 2 hours at 4°C in Karnovsky fixative, centrifuged at $500 \times g$ for 15 minutes, and rinsed in cacodylate buffer 0.1 M, pH 7.2. Sperm were then postfixed in 1% buffered OsO_4, dehydrated, and embedded in Epon Araldite. The ultrathin sections, obtained with a Supernova ultramicrotome (Reickert-Jung, Vienna, Austria), were collected on copper grids and stained by uranyl acetate and lead citrate. Observation was performed with a Philips CM10 electron microscope (Philips Scientific, Eindhoven, the Netherlands). Several ultrastructural features were evaluated: position, shape, content, and dimension of acrosome; shape of nucleus; arrangement and appearance of chromatin (ie, uncondensed, condensed, disrupted); presence of cytoplasmic residues with or without vacuoles; shape of mitochondria; helix assembly, pattern, arms, and shape of axoneme; fibrous sheath; and accessory fibers of flagellum. Each characteristic was evaluated by examining 100 ultrathin sections of spermatozoa and calculating the percentage of the presence of the defects in the observed cells (Baccetti et al, 1995).

Statistical Analysis

All variables were checked for normal distribution. Data were analyzed after logarithmic transformation, when appropriate. Bivariate correlation was evaluated by calculating the Pearson's correlation coefficient (r). Cases with missing values, for 1 or both of a pair of variables, were excluded from the analysis. Multiple linear regression analysis was performed to establish which variable could explain a significant amount of variation in sperm DNA fragmentation. A stepwise procedure was used to select variables to be included into the model. A variable was entered into the model if the probability of F was smaller than 0.05 and removed if it was equal to or greater than 0.1.

All statistical analyses were carried out using the SPSS version 6.0 software for Windows (SPSS Inc, Chicago, Ill).

Results

In this study we used TUNEL-coupled flow cytometry to investigate DNA fragmentation in swim-up selected spermatozoa. Figure 1 shows typical frequency histograms obtained by analyzing sperm samples from 3 patients with low (Figure 1A), medium (Figure 1B), and high (Figure 1C) percentages of DNA-fragmented spermatozoa. Note that in the negative control there was a consistent intersample variability. Final sample washing was able to remove fluorescence up to the basal level (data not shown). Hence, we derive that such variability is the result of a variable amount of naturally occurring fluorescence material present in sperm cells (autofluorescence). Generally, samples with a high percentage of DNA-fragmented sperm also have a high level of autofluorescence (data not shown).

To evaluate whether or not the percentage of DNA-fragmented sperm corresponded to the number of TU-NEL-positive cells, as classically detected by fluorescence microscopy, we simultaneously analyzed 13 swim-up selected sperm samples by both procedures. We found a positive linear relationship between results from the 2 techniques ($r = .78$, $P < .002$) with the null value of y for x = 0 included in the confidence interval (Figure 2). Hence, the rate of DNA-fragmented sperm population detected by flow cytometry corresponded to the percentage of TUNEL-positive sperm as detected by fluorescence microscopy. This indicates that the flow cytometric method and the algorithm used for data processing is suitable and useful to study DNA breakage in human sperm.

TUNEL-coupled flow cytometry showed the presence of a variable amount of DNA fragmentation in almost all the swim-up selected semen specimens examined (mean $= 11.07\% \pm 8.00\%$, range $= 0.79\%$–42.64%, n = 140). These values are in close agreement with previously reported percentages of TUNEL-positive cells in swim-up selected sperm in similar patient population (Sun et al, 1997; Lopes et al, 1998b).

Because we found a great variability in the extent of DNA breakage among the different samples, we started investigations on the functional significance of this phenomenon. We found that DNA fragmentation correlated with several routine semen parameters, as evaluated before and after swim-up selection. We found a negative correlation between DNA breakage and progressive motility in both unselected (Figure 3; Table 1) and swim-up selected sperm population (Figure 3; Table 2). Accordingly, in both populations, a positive correlation was observed between the percentage of immotile sperm and the percentage of sperm with fragmented DNA (Figure 3, Tables 1 and 2). No correlation was found between the rate of DNA breakage and the number of sperm with nonprogressive motility, either in unselected or in swim-up selected sperm (Tables 1 and 2). These data are in contrast with the study carried out in unselected sperm by Gorczyca et al (1993b) but in agreement with later reports (Sun et al, 1997; Lopes et al, 1998b) that correlated the percentages of swim-up selected DNA-fragmented cells with unselected sperm parameters. Similar to the latter studies, we also found a significant relationship between the rate of DNA breakage and the percentage of abnormal morphology in both unselected sperm (Figure 4; Table 1) and swim-up selected sperm (Figure 4; Table 2). Among the different morphological defects examined, only sperm tail anomalies correlated with DNA fragmentation (Figure 4; Tables 1 and 2).

We did not find any correlation between percentage of DNA breakage and either sperm count or sperm concentration (Table 1). Although these results contrast with the report of Sun et al (1997), they agree with a subsequent study by the same group (Lopes et al, 1998b) and with the study by Gorczyca et al (1993b). Finally, DNA frag-
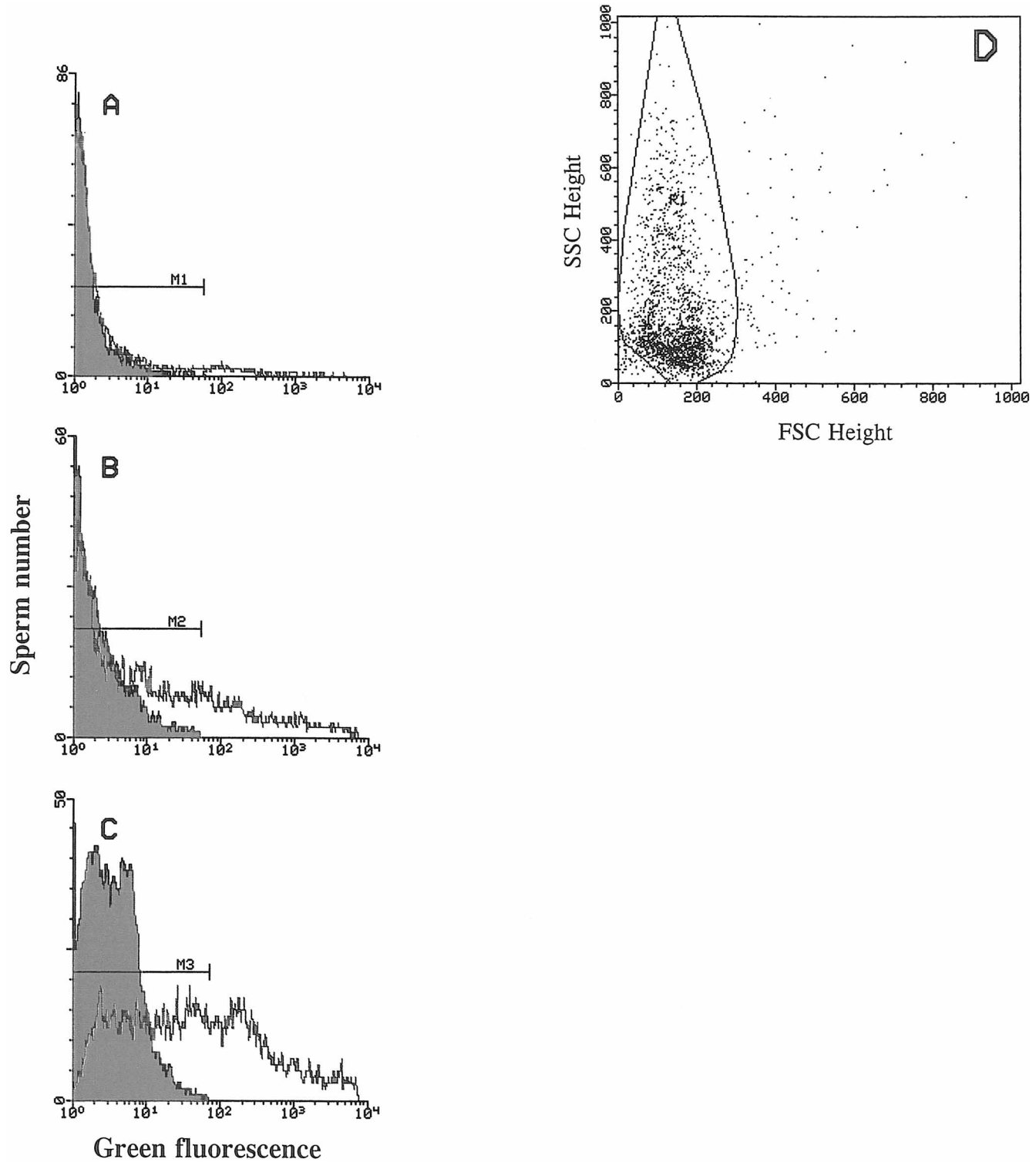
Figure 1. Frequency distributions of DNA-fragmented sperm in samples from 3 patients characterized by low **(A)**, medium **(B)**, and high **(C)** percentage of TUNEL-positive spermatozoa. For each sample (open histograms), the corresponding negative control (solid histograms) is used to set a region (M1, M2, M3) including all TUNEL-negative cells. **(D)** A forward scatter/side scatter dot plot from a swim-up selected sperm sample and the characteristic flame-shaped region, including the population of interest. See "Materials and Methods" for details.
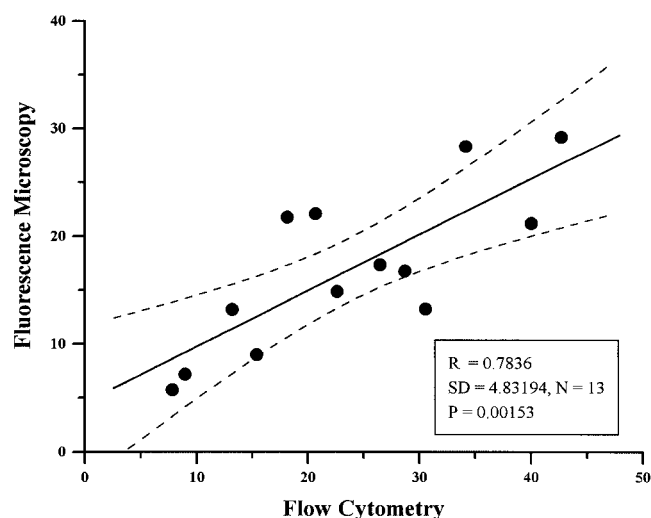
Figure 2. Linear regression analysis between the measures of TUNEL-positive sperm evaluated by fluorescence microscopy and flow cytometry.

Table 1. *Linear regression anaylsis of the relationship between percentage of DNA fragmented sperm (mean = 11.07 ± 8%, n = 140) in swim-up selected population and routine semen parameters in unselected sperm*

Variable	Mean ± SD	n	r	P Value
Sperm count (million sperm/ejaculate)	258.6 ± 247.1	139	−0.11	NS
Sperm concentration (million/mL)	76.7 ± 57.2	139	−0.05	NS
Volume of the ejaculate (mL)	3.4 ± 1.5	140	0.11	NS
pH	7.6 ± 0.3	136	0.08	NS
Morphology defects, %				
General	72.1 ± 11.9	137	0.31	≤.0001
Head	40.8 ± 11.5	104	0.11	NS
Neck/midpiece	19.3 ± 6.2	104	0.17	NS
Tail	10.3 ± 6.2	104	0.19	0.05
Cytoplasmic droplets	2.1 ± 1.9	104	0.01	NS
Motility, %				
Progressive	48.3 ± 16.1	140	−0.28	≤.001
Nonprogressive	12.5 ± 6.5	140	0.07	NS
Immotile sperm	39.1 ± 13.4	140	0.32	≤.0001

* NS indicates not significant.

mentation did not correlate either with volume or pH of semen samples (Table 1).

To identify which independent variable (among the selected and unselected routine sperm parameters) explained a significant amount of the variation in DNA fragmentation, we used a stepwise multiple linear regression model. This analysis demonstrated that only the percentage of immotile spermatozoa satisfied the criteria to be included in the model.

To investigate whether DNA-fragmented spermatozoa were still viable and whether the cell membrane exhibited functional integrity, we simultaneously performed TUNEL-coupled flow cytometry and the hypo-osmotic swelling test in a limited series of swim-up sperm samples (n = 35). We found a negative correlation between percentage of curly sperm and the extent of DNA fragmentation (Figure 4; Table 2).

In the second part of this study, we verified whether DNA fragmentation as assessed by TUNEL was related to the apoptotic-like ultrastructural characteristics of ejaculated sperm. Figure 5 shows that these sperm features resemble the morphology of somatic apoptosis: aggregation of the chromatin in large masses close to the nuclear envelope and within an abnormal-shaped nucleus, presence of translucent vacuoles in persistent cytoplasmic residues, and abnormality in the shape of mitochondria. Sperm exhibiting such features usually also display anomalies of axoneme and of the content and position of acrosome (Figure 5; Baccetti et al, 1996, 1997). We used germ cells and somatic cells to study whether the extent of DNA fragmentation, as assessed by TUNEL, correlated with the submicroscopic characteristics of apoptosis. In the 24 samples examined, we did not find any relationship among rate of DNA breakage and an abnormal shape of

nucleus, presence of marginated chromatin, acrosome with irregular content and position, or occurrence of cytoplasmic vacuoles. Conversely, we found a positive relationship between DNA fragmentation and both the presence of cytoplasmic residues ($r = .4$, $P < .05$) and mitochondrial swelling ($r = .48$, $P < .02$). Although it is possible that the DNA fragmentation we observed in ejaculated sperm reflects not only breakage occurring in nuclear DNA but also in mitochondrial DNA, it is important to note that we found only the nuclear region to be positive by fluorescence microscopy (data not shown). We also found a negative correlation between DNA breakage and normal shape of the axoneme ($r = −.48$, $P < .02$).

Table 2. *Linear regression analysis of the relationship between percentage of DNA fragmented sperm (mean = 11.07 ± 8%, n = 40) in swim-up selected population and motility, morphology, and viability in swim-up selected sperm*

Variable	Mean ± SD	n	r	P Value
Morphology defects, %				
General	54.8 ± 18.3	102	0.33	≤.001
Head	31.9 ± 14.4	62	0.2	NS
Neck/midpiece	14.3 ± 6.2	62	0.17	NS
Tail	6.6 ± 5.2	62	0.3	≤.01
Cytoplasmic droplets	0.52 ± 1.1	62	0.14	NS
Motility, %				
Progressive	65.8 ± 18.4	117	−0.46	≤.0001
Nonprogressive	8.9 ± 5.7	117	0.11	NS
Immotile sperm	25.8 ± 17.5	117	0.54	≤.0001
Viability, %	81.9 ± 10.5	35	−0.57	≤.0001

* NS indicates not significant.

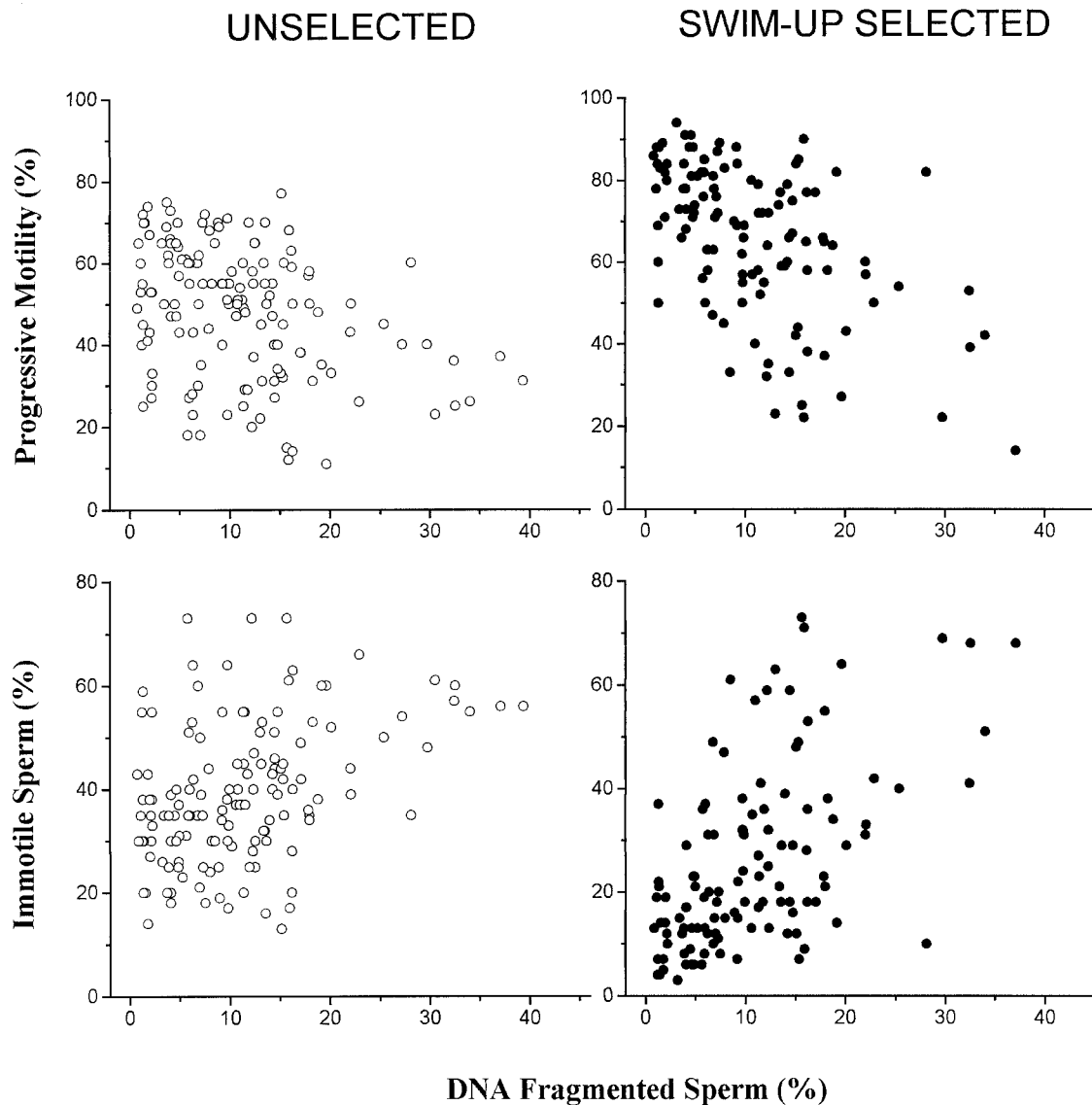UNSELECTED   SWIM-UP SELECTED

DNA Fragmented Sperm (%)

Figure 3. Dot plots between DNA fragmentation as determined by TUNEL assay in swim-up selected sperm and the percentages of progressive motile (upper panels) and immotile (lower panels) sperm evaluated in unselected (open circles, left panels) and swim-up selected populations (solid circles, right panels).

Discussion

In the last decade an increasing awareness of the importance of studying sperm DNA integrity has been developing and, up to now, several techniques have been set up to investigate DNA damage and, in particular, DNA breakage in germ cells. TUNEL assay is one of these techniques. This particular assay is based on the ability

of the enzyme, TdT, to detect the formation of 3′OH-free ends from single- and double-strand DNA breakage. In somatic cells, the highly frequent double-strand DNA breaks, as detected by TUNEL assay, are considered typical of apoptotic DNA degradation (Gorczyca et al, 1993a; Gold et al, 1994; Nakamura et al, 1995). Cells showing ultrastructural features similar to those of somatic apoptotic cells have been demonstrated in ejaculat-

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Figure 4. Dot plots between DNA fragmentation as determined by TUNEL assay in swim-up selected sperm and the percentages of abnormal morphology (upper panels) and tail defects (middle panels) evaluated in unselected (open circles, left panels) and swim-up selected population (solid circles, right panels). Lower panel shows dot plot between DNA fragmentation and the percentage of positive cells by the hypo-osmotic swelling test, as evaluated in swim-up selected sperm.
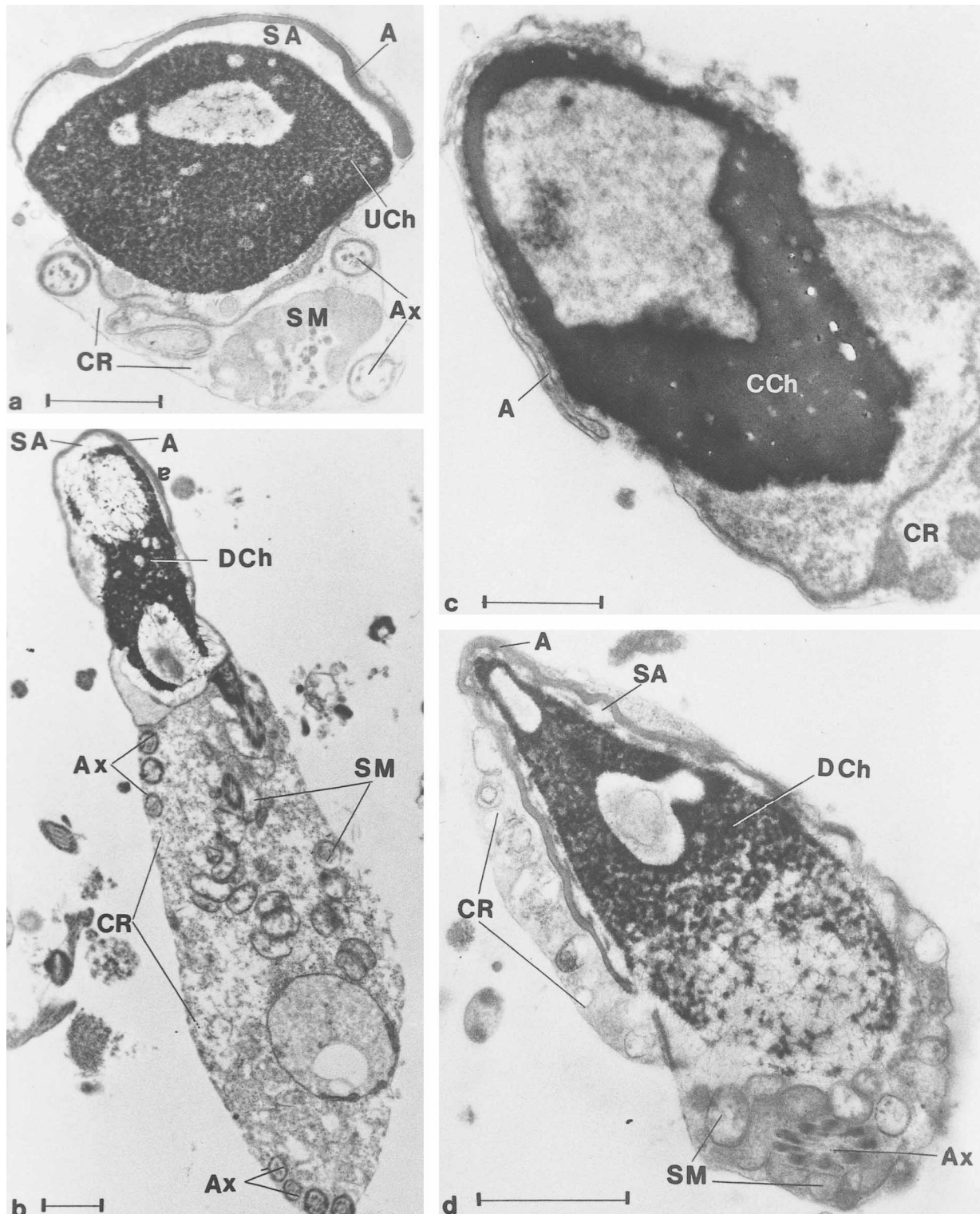
UNSELECTED   SWIM-UP SELECTED
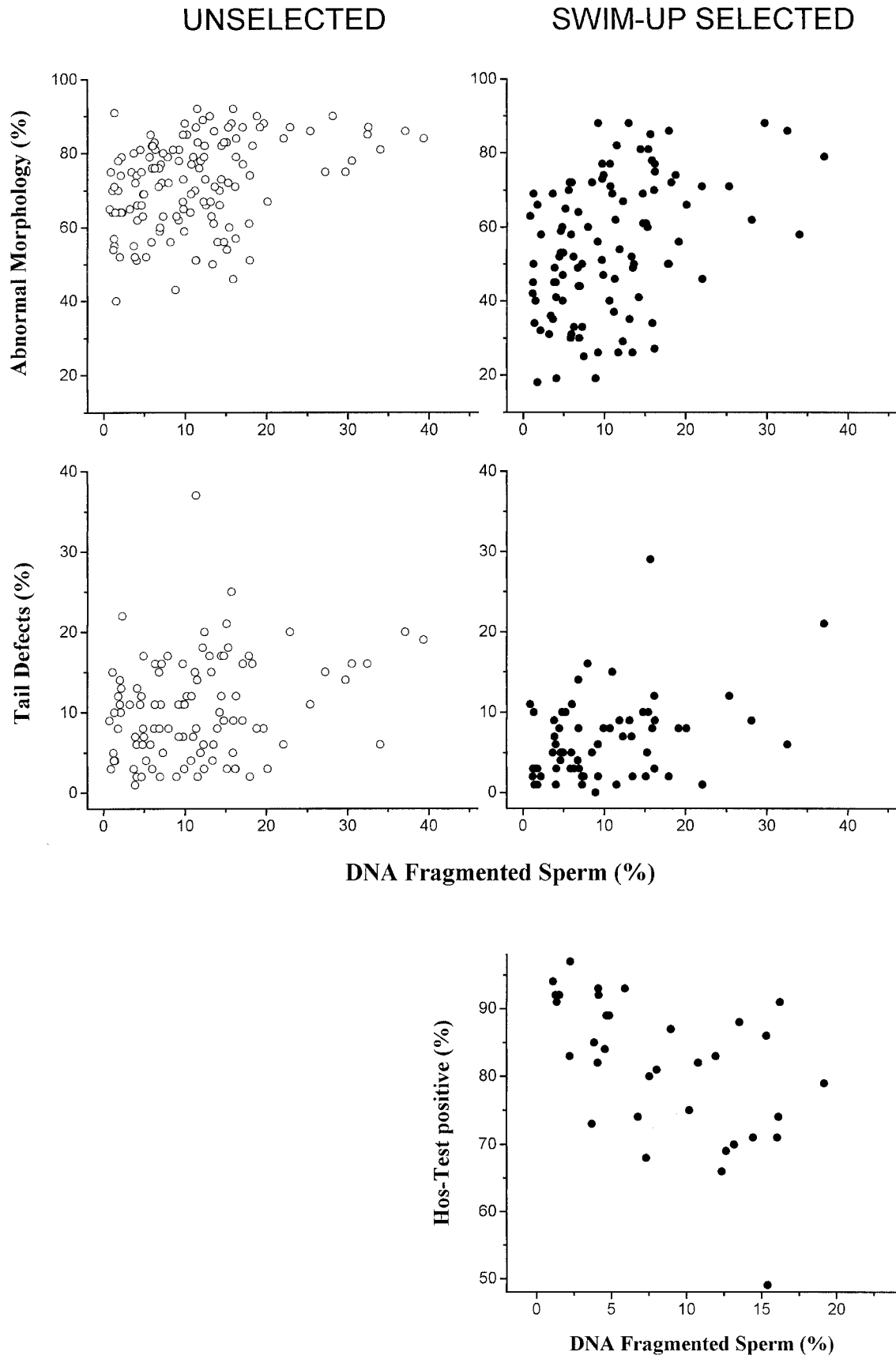
Figure 5. Transmission electron microscopy micrographs of longitudinal sections of human spermatozoa. They show ultrastructural sperm characteristics suggesting apoptosis according to Baccetti et al, 1996. The nuclear shape appears altered and the chromatin is disposed in uncondensed **(a)**, condensed **(c)**, or disrupted **(b**, **d)** masses. In the 3 conditions, the chromatin is frequently marginated. The acrosomes are generally located far from nuclei, and the subacrosomal spaces appear enlarged **(a**, **b**, **d)**. In the ample cytoplasmic residues **(a–d)**, the axonemes appear disorganized and coiled; swollen and badly assembled mitochondria are present. A indicates acrosome; Ax, axoneme; CCh, condensed chromatin; CR, cytoplasmic residue; DCh, disrupted chromatin; SA, subacrosomal space; SM, swollen mitochondria; UCh, uncondensed chromatin.

ed human sperm (Baccetti et al, 1996; Baccetti et al, 1997). These features were strictly associated with a poor morphological appearance (Baccetti et al, 1996). Although it has never been demonstrated, it has been assumed that in mature germ cells, DNA fragmentation, as detected by TUNEL assay, corresponds to apoptosis (Gorczyca et al, 1993b) and that both phenomena are indicators of poor sperm quality and low fertility. However, Manicardi et al (1998) demonstrated that TUNEL assay cannot itself be considered as indicative of apoptotic cleavage in mature spermatozoa, which is at variance with somatic cells. In the present study, we showed that TUNEL positivity is only partially associated with the ultrastructural sperm features that resemble typical somatic apoptosis. Indeed, we did not find any significant correlation between DNA fragmentation and those submicroscopic features that are considered hallmarks of apoptosis in somatic cells (and which have recently been extended to germ cells; Baccetti et al, 1996, 1997), such as nuclear shape, arrangement of the chromatin, or presence of translucent vacuoles in cytoplasmic residues. A positive correlation was found only with mitochondrial swelling. Mitochondria are now considered as the central executioners of the process of apoptosis (Mignotte and Vayssiere, 1998; Bernardi et al, 1999). However, whether or not mitochondrial swelling actually occurs in the progression of programmed cell death is still a controversial matter (Bernardi et al, 1999). Considering the pivotal importance of these organelles for sperm motility, the direct correlation between DNA fragmentation and mitochondrial swelling observed in this study is more indicative of impaired motility than of an occurrence of apoptosis. This conclusion is also substantiated by the finding that DNA fragmentation correlates with defects of the axoneme (as detected by transmission electron microscopy) and tail anomalies (as detected by optical microscopy). In addition, a stepwise multiple linear regression analysis shows that among all the correlating variables tested in this study, the percentage of immotile spermatozoa is the most important parameter that significantly affects variation of DNA breakage.

DNA-fragmented sperm could be interpreted as a fraction of cells that have failed to complete maturation and, in particular, to complete the packaging of chromatin during spermiogenesis. Indeed, in the elongating phase, protamines substitute histones (Balhorn et al, 1982; Bucci et al, 1982; Poccia 1986) and associate with DNA to form a very condensed complex that is stabilized by disulfide bonds among the same protamines (Rosseaux and Rosseaux-Prevost, 1995). Because of this unique packaging mechanism, sperm DNA develops an extreme stability and resistance to standard lysis agents and enzymatic digestion (Adolph et al, 1990; Bianchi et al, 1993). During spermiogenesis, McPherson and Longo (1992) observed

the presence of DNA strand breaks and proposed that they may have a role in favoring the histone-to-protamine substitution. Later on in the maturation process, DNA strand breaks are likely to be ligated by the action of nuclear enzymes, such as topoisomerase II (McPherson and Longo, 1993). Sperm that fail to complete ligation and maturation may retain DNA strand breaks. Hence, ejaculated spermatozoa showing DNA breakage may be considered as more immature. In keeping with this speculation, a strong direct correlation has been observed between DNA breakage in sperm and both poorly protaminated chromatin (Manicardi et al, 1998) and increased sensitivity of DNA to denaturation (an index of less stable and resistant sperm chromatin; Gorczyca et al, 1993b; Sailer et al, 1995; Aravindan et al, 1997). Furthermore, in the present study we found that the presence of DNA fragmentation in ejaculated sperm is associated with the ultramicroscopic appearance of the presence of cytoplasmic residues.

In conclusion, our data indicate that DNA fragmentation in ejaculated sperm should be considered as a marker of poor functional activity rather than an index of apoptosis. In other words, DNA-fragmented sperm are not cells that are committed to death, but rather, they retain several abnormalities that are compatible with a lower degree of maturation. DNA-fragmented sperm are in fact less motile, more immature, and even less susceptible to hypo-osmotic swelling, which indicates a lower functional integrity of the sperm membrane (Jeyendran et al, 1984). Identification and selection of these DNA-damaged sperm may improve the fertilization capacity even in ICSI programs.

Acknowledgments

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