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Nuclear staining identifies two populations of human sperm with different DNA fragmentation extent and relationship with semen parameters

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BACKGROUND: Sperm DNA fragmentation is a possible predictive parameter for male fertility status. The occurrence of M540 bodies in semen of subfertile subjects affects flow cytometric investigations in sperm. We set up a new method to evaluate DNA fragmentation excluding M540 bodies. METHODS: DNA fragmentation was evaluated by flow cytometry in semen of 75 subjects both by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL, traditional method) and by double staining with TUNEL and propidium iodide (PI, new method). RESULTS: The use of the new method revealed that TUNEL underestimates sperm DNA fragmentation in flow cytometry and showed two sperm populations stained with low (PI^{dim}) and high (PI^{br}) avidity for PI. The PI^{dim} population is entirely composed of DNA fragmented sperm and its incidence shows highly significant negative correlations with morphology, motility, sperm count and concentration (respectively, $r = -0.51$, -0.52, -0.46 and -0.32, $n = 75$). DNA fragmentation in the PI^{br} sperm population is independent from semen quality. CONCLUSIONS: The correlations between sperm DNA breakage and semen quality previously reported are mainly driven by the occurrence of the PI^{dim} population. DNA fragmented sperm in this population are more likely to have poorer morphology, reduced motility and thus a reduced chance to fertilize an oocyte than DNA damaged sperm in PI^{br} population. Distinguishing between the two types of sperm DNA fragmentation appears to be important in clinical investigations.

Keywords: sperm; DNA fragmentation; terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling; nuclear staining; M540 bodies

Introduction

Sperm DNA fragmentation consists of single and double-stranded DNA breaks, frequently occurring in semen of subfertile patients (Lopes *et al*., 1998; Irvine *et al*., 2000; Muratori *et al*., 2000). Despite the origin and the mechanisms responsible for such genomic anomaly are not yet clarified, it has been proposed that sperm DNA fragmentation could be a good parameter to predict the male fertility status as an alternative or in addition to poorly predictive standard parameters presently determined in routine semen analysis (Lewis, 2007; Erenpreiss *et al*., 2006). Indeed, sperm DNA breakage reflects, but not exactly overlaps, standard semen parameters (Lopes *et al*., 1998; Irvine *et al*., 2000; Muratori *et al*., 2000) suggesting that it is partially independent from semen quality. Results of studies aimed to establish whether the amount of sperm DNA fragmentation could predict the outcome of Assisted Reproduction Techniques (ARTs) are conflicting. The fact whether or not the amount of sperm DNA fragmentation negatively impacts on fertilization, embryo development and pregnancy rate is still matter of controversy (for review see O'Brien and Zini, 2005; Li *et al*., 2006; Tarozzi *et al*., 2007). Such conflicting results have been ascribed to different causes (Makhlouf and Niederberger, 2006), including poor criteria for couples recruitment, different sperm populations used for DNA fragmentation detection (unprocessed semen or selected sperm), and different techniques used to determine DNA damage (Evenson and Wixon, 2006; Li *et al*., 2006). Concerning the latter point, one of the most popular technique employed to detect DNA fragmentation is terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick end labeling (TUNEL) coupled to flow cytometry (O'Brien and Zini, 2005), which allows detection of the phenomenon in a great number of cells.

Our group has reported that the occurrence of M540 bodies (Muratori *et al*., 2004) in semen of subfertile patients may heavily affect flow cytometric investigations on sperm (Muratori *et al*., 2005). These elements are variable in size and density and occur in high level in men with poor quality semen (Muratori *et al*., 2004; Marchiani *et al*., 2007). In

addition, since most of them exhibit size and density properties similar to sperm heads they cannot be directly distinguished from sperm by using only the light scattering (FSC, Forward Scatter; SSC, Side Scatter) signals, and labeling the sample with a fluorescent probe (see below) to distinguish them from sperm is also needed (Muratori *et al*., 2005). Recently, we have demonstrated that M540 bodies are apoptotic bodies, as they contain apoptotic features including caspase activity, p53 and FAS (Marchiani *et al*., 2007). In addition, a fraction of M540 bodies contains fragmented DNA (Marchiani *et al*., 2007). Up to now, all the flow cytometric investigations on sperm DNA fragmentation in unprocessed semen included M540 bodies in the analysis and it is not known whether and how their presence affects the measures of this parameter. M540 bodies may be removed from the analysis following nuclear staining of the samples, as M540 bodies are devoid of nucleus (Muratori *et al*., 2004; Marchiani *et al*., 2007).

The aim of the present study was to re-evaluate sperm DNA fragmentation in unprocessed human semen, by TUNEL coupled to nuclear staining with propidium iodide (PI) to exclude M540 bodies from the fluorescence analysis. By using such technique, we revealed the presence, in semen of subfertile patients, of two sperm populations characterized by different staining for PI. We report that the two populations show different extents of DNA fragmentation with different relationships to semen parameters.

Material and Methods

Chemicals

Human tubal fluid (HTF) medium and Human Serum Albumin (HSA) were purchased from Celbio (Milan, Italy). Diff-Quick kit was purchased from CGA, Diasint (Florence, Italy). PureSperm was supplied by Nidacon, Gothenberg, Sweden. Bovine Serum Albumin (BSA) was purchased from ICN Biomedicals, Ohio, USA. Ribonuclease A (RNAse A) and the other chemicals were from Sigma Chemical.

Semen samples collection and preparation

Semen samples were consecutively collected [according to World Health Organization (WHO) criteria (World Health Organization, 1999)] from 75 subjects undergoing routine semen analysis for couple infertility in the Andrology laboratory of the University of Florence after the approval of the Hospital Committee for Investigations in Humans and after informed patient's consent. Semen samples with any detectable leukocytes, evaluated by assessing non-sperm components of semen after Diff-Quik staining (World Health Organization, 1999), were excluded from the study. Sperm morphology and motility were assessed by optical microscopy, according to WHO criteria (World Health Organization, 1999). Sperm morphology was evaluated by determining percentage of normal and abnormal forms after Diff-Quik staining, by scoring at least 100 sperm/slide. Sperm motility was scored by determining percentage of progressive motile, non-progressive motile (total motility) and immotile spermatozoa by scoring at least 100 sperm/slide. Semen samples were collected from normozoospermic (N, $n = 8$), asthenozoospermic (A, $n = 5$), teratozoospermic (T, $n = 25$), asthenoteratozoospermic (AT, $n = 26$) and oligoasthenoteratozoospermic (OAT, $n = 11$) subjects (World Health Organization, 1999).

Semen samples preparation

In the present study, experiments were performed in unprocessed semen, swim-up selected and gradient processed sperm samples. Unprocessed semen samples were washed twice with HTF medium and then fixed with paraformaldehyde [200 μl, 4% in phosphate-buffered saline (PBS) pH 7.4] for 30 min at room temperature. Swim-up selection was performed by layering 1 ml of HTF medium supplemented with 10% HSA on the top of an equal volume of semen fluid. After 1 h of incubation at 37°C in 5% CO_2 atmosphere, 900 μl of medium were carefully collected. After centrifugation (500 × g for 10 min) sperm were fixed as described above. For sperm selection by gradient separation, semen samples were layered on 50, 70 and 95% PureSperm fractions (prepared in HTF/HSA medium) and centrifuged at 500 × g for 30 min at 26°C. The resulting 95% pellet was collected, washed with 1 ml of HTF/HSA medium and finally fixed as described above.

TUNEL coupled to nuclear staining and flow cytometry

Sperm DNA fragmentation was determined in unprocessed semen samples. Samples were processed by TUNEL as described elsewhere (Muratori *et al*., 2000). Briefly, fixed spermatozoa (10×10^6) were centrifuged at 500 × g for 10 min and washed twice with 200 μl of PBS with 1% BSA. Then, spermatozoa were permeabilized with 0.1% Triton X-100 in 100 μl of 0.1% sodium citrate for 2 min in ice. After washing two 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 h at 37°C in the dark. Finally, samples were washed twice, resuspended in 500 μl of PBS, stained with 10 μl of PI (30 μg/ml in PBS) and incubated in the dark for 10 min at room temperature. Samples were acquired by a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA, USA) equipped with a 15-mW argon-ion laser for excitation. For each test sample, three sperm suspensions were prepared for instrumental setting and data analysis, by omitting (i) both PI staining and TdT; (ii) only TdT (negative control) and (iii) only PI staining (for fluorescence compensation). Green fluorescence of nucleotides, was revealed by an FL-1 (515–555 nm wavelength band) detector; red fluorescence of PI was detected by an FL-2 (563–607 nm wavelength band) detector. For each sample, 10 000 events were recorded within the characteristic flame shaped region in the FSC/SSC dot plot which excludes debris and large cells (Muratori *et al*., 2003, 2004). We determined sperm DNA fragmentation, within the (i) R1 region (containing sperm and M540 bodies, traditional method, herein indicated as TUNEL) and (ii) PI positive events of the R1 region (i.e. only sperm, new method, herein indicated as TUNEL/PI). In order to exclude PI negative events, a marker was set in the PI axis of the dot plot of the sample in which PI staining and TdT were omitted, including 99% of total events. All the events outside this marker were considered PI positive events in the corresponding test sample. In both the procedures described above, a marker was established in the TUNEL axis dot plot of negative control (TdT omitted), including 99% of total events. This marker was translated in the corresponding test sample and all the events beyond the marker were considered positive for TUNEL.

TUNEL coupled to nuclear staining and fluorescence microscopy

Double stained (TUNEL/PI, see above) samples for DNA fragmentation and nuclei, were smeared on slides and examined using a fluorescence microscope (Leitz, Type 307-148002, Wetzlar, Germany), equipped with E4 and N2.1 filters (Leica, Milan, Italy) by an oil immersion 100× magnification objective. Images were captured by

a Canon digital camera using Remote Capture software (provided by Canon, Japan) and edited by Adobe photoshop version 5.0 (Adobe Systems Inc., CA, USA).

Statistic analysis

Bivariate correlations were evaluated by calculating the Spearman's correlation coefficient (ρ). In experiments aimed to compare DNA fragmentation/cell in the two sperm populations with different PI staining (see Results), results were expressed as mean values of nucleotide fluorescence distribution. Analysis of variance and the Student's *t*-test were used to assess statistically significant differences between (i) the mean values of DNA fragmentation distribution in the two sperm populations (paired data), (ii) the percentages of the two populations before and after sperm selection (paired data), (iii) the differences between the values of DNA fragmentation obtained by TUNEL/PI and TUNEL in A, AT, T and OAT versus N subjects (independent data) and (iv) the percentages and the mean values of PI fluorescence distribution of the two sperm populations before and after treatment with RNAse (paired data). All statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS), version 11.5, software for Windows (SPSS Inc., Chicago, IL, USA).

Results

Evaluation of sperm DNA fragmentation with TUNEL/PI method

Sperm DNA fragmentation was investigated in human semen samples by double staining with TUNEL and PI and detecting fluorescence by flow cytometry. Fig. 1A shows the FSC/SSC dot plot of a semen sample in which the characteristic region (R1) containing sperm and M540 bodies (Muratori *et al*., 2004, 2005) is drawn. Typical dot plots of green (TUNEL) and red (PI) fluorescence in R1 region, corresponding to the negative control (left panel) and the test sample (right panel), are also shown (Fig. 1B). PI negative events are M540 bodies (Fig. 1B, highlighted in grey), whereas PI positive events are spermatozoa. As shown in Fig. 1B, within sperm, two populations with different PI labeling are present: a brighter one (from herein indicated as PI^{br} population) and a dimmer one (PI^{dim} population). We confirmed that both the populations were formed by sperm, by observing TUNEL/PI stained samples by fluorescence microscopy (Fig. 1C and D). As can be observed, pale PI stained spermatozoa (arrowheads) are present, beside brilliant ones (arrows). To check whether the different PI staining could be due to PI binding to RNA, whose content might be different in the two populations, semen samples ($n = 5$) were treated with RNAse (1 mg/ml). The obtained results show that both the percentages (87.0 and 87.1%, respectively, in untreated and treated PI^{br} population, $P = 0.9$; 13.2 and 13.0%, respectively, in untreated and treated PI^{dim} population, $P = 0.9$) and the mean values of the PI fluorescence intensity (359.2 and 391.0, respectively, in untreated and treated PI^{br} population, $P = 0.8$; 106.8 and 110.2, respectively, in untreated and treated PI^{dim} population, $P = 0.9$) did not change after RNAse treatment. FACScan analysis demonstrated that sperm in the PI^{dim}population were always DNA fragmented, as revealed by the complete shift towards the high values of DNA fragmentation of the PI^{dim} population (right panels in Figs 1B and 2). Such finding was confirmed by fluorescence microscopy observation (Fig. 1D).

Comparison of values of sperm DNA fragmentation obtained by TUNEL and TUNEL/PI methods

Sperm DNA fragmentation was detected in unprocessed semen samples from 75 patients, whose mean values of standard semen parameters, age and abstinence are indicated in Table I. To compare values of DNA fragmentation obtained by TUNEL (unable to distinguish sperm from M540 bodies and, within sperm, between the PI^{dim} and the PI^{br} populations) and by TUNEL/PI (i.e. in the population formed by solely sperm, see also material and methods section), we first determined DNA fragmentation in all the events included in the R1 region, containing sperm and M540 bodies (traditional method), and then within the PI positive events of R1 region (i.e. solely sperm, Fig. 2B, new method). In some semen samples (13 out of 75), the two sperm populations showed different levels of non-specific green fluorescence (Fig. 2B, inset in the left panel) as found in negative controls prepared by omitting TdT. Hence, DNA fragmentation was determined separately in the two populations and then the two values were added.

Comparison between the two values of DNA fragmentation obtained in the 75 semen samples is reported in Fig. 3, where each bar indicates the difference (mean $\pm$ SD $= 6.9 \pm 10.0$; range: from -5.5 to 51.1%; $n = 75$) between the value obtained by TUNEL/PI and that obtained by TUNEL in each sample. In most of the semen samples, TUNEL method underestimates sperm DNA fragmentation. As shown in Fig. 3, the differences between the new and the traditional method of calculation are not always the same and appear to be independent from semen quality: no statistical significance was indeed found within the several semen categories (N, OAT, AT, T and A subjects) in the differences between the two techniques (data not shown). Such finding is not surprising, since the differences between values yielded by the two techniques are due to combinations of several factors related to the variable occurrence and characteristic of semen M540 bodies and of the PI^{dim} sperm population. Fig. 2 shows examples of how such factors may affect the determination of sperm DNA fragmentation. Note that the marker set in Fig. 2A (where M540 bodies are considered) is shifted toward right with respect to the marker in Fig. 2B (where only sperm are considered) due to the higher level of non-specific green fluorescence of M540 bodies (as found in negative controls prepared by omitting TdT) versus that of sperm. As a consequence, a fraction of sperm DNA fragmentation (included between the solid and the dash lines in Fig. 2B) results are masked by TUNEL method (Fig. 2A). Also note that only a small fraction of M540 bodies are positive for TUNEL (Marchiani *et al*., 2007) and thus most of them (TUNEL negative) contribute to increasing the percentage of TUNEL negative events when calculated with TUNEL. Further, in those semen samples (13 out of 75) where the PI^{dim} sperm population shows higher level of non-specific fluorescence than the PI^{br} one, a fraction of DNA fragmented sperm in the latter population was masked (included between the solid and the dash lines in the inset of Fig. 2B, right panel).

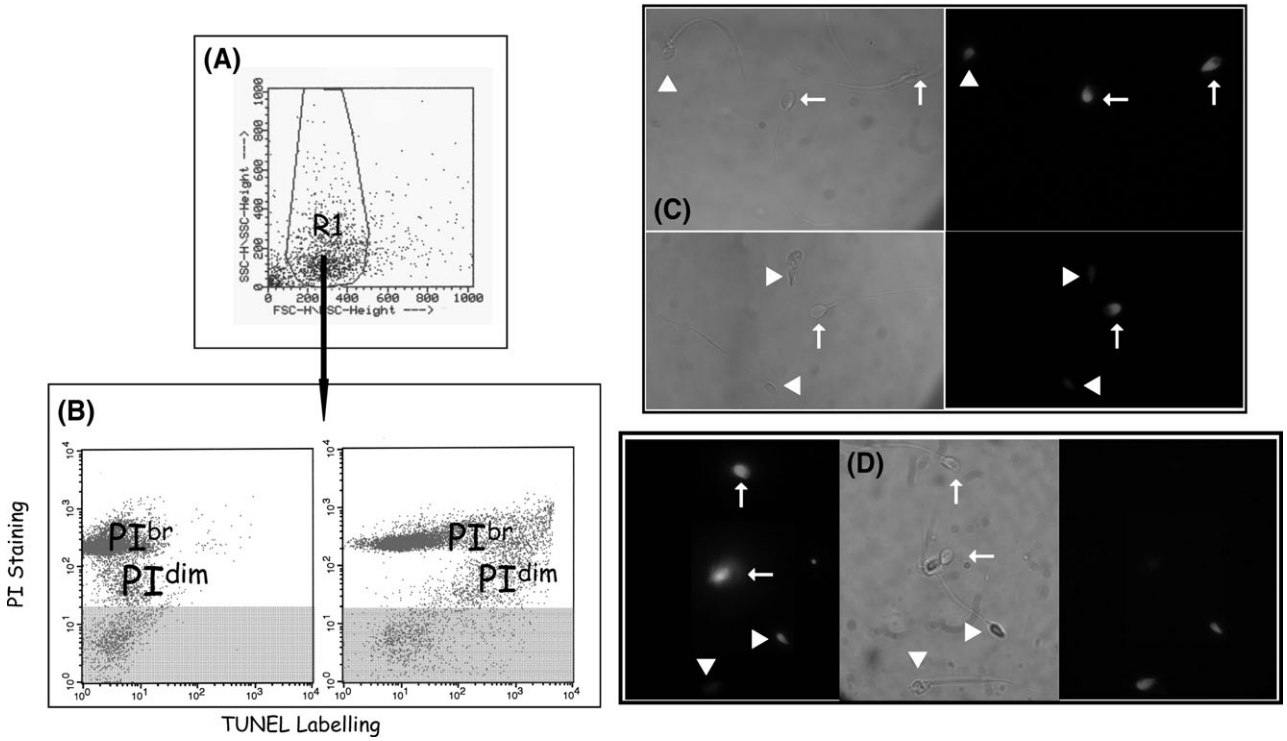

Figure 1: (**A**) Forward scatter and side scatter (FSC/SSC) dot plot obtained by FACScan acquisition of a semen sample. A region (R1) is established to exclude debris and large cells. (**B**) Terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick end labeling/propidium iodide (TUNEL/PI) fluorescence dot plots of a semen sample obtained within R1 region. The left panel shows the negative control (TdT omitted), the right one the test sample. The events negative for PI staining (grey highlighted region) are M540 bodies (devoid of nuclei) whereas the events positive for PI staining are sperm. Note the presence of two sperm populations (indicated by PI^{br} and PI^{dim}), which differ in the intensity of PI staining. C. Micrographs of samples stained with PI (right) obtained by fluorescence microscopy and of the corresponding bright fields (left) are shown. D. Micrographs of samples double stained with TUNEL/PI obtained by fluorescence microscopy and showing the red fluorescence of PI (left), the corresponding bright field (middle) and the green fluorescence of TUNEL (right). Note the presence of bright (arrows) and pale (arrowheads) fluorescent sperm in both (**C**) and (**D**)

Relationship between sperm DNA fragmentation and semen quality

To investigate the relationship between sperm DNA fragmentation and semen quality, we correlated the percentages of DNA fragmented sperm as determined by the two methods described above with the standard semen parameters in the patients included in the study. The Spearman's correlation coefficients and the corresponding *P*-values are reported in Table II. As shown, sperm DNA fragmentation as determined by TUNEL (mean $\pm$ SD = 33.9 $\pm$ 14.6%; range: 14.5–96.1%; $n = 75$), does not significantly correlate with the main semen parameters, although a trend to statistical significance is present for total and progressive motility and normal morphology. In contrast, when TUNEL/PI method is used (mean $\pm$ SD DNA fragmentation: 40.8 $\pm$ 16.3%; range: 13.4–94.6%; $n = 75$), a significant negative correlation was found with normal morphology, progressive and total motility, number and concentration (Table II).

As stated above, PI^{dim} sperm population was entirely composed of DNA fragmented sperm, in each of the subjects included in the study. However, the incidence of such a population is highly variable among semen samples (mean $\pm$ SD = 15.0 $\pm$ 11.3%; range: 1.6–52.2%; $n = 75$), as shown in Fig. 4 where examples of samples with low (upper panel), medium (middle panel) and high (lower panel) levels of this population (highlighted in grey in Fig. 4) are reported. Hence, we investigated whether the different incidence of PI^{dim} sperm population was related to that of semen parameters. We found that the percentages of this PI^{dim}, DNA fragmented population, show highly significant correlations with semen parameters compared to the global sperm DNA fragmentation (i.e. that observed by TUNEL/PI) (Fig. 5 and Table II). Consistently, when we investigated the relationship between standard semen parameters and DNA fragmentation in the PI^{br} sperm population, we did not find any significant association (data not shown), strongly indicating that DNA fragmentation in such a population is independent from quality of semen.

In agreement with the close relationship between the percentages of PI^{dim} sperm population and poor semen parameters, we found that this population was reduced after sperm selection by swim-up (mean $\pm$ SD = 6.2 $\pm$ 4.6% versus 16.7 $\pm$ 8.5% in corresponding unprocessed semen samples; $P < 0.001$, $n = 6$) and PureSperm gradient separation (mean $\pm$ SD = 13.8 $\pm$ 8.6% versus 18.1 $\pm$ 9.5 in corresponding unprocessed semen samples; $P < 0.01$, $n = 7$).

Comparison of DNA fragmentation in the PI^{dim} and PI^{br} sperm populations

To compare the intensity of sperm DNA fragmentation in the two sperm populations, we calculated the mean values of

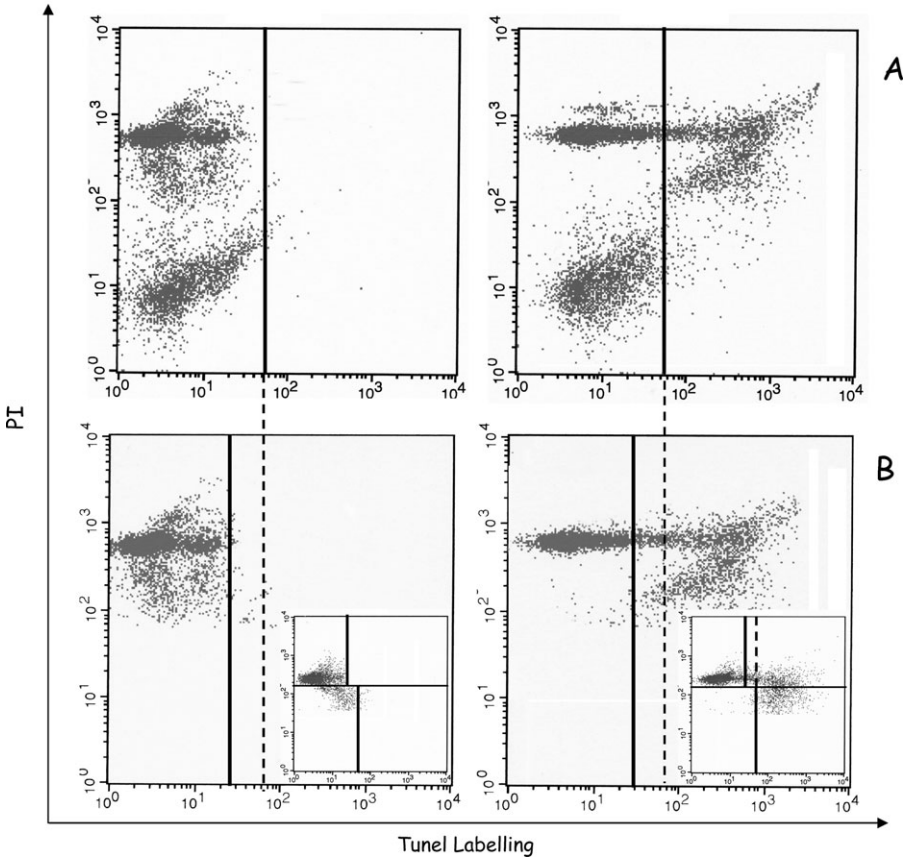

Figure 2: Different methods of calculating DNA fragmentation in sperm.
(**A**) Fluorescence analysis is conducted within R1 region (see Fig. 1A). (**B**) Fluorescence analysis is conducted within the PI positive events of the R1 region, thus in the population formed solely by sperm. In the TUNEL/PI dot plots of the negative controls a region is established that includes more than 99% of the events (A and B, left panels). Such a region is translated into the corresponding test samples (A and B, right panels). Dash lines mimic in (B), the region established in (A). Note that a fraction of sperm DNA fragmentation (included between the solid and the dash lines) would be masked when it is calculated in R1 region (TUNEL method). In the insets, an example of those samples (13 out of 75) in which there was a different level of non-specific green fluorescence in the PI^{br} and PI^{dim} populations. In these cases, different negative regions were established, one for each population. Hence, DNA fragmentation was calculated separately in the two populations and the two values were added. Dash lines in the insets mimic in PI^{br} population the region established in PI^{dim} one. Note that a fraction of sperm DNA fragmentation in PI^{br} population (included between the solid and the dash lines) would be masked by using the region established for PI^{dim} population (TUNEL method).

Table I. Semen parameters, age and abstinence (mean ± SD) of subjects included in the present study

Parameter	Mean ± SD	*n*
Sperm count ($\times 10^6$)	187.5 ± 170.1	75
Sperm concentration (sperm/ml)	63.9 ± 56.9	75
Total motility (%)	62.6 ± 16.1	75
Progressive motility (%)	48.1 ± 17.5	75
Normal morphology (%)	18.2 ± 1.0	75
Volume (ml)	3.2 ± 1.3	75
pH	7.4 ± 0.1	75
Age (years)	34.7 ± 6.8	75
Abstinence (days)	3.8 ± 1.5	75

DNA breakage distribution (i.e. a measure of DNA fragmentation/cell) in fragmented sperm of each population (i.e. in the entire PI^{dim} sperm population and in the DNA fragmented sperm fraction of the PI^{br} sperm population). We found that DNA damage per cell was greater in PI^{dim} sperm population than in PI^{br} one (mean ± SD = 567.0 ± 355.5 versus 478.1 ± 295.2, respectively; $P < 0.05$).

Discussion

Two main messages emerge from the present study: (i) that TUNEL coupled to flow cytometry technique, frequently used to evaluate sperm DNA fragmentation in human semen, leads to a heavy underestimation of the phenomenon; (ii) that there are two types of sperm DNA fragmentation, one dependent and the other independent of semen quality. We believe that these results may greatly impact future studies on the relationship between sperm DNA fragmentation and ART outcome.

TUNEL is a popular assay to investigate sperm DNA breakage. One of the advantages of this technique is the fact that it can be coupled to flow cytometry, a rapid and objective technology relying on large numbers of observations. However, the reliability of flow cytometry deeply depends on how precisely the population of interest (in this case sperm) is defined. In a recent paper (Muratori *et al*., 2005), our group has shown that the exclusion of semen elements other than sperm (such as M540 bodies) from flow cytometric analysis of ubiquitination yields a positive relationship between

semen quality and ubiquitination, unlike previous studies in which ubiquitination was investigated in global semen (Sutovsky *et al*., 2004). In the present study, we show that measuring sperm DNA fragmentation by TUNEL method in a FSC/SSC region excluding debris and large cells is not sufficiently precise. The use of TUNEL/PI double staining technique not only allowed us to distinguish between M540 bodies and sperm, but also revealed the occurrence, within sperm, of two populations with different PI staining properties and different levels of DNA fragmentation. Overall, omitting subtraction of M540 bodies and lack of recognition of differently PI-stained populations of sperm lead to an underestimation of DNA fragmentation (Fig. 3). More important, such an underestimation is not always the same in the different samples and is not predictable from standard semen parameters. In fact, it is due to the variable combinations of two factors: (i) M540 bodies that are included in the same FSC/SSC region of sperm, that may contain a variable percentage of DNA fragmentation and may exhibit high levels of non-specific fluorescence, masking a fraction of DNA fragmented sperm (Fig. 2); (ii) the presence of a PI^{dim} sperm population, whose level of non-specific fluorescence may be very large thus masking a fraction of DNA damaged cells in the PI^{br} population (Fig. 2).

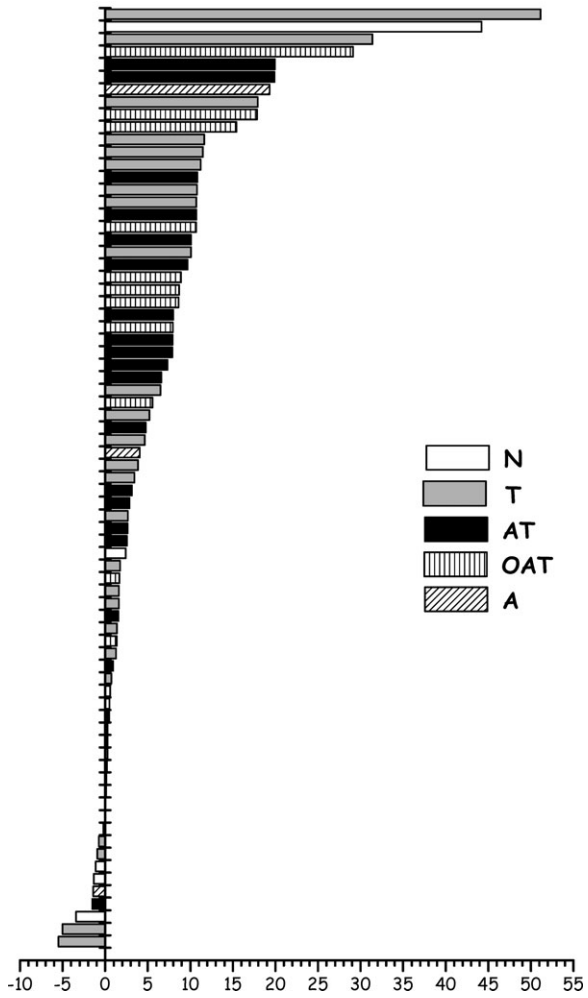

Figure 3: Differences between the percentages of DNA fragmented sperm calculated by the TUNEL/PI technique and by TUNEL in the 75 samples included in the study.
N, normozoospermic, A, asthenozoospermic-, T, teratozoospermic-, AT, asthenoteratozoospermic, OAT, oligoasthenoteratozoospermic subjects

In the present study, we report that the relationship between semen quality and levels of DNA fragmentation in our cohort of subjects becomes significant when the DNA damage is calculated using the TUNEL/PI method and shows even more strict and highly significant correlation coefficients when calculated within the PI^{dim} population which is formed only by DNA fragmented sperm. Conversely, evaluation of sperm DNA fragmentation with TUNEL, did not result in any statistically significant correlation, albeit showing a trend towards an association between sperm DNA fragmentation and poor morphology and motility. Not surprisingly, other studies performed with comparable techniques (i.e. TUNEL coupled to flow cytometry in unselected sperm) reported conflicting results: whereas Varum *et al*. (2007) found a negative association between semen quality and percentage of TUNEL positive sperm, others found that DNA damaged sperm did not correlate to standard semen parameters (Sepaniak *et al*., 2006) or correlated only to sperm concentration (Oosterhuis *et al*., 2000). On the other hand, the clear correlation we found between sperm DNA fragmentation, as determined by TUNEL/PI, and poor semen quality is in agreement with studies employing different techniques (Irvine *et al*., 2000; Saleh *et al*., 2003) or selected sperm preparations (Sun *et al*., 1997; Muratori *et al*., 2000) to reveal DNA breakage. Determination of DNA fragmentation in selected sperm should be less affected with respect to investigation in unselected sperm samples, since both M540 bodies (Muratori *et al*., 2004) and PI^{dim} sperm population (present

Table II. Spearman correlations between sperm DNA fragmentation as calculated by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL), TUNEL/propidium iodide (PI) and within PI^{dim} sperm population (see Results) and semen parameters

Parameters	*n*	TUNEL (Sperm + bodies)		TUNEL/PI (Sperm)		TUNEL/PI (PI^{dim} population)	
		ρ	*P*	ρ	*P*	ρ	*P*
Sperm count (sperm/ejaculate)	75	0.05	0.331	−0.25	0.016	−0.46	0.000
Sperm concentration (sperm/ml)	75	−0.07	0.265	−0.35	0.001	−0.52	0.000
Total motility	75	−0.17	0.068	−0.19	0.050	−0.32	0.003
Progressive motility	75	−0.17	0.066	−0.29	0.005	−0.41	0.000
Normal morphology	75	−0.18	0.056	−0.37	0.001	−0.51	0.000

PI^{dim}, sperm population with low avidity for PI staining.

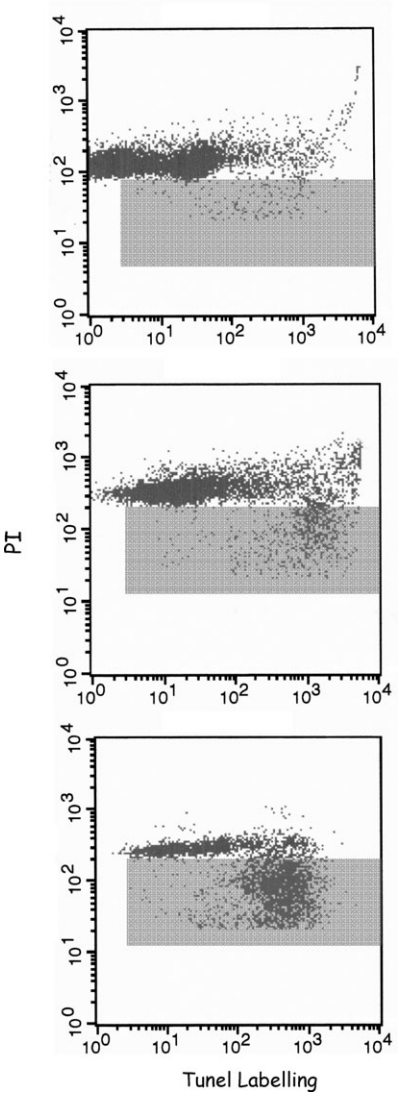

Figure 4: TUNEL/PI dot plots of semen samples from patients with low (upper panel), medium (middle panel) and high (lower panel) incidence of the PI^{dim} sperm population (highlighted in grey)

study) were reduced after sperm selection by swim-up and/or migration in discontinuous density gradient. However, selected sperm samples are poorly representative of the entire sperm population.

An interesting and novel result of the present study is the demonstration, in semen of subfertile patients, of a fraction of DNA fragmented sperm correlating more closely to semen quality than sperm DNA fragmentation as a whole. Such a fraction of sperm belongs entirely to a sperm population which can be clearly distinguished from the rest of spermatozoa because of a different avidity for PI (PI^{dim} population). Interestingly, although DNA fragmentation is found in both sperm populations, only that found in PI^{dim} sperm is strictly correlated to semen parameters, allowing us to conclude that the negative association between semen quality and DNA damage, found by many studies in the last decades, is mainly driven by the occurrence of such a sperm population.

As mentioned above, the PI^{dim} sperm population is stained with lesser avidity by the nuclear dye PI. The reason for such a phenomenon, as well as the origin of DNA damage in the PI^{dim} population, needs to be fully investigated. Presently, we can only speculate about these issues, considering the peculiar feature that PI^{dim} population consists entirely of DNA fragmented sperm. Even if the origin of sperm DNA fragmentation is not yet definitively clear, several mechanisms, alternative or concurrent, have been hypothesized. They include the failure of germ cell apoptosis to complete (i.e. abortive apoptosis, Sakkas *et al*., 1999), an impairment in sperm chromatin packaging during spermiogenesis (Sakkas *et al*., 1995; Marcon and Boissonneault, 2004) and the imbalance between reactive oxygen species production and antioxidant defence in semen (Agarwal *et al*., 2003). DNA fragmentation in PI^{dim} and PI^{br} populations might be induced by any of these mechanisms and it is possible that PI^{dim} sperm might derive from DNA fragmented PI^{br} sperm through progressive DNA damage. Alternatively, it can be hypothesized that different mechanisms produce the damage in the two populations. Whatever the mechanism inducing DNA fragmentation in PI^{dim} population, it appears that it leads to a more intensive DNA breakage/cell than in PI^{br} population and possibly to loss of DNA fragments. It is worth noting that if PI^{dim} sperm had lost part of their DNA, the value of DNA breakage would be greater if measured versus total content of DNA/cell. In this view, the lower nuclear staining of PI^{dim} population may result from both a lower content of DNA and a decreased binding of DNA to the intercalating compound, PI. Indeed, the affinity of PI for DNA is based on its ability to reduce free energy of torsion stress in coiled DNA, by intercalating between bases. Such an ability is much decreased if many breaks are present. Hence, it is expected that the more fragmented PI^{dim} sperm (mean of DNA fragmentation distribution is greater than in PI^{br} sperm) has less avidity for PI.

In studies investigating the impact of sperm DNA fragmentation on reproduction, the prevailing idea is that sperm with damaged DNA, even if retaining the ability to fertilize the oocyte (Ahmadi and Ng, 1999), affect the subsequent steps resulting in increased failure of embryo development and miscarriage (Agarwal and Allamaneni, 2004; Lewis and Aitken, 2005; Li *et al*., 2006). However, data on the relationship between DNA damage and ART outcome are very conflicting (O'Brien and Zini, 2005; Li *et al*., 2006). In this controversial scenario, Alvarez (2005) suggested that only a deeper knowledge of the phenomenon of sperm DNA fragmentation can help in solving the issue. This author suggests that it is the time to consider, beside the amount of sperm DNA damage as a whole, if, and which, other variables affect the outcome of ART, including the DNA regions that are damaged (i.e. introns versus exons), the efficacy of the oocyte DNA repairing systems and the types of DNA damage (and thus the mechanism responsible for it) (Alvarez, 2005). The latter issue is important, as the oocyte repair systems (Genesca *et al*., 1992) may not have the same efficacy to remove different types of DNA damage such as double or single-stranded DNA breaks and occurrence of modified bases (Alvarez, 2005; Derijck *et al*., 2007). Results of the present study further support the emerging idea that more detailed investigation of sperm DNA fragmentation is needed to progress in

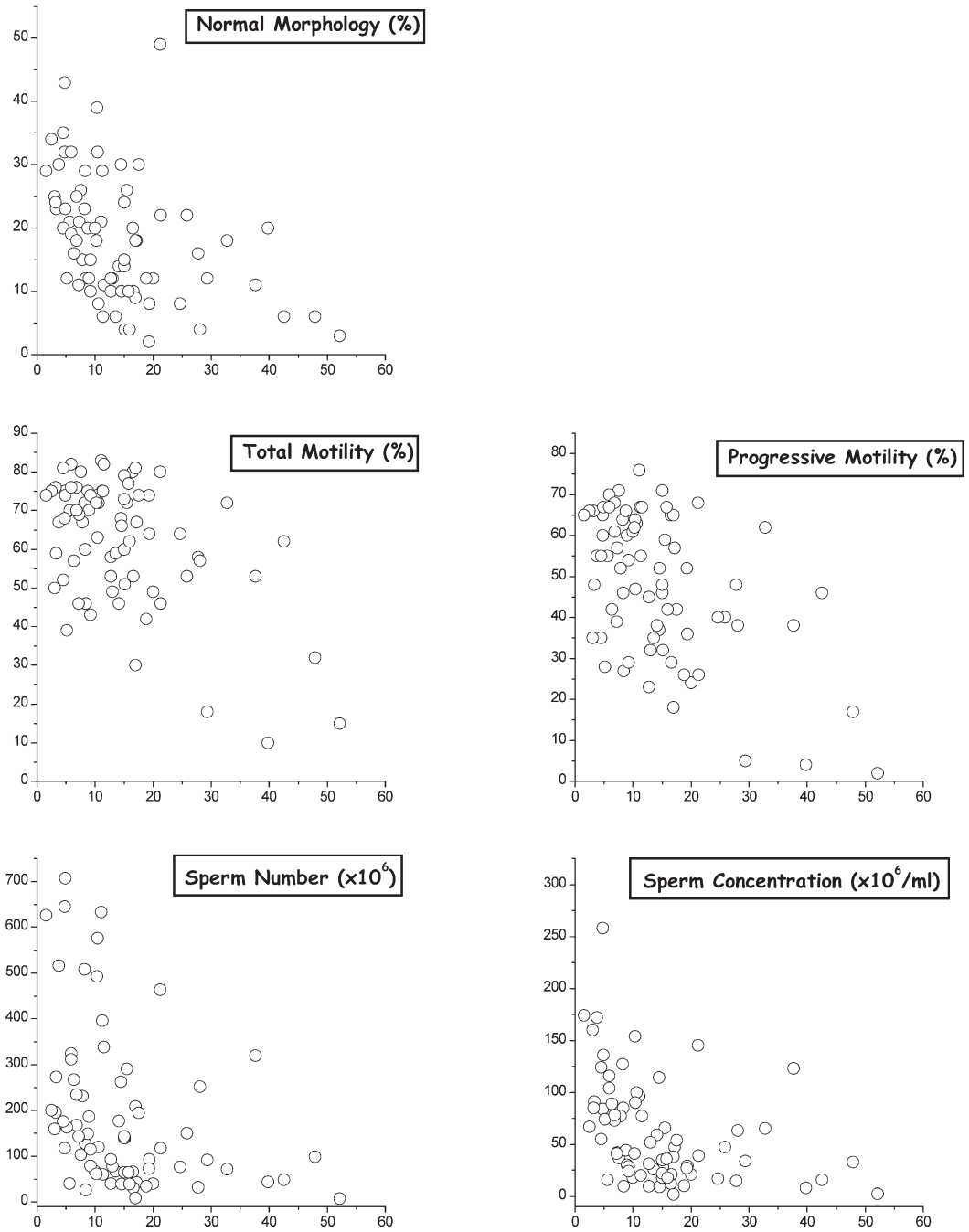

Figure 5: Scatter plots between percentages of DNA fragmentation in PI^{dim} sperm population from 75 semen samples, and normal morphology, total and progressive motility, sperm count and sperm concentration. Spearman's correlation coefficients are reported in Table II

this field of research and to render this parameter more powerful in predicting the impact on reproduction. Our data, indeed, suggest that DNA fragmented sperm in the PI^{dim} population are more likely to have reduced motility and abnormal morphology than those in the PI^{br} population. It can be argued that also the chance to fertilize the oocyte naturally or in IVF, as well as to be chosen by the operator for ICSI, is reduced in PI^{dim} sperm. In addition, if the DNA damage in the two sperm populations is different also its chance to be rescued by oocyte repair mechanisms and thus to support subsequent embryo development, might be different (Derijck *et al*., 2007). The impact of sperm DNA fragmentation in each of the two differently PI-stained sperm populations on reproduction outcome needs to be evaluated in clinical settings.

In conclusion, our study demonstrates that assessment of sperm DNA fragmentation in semen by TUNEL coupled to flow cytometry results in only a rough measure of the phenomenon unless a nuclear staining is also simultaneously performed, allowing to distinguish between sperm and M540 bodies and, within sperm, between two populations with

different extents of DNA fragmentation and relationship with semen parameters.

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