Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation.

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Spontaneous DNA Fragmentation in Swim-Up Selected Human Spermatozoa During Long Term Incubation

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ABSTRACT: The origin and the meaning of DNA fragmentation in ejaculated human spermatozoa are not yet clear, although some hypotheses have been proposed. In the present study, we used investigated sperm DNA fragmentation by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL)-coupled flow cytometry to investigate DNA fragmentation in spermatozoa that were selected by the swim-up procedure and incubated long-term. In addition, using flow cytometry we detected annexin V binding assay and propidium iodide staining, and we also studied membrane phosphatidylserine translocation and the loss of membrane integrity in the same sperm populations that we used in the TUNEL investigation. We found that in vitro sperm DNA fragmentation 1) occurs after ejaculation under experimental conditions without the involvement of any external factor, 2) is not affected by treatment with the nuclease inhibitor aurintricarboxylic acid, 3) is increased by treatment with the glutathione peroxidase inhibitor mercaptosuccinate, 4) correlates with basal values (ie, just after swim-up selection) of DNA fragmentation in teratozoospermic but not in normospermic semen samples, 5) develops in a sharply associated manner with the in vitro occurrence of sperm necrosis, and 6) is predicted by the basal value of annexin V binding in viable spermatozoa. These findings suggest an involvement of endogenously produced reactive oxygen species as the possible cause of in vitro sperm DNA fragmentation.

Key words: DNA damage, necrosis, phosphatidylserine translocation, reactive oxygen species, sperm.

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The presence of DNA fragmented sperm in human ejaculate is well documented, in particular in men with poor semen quality (Sun et al, 1997; Lopes et al, 1998a, Irvine et al, 2000; Muratori et al, 2000). The greater occurrence of this DNA anomaly in subfertile men has given rise to concerns about using assisted reproductive technology (ART) with these patients because the fertilizing ability of a DNA damaged spermatozoon and the consequences of embryo development are not entirely known. Indeed, some ART protocols override the physiological sperm selection processes, which could increase the risk that a DNA damaged sperm participates in fertilization. Moreover, a recent report indicates that a greater risk of major birth defects is present in children conceived via in vitro ferilization (IVF) and intracytoplasmic sperm injection (ICSI) techniques (Hansen et al, 2002). This increased risk might also be coupled with the poor criteria for sperm selection during fertilization protocols.

Besides the effects on fertilization and embryo development, the meaning and origin of DNA fragmentation in human sperm are not yet clear, even though three main hypotheses have been proposed. The first hypothesis (Sailer et al, 1995) attributes sperm DNA fragmentation to the failure of the DNA breaks to physiologically religate during chromatin packaging (McPherson and Longo 1993a,b). Another hypothesis considers DNA breaks as the result of an apoptotic DNA cleavage in germ cells (Sakkas et al, 1999). In particular, it has been suggested that the process of cell death fails to complete and thus the dying cells are not eliminated from the testis (ie, abortive apoptosis). A third hypothesis is that human sperm DNA fragmentation occurs during ejaculation and is caused by an excessive production of reactive oxygen species (ROS). ROS production has an important role in spermatozoan capacitation and acrosome reaction processes (De Lamirande and Gagnon, 1999). The speculation is that an excessive level of these aggressive compounds may be responsible for cellular damage and, in particular, DNA damage (De Lamirande and Gagnon, 1999; Aitken and Krausz, 2000). One of the main differences between the first two hypotheses and the ROS hypothesis is the site of origin of DNA fragmentation. Whereas abortive apoptosis and the failure of DNA breaks to religate would occur in the testis, oxidative DNA damage could originate in testicular as well as post-testicular sites.

In order to gain an insight into whether sperm DNA fragmentation can occur independent of testicular chromatin rearrangement or testicular apoptosis, we investigated DNA fragmentation by TUNEL assay in swim-up selected spermatozoa incubated in vitro for up to 24

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hours. To investigate the relative role of sperm endonuclease activity and sperm ROS production in the development of sperm DNA damage, we used the nuclease inhibitor, aurintricarboxylic acid (ATA), and the ROS scavenging inhibitor, mercaptosuccinate. In addition, we investigated whether DNA fragmentation and translocation of membrane phosphatidylserine accompanies the loss of viability that occurs during prolonged incubation of spermatozoa in vitro (Aitken et al, 1996; Weil et al, 1998). To this aim, we detected annexin V (Ann V) binding in live and dead sperm with flow cytometry in the same sperm populations in which we investigated DNA fragmentation.

Materials and Methods

Semen Collection, Preparation, and Treatment

Semen samples were obtained from patients undergoing routine semen analysis for couple infertility in the andrology laboratory of the University of Florence, and analyzed according to World Health Organization (WHO, 1992) criteria. Samples with any detectable leukocytes in semen were excluded from the study. The 43 samples in the study were classified as follows: normozoospermia, 18; asthenozoospermia, 2; oligozoospermia, 1; teratozoospermia, 17; and asthenoteratozoospermia, 5 (WHO, 1992).

Swim-up selection was performed by layering 1 mL of human tubal fluid (HTF, Celbio, Milan, Italy) medium with 1% human serum albumin (Celbio) on top of an equal volume of semen sample. After 1 hour of incubation at 37°C, 900 μL of medium was carefully collected.

Sperm motility was assessed with light microscopy and scored by determining the percentages of progressive motile, nonprogressive motile, and immotile spermatozoa (WHO, 1992).

Selected cells were centrifuged at 250 × *g* for 10 minutes and resuspended in aliquots containing 10^6 sperm/100 μL of modified HTF medium (Celbio). Incubation was performed for the indicated times at 37°C. To estimate the normalized rate of de novo–generated sperm DNA fragmentation and necrosis during the 24 hours of incubation, we used the following calculation: [(24 hours incubation value − preincubation value)/(100 − preincubation value)] × 100.

Mercaptosuccinate (Sigma, Milan, Italy), an inhibitor of glutathione peroxidase (GPX), was added to the incubation medium from a freshly prepared stock solution (in distilled water, 200 mM). Two microliters of the stock solution of the inhibitor was added to the incubation medium in order to obtain the final concentration of 2 mM.

For treatment with ATA (25 μM, Sigma), a fresh stock solution was prepared in ethanol (25 mM). The compound was added to the incubation medium from a diluted solution (2.5 mM in distilled water). Controls were treated with an equal percentage of ethanol (1 ‰).

TUNEL Assay

DNA fragmentation was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick end labeling (TUNEL) as described elsewhere (Muratori et al, 2000). Briefly, spermatozoa (2×10^6) were centrifuged at 500 × *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 100 μL of 0.1% sodium citrate for 2 minutes in ice. After washing 2 times, the labeling reaction was performed by incubating sperm in 50 μL of labeling solution (supplied with the In Situ Cell Death Detection Kit, fluorescein, Roche Molecular Biochemicals, Milan, Italy) containing the TdT enzyme for 1 hour at 37°C in the dark. For each experimental set, a negative control was prepared by omitting TdT from the reaction mixture. After labeling, 2 subsequent washes were performed and sperm were resuspended in PBS for flow cytometry analysis. Positive controls were prepared as described before but with an additional treatment with 2 IU DNAse I (Pharmacia Biotech Italia, Milan, Italy) for 20 minutes at 37°C before the labeling reaction.

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/s. Based on the light scatter characteristics of swim-up selected sperm (Muratori et al, 2000), debris were gated out by establishing a region around the population of interest in the forward scatter/side scatter (FSC/SSC) dot plot (Figure 1A). Data were processed with the MANL model of CELL FIT 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 outside this region were considered positive.

Annexin V Binding Assay

The Ann V binding assay was used to detect the translocation of membrane phosphatidylserine to the outer side of the plasma membrane in dead and live spermatozoa (Barroso et al, 2000). The assay was performed by using the Annexin V-FITC Apoptosis Detection Kit (Oncogene Research Products, Boston, Mass) after optimizing for spermatozoa. At the indicated times, 10^6 sperm cells were centrifuged at 250 × *g* for 10 minutes and resuspended in 250 μL of modified HTF medium supplemented with $CaCl_2$ up to 2.5 mM. Then, 1.25 μL of Ann V conjugated to fluorescein isothiocyanate (FITC) solution (Ann V-FITC, 200 μg/mL in 50 mM Tris pH 7.4; 0.1 M NaCl; 1% BSA; 0.02% NaN_3) and 5 μL of media binding reagent [supplied by the manufacturer]) were added, and samples were incubated for 30 minutes in the dark at room temperature. After centrifugation at 250 × *g* for 10 minutes, sperm were resuspended in 500 μL of 1× binding buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 4% BSA) and kept in ice until analysis by flow cytometry. Before cytometric analysis, 10 μL of propidium iodide (PI, 30μg/mL in PBS) were added and samples were acquired within 10 minutes. For each experimental set, 2 sperm suspensions were prepared for instrument setting and data analysis 1) by omitting both Ann V-FITC and PI staining (the autofluorescence sample), and 2) by omitting only the PI staining (the sample for compensation, see below). Positive controls were

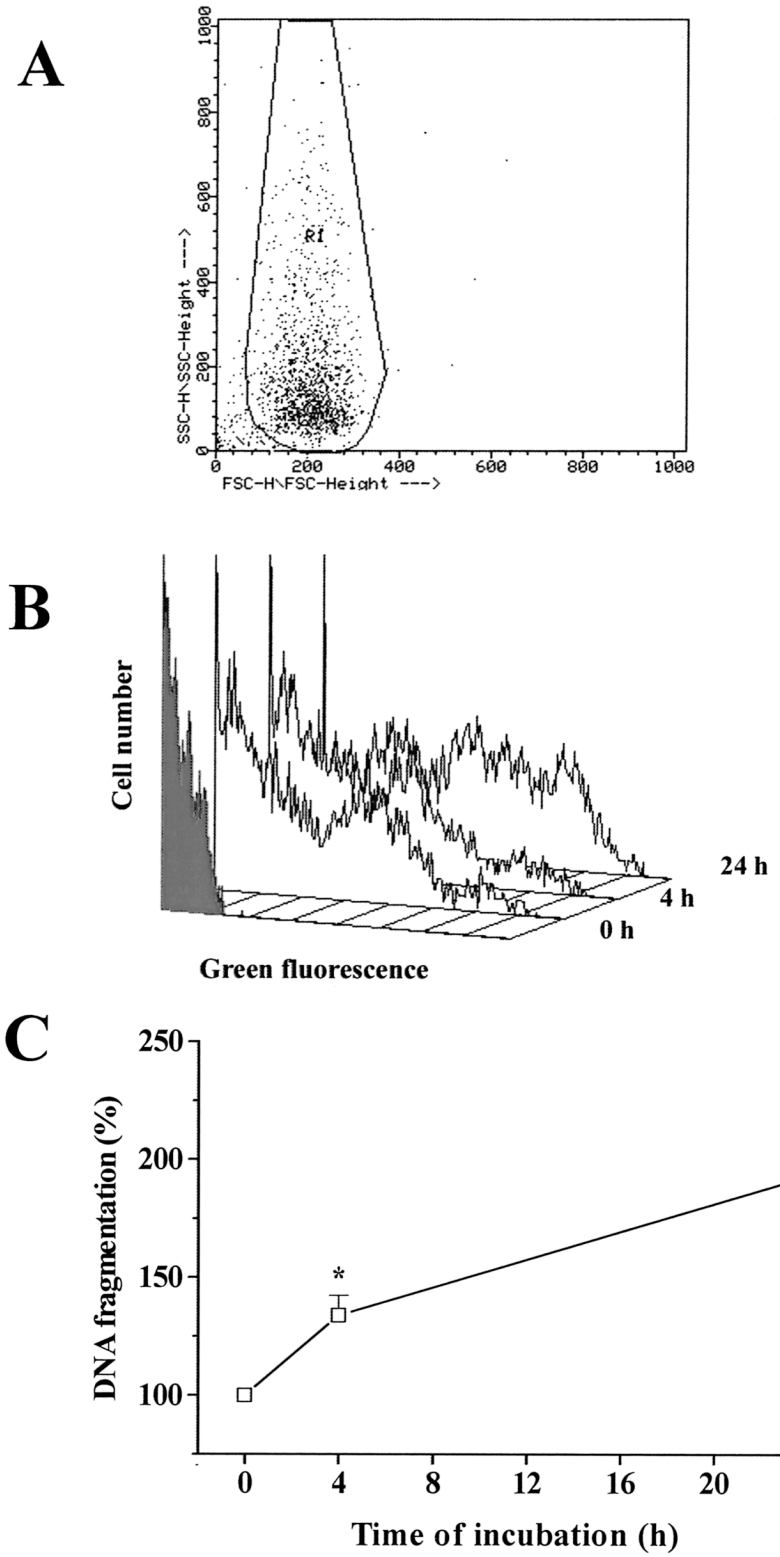

Figure 1. Development of in vitro sperm DNA fragmentation. **(A)** FSC/SSC dot plot from a swim-up selected sperm sample and the characteristic flame-shaped region that includes the population of interest. **(B)** Frequency distributions of DNA fragmented sperm from a population incubated for 0, 4, and 24 hours. A histogram corresponding to the negative control (solid histogram) is also shown. **(C)** Sperm DNA fragmentation expressed as a percentage (mean ± SE) of basal value in spermatozoa incubated for 0, 4, and 24 hours. *$P < .001$ vs time 0.

prepared by treating sperm samples with H_2O_2, 25 μM for 1 hour (Ramos et al, 2001).

Ann V-FITC green fluorescence and PI red fluorescence were revealed by using, respectively, FL-1 and FL-2 (563–607 nm wavelength band) detectors. Fluorescence compensation was set by acquiring sperm labeled with the only Ann V-FITC. For each sample, 10 000 events were recorded and percentages of subpopulations were determined after gating out debris as described above. Quadrant setting was established in the FL-1/FL-2 dot plot corresponding to the autofluorescence sample by including more than 99% of total events in the lower left quadrant.

Statistical Analysis

Data were analyzed with MicroCal Origin software 3.5 version (MicroCal Software Inc, Northampton, Mass) and SPSS 6.0 version (SPSS Inc, Chicago, Ill). Results are presented as means ± SE (unless otherwise stated) and were obtained in at least three experiments. We used the paired Student *t* test and the Wilcoxon signed rank test to assess whether statistically significant differences occurred over time in the various investigated parameters by comparing mean values for each parameter at the different time points. The paired Student *t* test and the Wilcoxon signed rank test were also used to compare groups with and without treatment (ie, mercaptosuccinate and ATA). Differences were considered significant if the probability of their occurrence was $< .05$. Correlation tests were performed by linear regression analysis.

Results

Increase of DNA Fragmentation With Time in Ejaculated Sperm

Figure 1B shows the fluorescence histograms of TUNEL-labeled sperm incubated for 0, 4, and 24 hours from a typical experiment. The average percentages of DNA fragmented sperm at 4 and 24 hours with respect to basal values (time 0) are reported in Figure 1C. As shown, sperm DNA fragmentation continued after ejaculation during the 24 hours of in vitro incubation, increasing from 133.9% ± 8.4% (n = 24) after 4 hours to 193.2% ± 12.6% (n = 43) at 24 hours. As previously observed (Muratori et al, 2000), the percentage of TUNEL-labeled sperm detected immediately after swim-up selection was highly variable (mean ± SD, 14.9% ± 10.7%, n = 43). The increase in sperm DNA fragmentation rate with increasing time was also variable (mean ± SD, 16.8% ± 10.6%, after 24 hours of incubation, n = 43). Furthermore, we found a linear relationship between the basal values of DNA damage and the corresponding percentage rates of de novo–generated DNA fragmentation in 24 hours of incubation ($r = 0.63$, $P < .001$, n = 43; Figure 2A). The correlation between basal rates of DNA fragmentation and subsequent DNA fragmentation rates suggested that some intrinsic sperm feature of the basal populations had affected the development of the subsequent in vitro DNA damage. To verify whether morphological anomalies could represent such an intrinsic sperm feature, semen samples were divided according to their corresponding seminal standard parameters. We found that when teratozoospermic samples were selected (22 out of 43), the above relationship became higher ($r = 0.72$, $P < .001$, n = 22; Figure 2B), whereas it was completely lost in the remaining samples (data not shown).

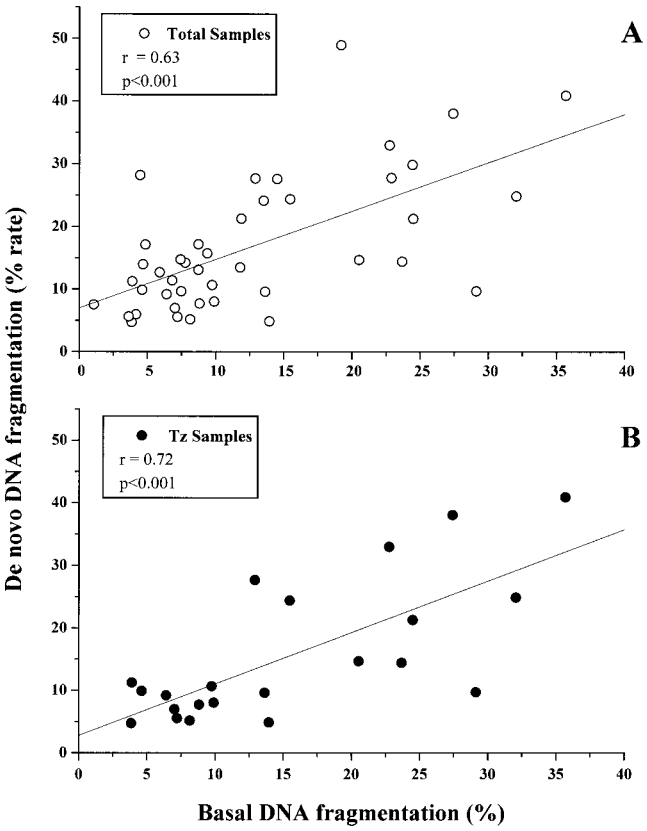

Figure 2. Linear regression analysis between basal DNA fragmentation values and the corresponding percentage rates of de novo DNA fragmentation generated in 24 hours of incubation. **(A)** Total samples (n = 43). **(B)** Samples from patients with teratozoospermia (Tz, n = 22).

Effects of Nuclease and GPX Inhibition on Sperm DNA Fragmentation

We next performed a series of experiments aimed at understanding the mechanisms that were responsible for sperm DNA fragmentation. Activation of a nuclease activity has been reported to occur in mature spermatozoa, leading to sperm chromosomal DNA cleavage (Maione et al, 1997); such nuclease activity is inhibited by ATA (Maione et al, 1997). To study whether in vitro spontaneous sperm DNA fragmentation was due to activation of sperm nucleases, we incubated spermatozoa for 4 and 24 hours in the presence or absence of 25 μM ATA. As shown in Figure 3A, treatment with ATA did not signif-

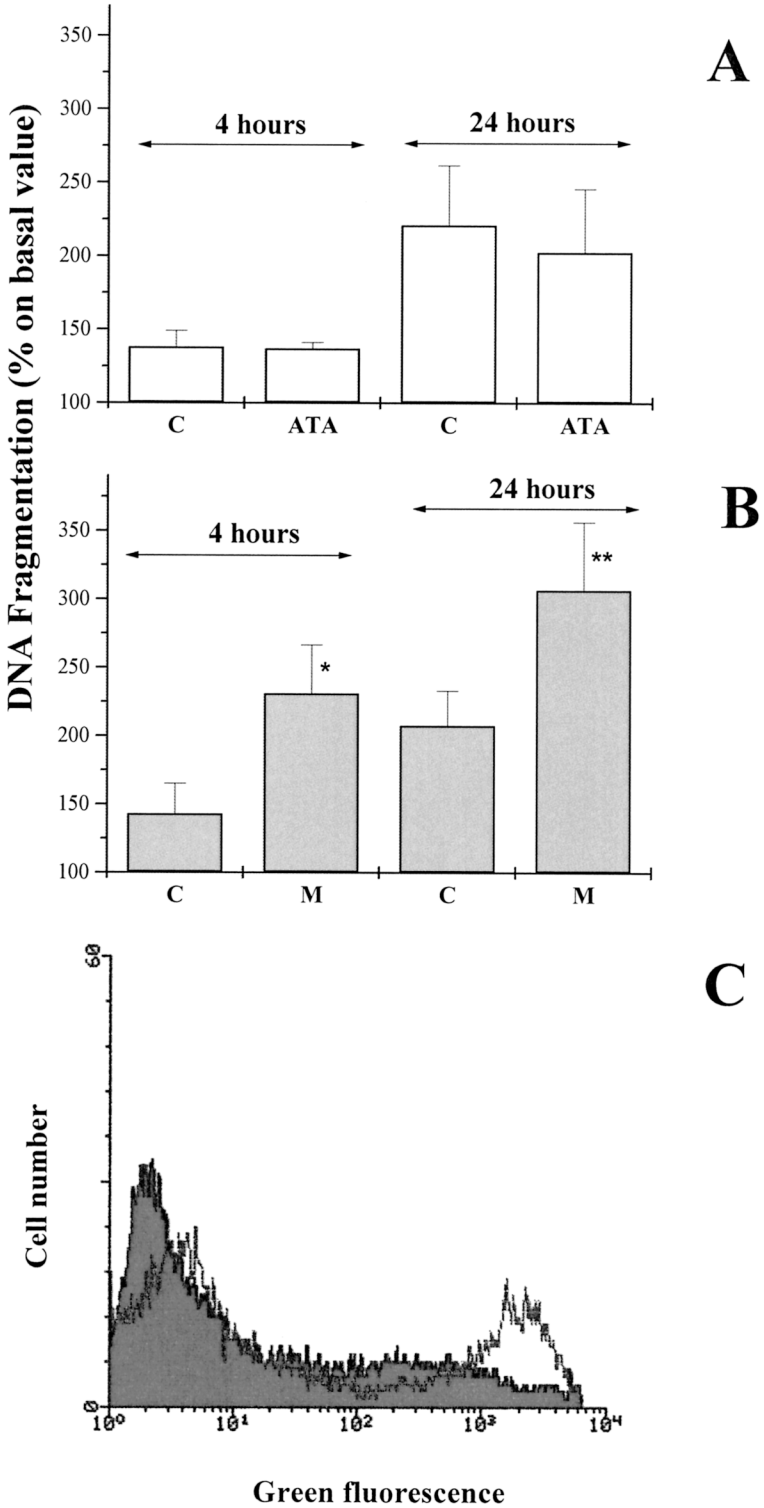

Figure 3. Effects of 25 μM ATA **(A)** and 2 mM mercaptosuccinate (m) **(B)** on DNA fragmentation in spermatozoa after 4 hours (n = 5 and 6 in **A** and **B,** respectively) and 24 hours (n = 6 and 8 in **A** and **B,** respectively) of incubation. C, control. Data (mean ± SE) are percentages of basal values.*$P < .05$; **$P < .01$ vs basal values. **(C)** DNA fragmentation increase over control (solid histogram) in a sperm population treated for 24 hours with mercaptosuccinate (open histogram).

icantly affect sperm DNA fragmentation at any time of incubation.

Because it is well known that sperm exposure to ROS (Lopes et al, 1998b) or induction of sperm ROS genesis by nicotinamide adenine dinucleotide phosphate (NADPH) (Aitken et al, 1998; Twigg et al, 1998) causes DNA fragmentation, we verified whether inhibition of ROS scavenging affected the development of in vitro sperm DNA fragmentation. We treated spermatozoa with 2 mM mercaptosuccinate, an inhibitor of GPX activity (an enzymatic scavenger) for 4 and 24 hours. Figure 3B shows that the GPX inhibitor increased sperm DNA fragmentation development after both 4 and 24 hours of treatment. Figure 3C shows the increase in TUNEL fluorescence over controls in a typical experiment of sperm treatment (24 hours) with mercaptosuccinate.

Sperm DNA Fragmentation and Translocation of Membrane Phosphatidylserine

In this part of the study we investigated whether the development of in vitro sperm DNA fragmentation was associated with other signs of cell deterioration, such as translocation of membrane phosphatidylserine (an apoptotic marker for somatic cells; Martin et al, 1995), and loss of viability and motility. Translocation of phosphatidylserine in live and dead sperm was determined by the Ann V-FITC binding assay, followed by PI staining and cytometric fluorescence detection of both fluorochromes. Figure 4A and B show typical dot plots for staining with Ann V-FITC and PI in the sperm of two patients immediately after swim-up selection. A positive control of the technique, obtained by treating sperm with H_2O_2 (25 μM, 1 hour; Ramos et al, 2001) is reported in Figure 4C. Based on the quadrant setting in the autofluorescence dot plot, three sperm subpopulations are distinguishable in the samples (Figure 4A, B, and C): 1) viable cells, Ann V negative (Ann V^-/PI^-), lower left quadrant; 2) viable cells, Ann V positive (Ann V^+/PI^-), lower right quadrant; and 3) necrotic cells, Ann V positive (Ann V^+/PI^+), upper right quadrant.

We determined DNA fragmentation with TUNEL (n = 14), motility with microscopy (n = 13), and translocation of phosphatidylserine in live and dead sperm with an Ann V binding assay (n = 16) in swim-up selected sperm populations incubated for 0, 4, and 24 hours. The average values for results are reported in Figure 5, showing necrosis, DNA fragmentation, and percentage of immotile sperm, all with a similar pattern of increase over time. Indeed, linear regression analyses confirmed that a strong relationship existed between TUNEL-labeled sperm at each incubation time point and the percentages of 1) immotile sperm at basal time (as already reported in Muratori et al, 2000) and after 4 and 24 hours (data not shown), and 2) necrotic sperm at corresponding times (0 hours (r = 0.72, $P < .01$, n = 14; 4 hours, r = 0.74, $P < .01$, n = 14; 24 hours, r = 0.89, $P < .001$, n = 14; Figure 6A).

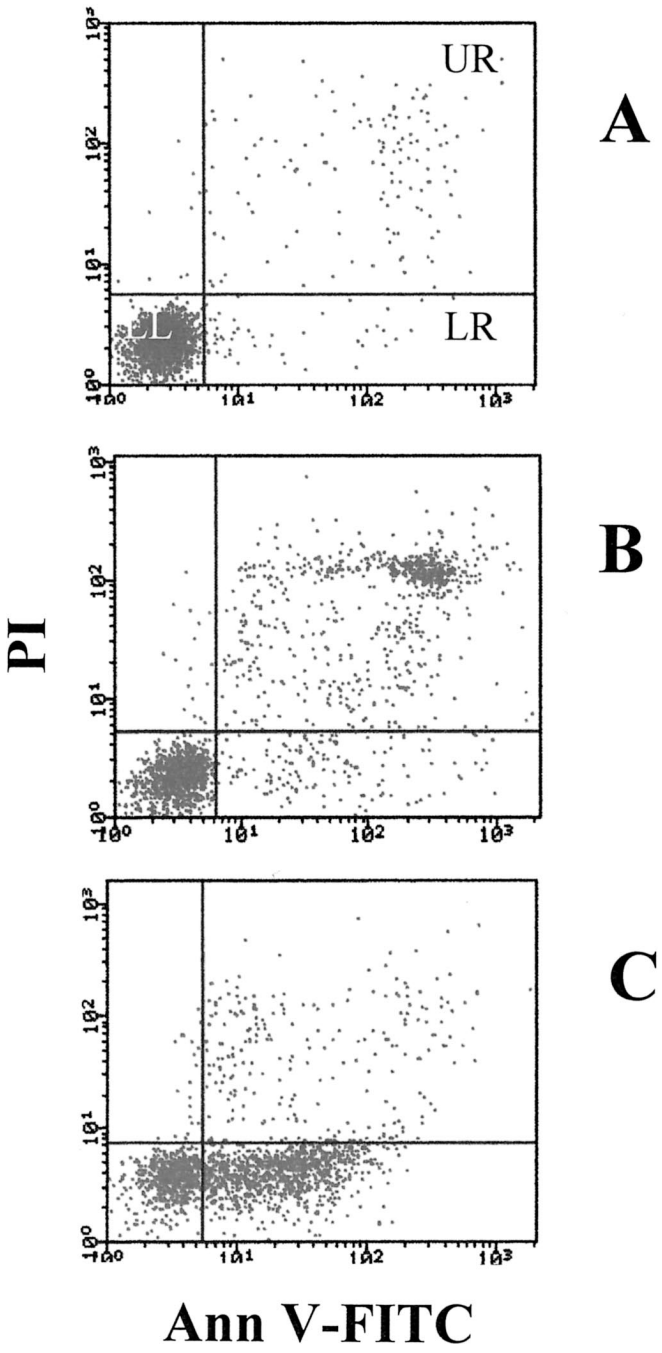

Figure 4. Flow cytometric dot plots of double staining with Ann V-FITC and PI. Patients with low **(A)** and high **(B)** percentages of Ann V^+/PI^- and Ann V^+/PI^+ sperm, stained immediately after swim-up. **(C)** Positive control obtained by treating a sperm suspension with 25 μM H_2O_2 for 1 hour (Ramos et al, 2001). Lower left quadrant, Ann V^-/PI^- sperm; lower right quadrant, Ann V^+/PI^- sperm; upper right quadrant, Ann V^+/PI^+ sperm. Data were analyzed after gating out debris from the population of interest (see Figure 1A).

As shown in Figure 5, whereas percentages of necrotic,

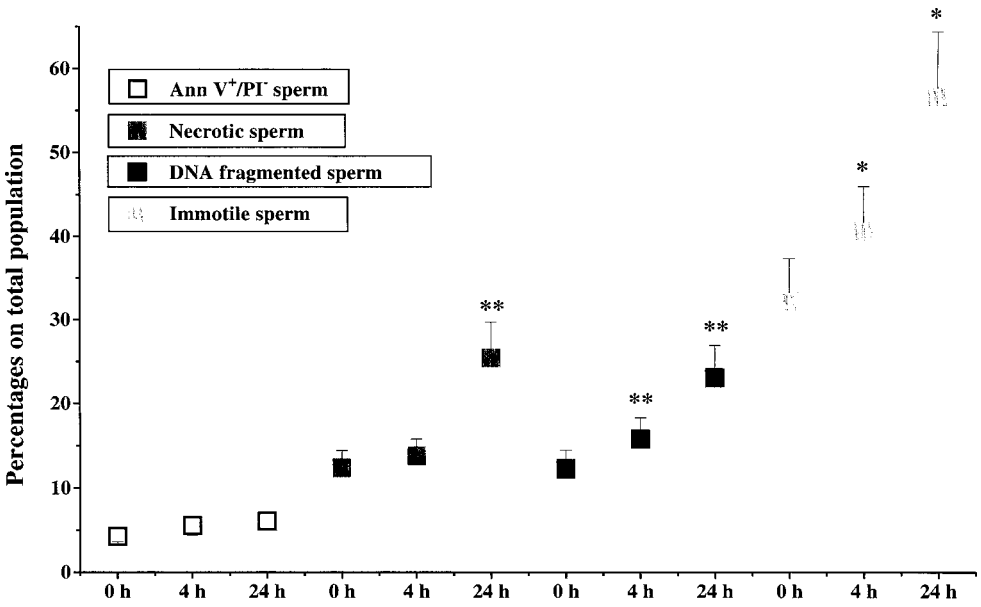

Figure 5. Time course of Ann V binding in viable spermatozoa (white squares, n = 16), necrosis (gray squares, n = 16), DNA fragmentation (black squares, n = 14), and percentage of immotile cells (light gray squares, n = 13) in sperm populations incubated for 0, 4, and 24 hours. Values are percentages of total population. *$P < .01$; **$P < 0.001$ vs time 0.

DNA fragmented, and immotile sperm increased during incubation times, the percentage of Ann V binding in viable sperm remained at about the same value. This result suggests that positive attraction to Ann V by viable sperm during incubation is transient and that Ann V^+/PI^- sperm may represent cells undergoing an initial and transient membrane damage, which later develops into a massive increase in permeability, and necrosis. To gain further insight to this interpretation, we tested whether the basal percentage of Ann V^+/PI^- sperm correlated with the increase in sperm necrosis in the following 24 hours of incubation. Figure 6B shows that basal Ann V^+/PI^- sperm percentages and the percentage rates of de novo–generated necrosis during the next 24 hours were highly correlated ($r = 0.69$, $P < .01$, n = 16), indicating that basal Ann V^+/PI^- values are predictive of subsequent sperm necrosis. The percentages of Ann V^+/PI^- sperm as determined in each sample at the indicated times were not correlated with the corresponding percentages of progressive motility and showed a negative correlation with total motility ($r = -0.57$, $P < .01$, n = 39).

Considering the sharp correlation between sperm DNA fragmentation and sperm necrosis (Figure 6A), we believed that if Ann V^+/PI^- sperm represented cells that would later die by necrosis, then basal Ann V^+/PI^- sperm should predict the development of DNA damage in the next time period of incubation. Indeed, basal Ann V^+/PI^- sperm correlated strictly ($r = 0.78$, $P < .001$, n = 14) with the rate of de novo DNA fragmentation, which was generated in the following 24 hours of incubation (Figure 6C).

Discussion

The present study shows that the process of DNA fragmentation in spermatozoa progresses even after ejaculation. Indeed, we demonstrate that in vitro incubation of swim-up selected human spermatozoa in HTF medium without the addition of external factors results in a progressive increase in the percentage of DNA fragmented sperm. This finding is consistent with previous reports that investigated chromatin integrity by the sperm chromatin structure assay (SCSA) in mammalian sperm incubated in vitro for a long time in similar conditions (Estop et al, 1993; Ellington et al, 1999).

To explain spontaneous DNA fragmentation during in vitro sperm incubation, the involvement of a sperm endonuclease activity can be postulated, as it occurs in somatic cells (Wyllie et al, 1980; Ishizaki et al, 1993, 1994, 1995). Indeed, Maione et al (1997) reported that a particular nuclease activity that is sensitive to the nonspecific deoxyribonuclease inhibitor ATA, is present in mature sperm. However, other authors (Krzyzosiak et al, 2000) have shown that ATA was ineffective and even detrimental to sperm chromatin integrity during long-term incubation of bovine sperm. In the present study, we showed that treatment of human sperm with ATA did not prevent the occurrence of DNA damage in vitro, suggesting that such nuclease activity cannot account for the phenomenon.

Another possible cause of spontaneous DNA fragmentation is ROS production by sperm. We showed that in-

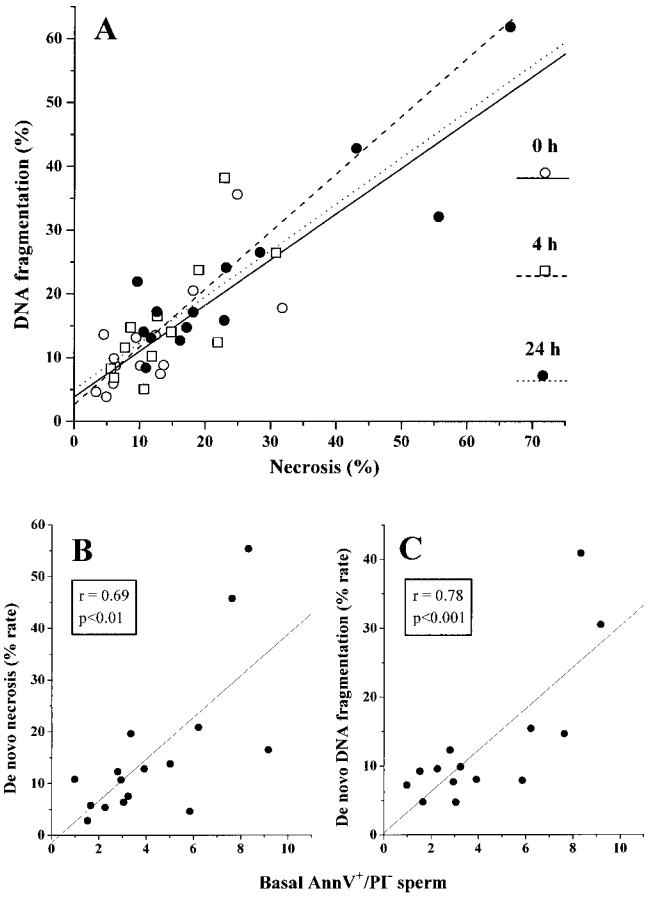

Figure 6. **(A)** Linear regression analyses between percentages of DNA fragmented sperm (at 0, 4, and 24 hours of incubation) and percentages of necrotic sperm at the corresponding time points (time zero: solid line, $r = 0.72$, SD = 5.9, n = 14, $P < 0.01$; 4 hours: dashed line, $r = 0.74$, SD = 6.7, n = 14, $P < 0.01$; 24 hours: dotted line, $r = 0.89$, SD = 6.4, n = 14, $P < 0.001$). **(B)** Linear regression analysis between percentages of basal Ann V^+/PI^- spermatozoa and the corresponding percentage rates of de novo (24 hours) sperm necrosis (n = 16). **(C)** Linear regression analysis between percentages of basal Ann V^+/PI^- spermatozoa and the corresponding percentage rates of de novo (24 hours) sperm DNA fragmentation (n = 14).

hibition of an enzymatic ROS scavenger, such as sperm GPX activity, markedly increased the phenomenon at each time investigated. This finding is consistent with a possible involvement of ROS genesis in determining the increase in DNA fragmentation. It must be noted that the use of swim-up selected spermatozoa allows us to exclude the presence of contaminating non-sperm cells (ie, immature round germ cells or leukocytes) in the incubation medium, suggesting that if ROS are involved in the phenomenon, they derive from sperm cells. Several reports support the possibility that spermatozoa produce ROS, and that ROS are involved in producing detrimental effects in sperm during in vitro incubation (for a review, see Storey, 1997), including DNA damage (Aitken et al, 1998; Twigg et al, 1998). In seminal fluid, ROS along with leukocytes and precursor germ cells, are produced by immotile or morphologically abnormal spermatozoa, and by morphologically normal but functionally abnormal spermatozoa (for a review see De Lamirande and Gagnon, 1999). It has been previously demonstrated that the amount of DNA fragmentation correlates with the levels of ROS generated in Percoll selected spermatozoa (Barroso et al, 2000). Moreover, NADPH-induced endogenous sperm ROS production causes DNA fragmentation and damage to the sperm plasma membrane (Aitken et al, 1998; Twigg et al, 1998). In addition, several reports have shown that detrimental effects to sperm chromatin/DNA accompany in vitro sperm aging, but they can be lessened in antioxidant conditions such as incubation in the presence of egg yolk extender (Karabinus et al, 1991) or coincubation with oviduct epithelial cells (Ellington et al, 1999). Although we cannot exclude that an unknown nuclease activity that is insensitive to ATA is involved in spontaneous sperm DNA fragmentation, or that such activity can be ultimately triggered by ROS as it occurs in somatic cells (Vinatier et al, 1996), overall, our data suggest an involvement of ROS in producing DNA fragmentation in sperm.

We show here that the rate of DNA fragmentation during in vitro incubation is characterized by a great variability among samples. On the other hand, the correlation between DNA fragmentation rates and the corresponding basal values suggests that the two phenomena are due to the same semen characteristic. Because such a correlation is greater when only the teratozoospermic samples are considered, and is lost in those with normal morphology, we suggest that sperm DNA damage is prevalent in morphologically abnormal spermatozoa. Indeed, a correlation between basal DNA fragmentation in swim-up selected sperm and abnormal morphology has already been reported by our group (Muratori et al, 2000) and by others (Sun et al, 1997; Lopes et al, 1998a; Irvine et al, 2000), whereas no correlation has been found with submicroscopic apoptosis-like sperm morphology (Muratori et al, 2000). On the other hand, DNA fragmented sperm also exhibit persistent cytoplasmic residues, as observed by electron microscopy (Muratori et al, 2000). The presence of cytoplasmic residues has been indicated as a feature of immature sperm (Huszar and Vigue, 1993) and is associated to an excessive production of ROS by sperm (Gomez et al, 1996). Recently, it has been reported that ejaculated sperm with diminished maturity display higher ROS production and DNA fragmentation as well as abnormal morphology of head (Ollero et al, 2001), midpiece, and tail (Gergely et al, 1999).

Because ROS addition induces sperm phosphatidylserine translocation and subsequent loss of viability (Duru et al, 2000; Ramos et al, 2001), our results from the Ann V binding assay and PI staining are also consistent with those that involve ROS in in vitro sperm deterioration and

DNA fragmentation. Indeed, we have shown that the basal Ann V^+/PI^- value, which is an index of sperm membrane damage (D'Cruz et al, 1998; Duru et al, 2000; Oosterhuis et al, 2000), predicts the development of DNA fragmentation. In addition, a PI-positive indication, which is an index of necrosis, is directly correlated with the phenomenon at each time of in vitro incubation.

The meaning of Ann V positive staining in viable sperm is controversial. Indeed, some authors have observed an increased presence of Ann V binding in the most motile fraction of sperm populations (Barroso et al, 2000), hypothesizing that this parameter may represent a membrane modification due to capacitation (Barroso et al, 2000; Duru et al, 2001). However, a variable percentage of Ann V positive cells has been also found in spermatozoa that were not selected (Oosterhuis et al, 2000; Anzar et al, 2002). In addition, Schuffner et al (2002) showed that phosphatidylserine externalization is decreased during a short incubation in capacitating medium, indicating that the phenomenon may not be exclusively associated with capacitation. In our experimental model, positive Ann V staining was negatively correlated with total motility. In addition, it is highly predictive of both DNA damage progression and necrosis. In this light, we speculate that the lack of an increase in Ann V^+/PI^- cells during incubation in vitro indicates that these cells evolve to necrosis (with a massive loss of membrane integrity). At the same time, a fraction of Ann V^- cells becomes positive, keeping the percentage of Ann V^+/PI^- sperm almost unvaried during incubation.

For patients with poor semen quality (and thus greater sperm DNA fragmentation) who participate in ART, two major issues have been raised. The first is whether ART increases the risk that a DNA fragmented sperm fertilizes the oocyte. If, as is suggested by our study, DNA fragmented sperm are also necrotic, it appears unlikely that these sperm can participate in fertilization in IVF procedures. On the other hand, selection of a single spermatozoon for injection into the oocyte in ICSI generally excludes the immotile (and thus the dead) cells.

The second point is whether sperm DNA damage represents a predictive parameter for the outcome of fertilization. Emerging findings show that sperm DNA/chromatin integrity is required for optimum fertility success (Evenson et al, 2002). Studies on sperm DNA integrity by SCSA have recently established a 30% DNA fragmentation index value in the sperm genome as a threshold over which there is a significantly reduced probability of a successful pregnancy (Evenson et al, 2002). To explain why the remaining 70% of sperm that are apparently not damaged are insufficient for fertility, the authors suggest that such a threshold could be considered as the tip of the iceberg (ie, an index of the presence of a DNA anomaly in the overall sperm population). This suggestion is somehow confirmed by the relationship between basal damage and the rate of de novo DNA fragmentation, which we observed in the present study. Indeed, such a relationship is consistent with the amount of basal DNA fragmentation serving as a sign of DNA anomaly, or the vulnerability of the entire sperm population, or both.

Overall, our data support the involvement of sperm ROS production in inducing detrimental effects during in vitro incubation of spermatozoa. Moreover, we observed that DNA fragmentation in spermatozoa does not appear to correspond strictly with the classic apoptotic process described for somatic cells, as was already suggested by previous studies (Weil et al, 1998; Muratori et al, 2000; Sakkas et al, 2002). Weil et al (1998) speculated that murine sperm lose their caspase-dependent death program in their final stages of development, because only a small number of sperm exhibit active caspase-3 during spontaneous death in vitro. In addition, it has been observed that DNA strand breaks are not associated with key apoptotic markers (FAS, Bcl-x, and p53; Sakkas et al, 2002) and apoptotic-like ultrastructural features (Muratori et al, 2000).

In conclusion, our data support the hypothesis that oxidative stress is a cause of DNA fragmentation in human spermatozoa and indicate an increase of this damage by incubating sperm in vitro. Other studies are necessary in order to address the etiology of excessive ROS production and the higher DNA susceptibility in sperm of infertile men as well as the effect of DNA fragmentation on pregnancy outcome with ART.

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