

Small Variations in Crucial Steps of Tunel Assay Coupled to Flow Cytometry Greatly Affect Measures of Sperm DNA Fragmentation

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ABSTRACT

Techniques assessing sperm DNA damage are numerous and heterogeneous. There are two main types of assays: direct and indirect ones. The former directly detect the amount of sperm DNA damage whereas the latter reveal the effect of an exogenous insult on sperm chromatin. In addition, even considering the same type of technique, different strategies to reveal and/or quantify sperm DNA damage are used. Finally, these techniques, except SCSA (Sperm Chromatin Structure Assay), lack standardized protocols to which adhere to minimize inter-laboratories variations. In this study we investigated the effects of some of the many variants by which TUNEL assay is performed on the measures of sperm DNA fragmentation by flow cytometry. In addition, by using an established procedure, we determined the precision of the technique by calculating intra-assay coefficients of variation (CVs). We found that the concentration of the fixative, the time of storage of fixed samples, the fluorochrome used to label DNA breaks and the method to analysis flow cytometric data, all greatly affect the measures of sperm DNA fragmentation. In particular we found that treatment with paraformaldehyde produced an additional damage in most of samples, suggesting that also TUNEL can be considered an indirect assay when performed in semen samples treated with such fixative reagent. We also showed that two different methods to analyse data yielded results that, albeit correlating, were different and differently associated to semen quality. On the contrary, TUNEL assay, as measured here, showed low intra-assay CVs, resulting in a quite precise technique when performed in established conditions.

Key words: Infertility, semen analysis, sperm DNA damage, TUNEL assay, flow cytometry

INTRODUCTION

Presently, diagnosis of male infertility mainly relies on routine semen analysis, a microscopic evaluation of several semen parameters including count, motility and morphology of sperm in the ejaculate. Routine semen analysis has a limited power when discrimination between fertile and infertile men is requested. (Wang C et al, 1988), and tests able to improve the predictability of male fertility status have been sought. Among such tests, those determining sperm DNA integrity appear promising. In principle, the integrity of sperm genome is a pivotal characteristic for successful reproduction function and it is expected that sperm DNA damage has a negative impact on male fertility and on the outcome of reproduction. Studies using SCSA have established a clinical threshold value above which the reproductive success is impaired (Evenson and Wixon, 2006; Erenpreiss et al, 2006; Giwercan et al, 2009). Nevertheless, a global consensus on the diagnostic and prognostic value of sperm DNA fragmentation has not been reached (Zini and Sigman, 2009), as the conclusions from these studies are often conflicting (Li et al, 2006; Collins et al, 2008). It has been proposed that several factors might be responsible for these controversies, since many variables can affect the predictive power of tests on sperm DNA integrity (Lewis et al, 2008). Disclosing how each of these variables (including the technique employed to detect the damage) affects the measures is crucial for the comparison of different studies.

Techniques revealing sperm DNA damage are numerous and often not equivalent (i.e. not revealing the same kind of DNA damage). In particular, it is believed that whereas direct assays [TUNEL, (Terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling), neutral COMET (or SCGE, single cell gel electrophoresis)] detect the real DNA breakage, the indirect ones [SCSA (Sperm Chromatin Structure Assay), alkaline COMET, SCD (Sperm chromatin dispersion) test] detect the effects of treatments inducing DNA damage (The Practice Committee of the American Society for Reproductive Medicine, 2008; Lewis et al, 2008). For instance, the most employed assays, TUNEL and SCSA, reveal, respectively, the amount of DNA breakage and the susceptibility of DNA to denaturation at sites of DNA strand breaks by acid treatment (Evenson, 2006). In addition, different strategies to reveal and/or quantify sperm DNA damage are used in the same type (direct or indirect) of assay. For instance, whereas TUNEL detects labeled DNA breaks and yields percentages of DNA fragmented sperm on a large number of events (when coupled to flow cytometry), neutral COMET visualizes the pattern of DNA fragments after electrically induced migration, providing qualitative, but poor quantitative (i.e. related to a low number of cells and to the difficulty of a precise measure of the COMET tail) information on DNA damage.

Finally, even when the same technique is used, an important pitfall is the lack of standardized protocols to which all users can adhere to minimize inter-laboratory variations. This is particularly true for TUNEL assay, which is performed with many variants and can be coupled to both fluorescence

microscopy and flow cytometry. On the contrary, SCSA is a long established and standardized technique used in combination with flow cytometry (Evenson et al, 2004; Virro et al, 2004).

Recently, our group has developed a new version of TUNEL assay (from herein indicated as TUNEL/PI) that couples the labelling of DNA breaks with nuclear staining by propidium iodide (PI) (Muratori et al, 2008a). TUNEL/PI improves the accuracy of the measures of sperm DNA fragmentation by flow cytometry, with respect to the traditional version of TUNEL (Muratori et al, 2008b). Indeed, nuclear staining allows the exclusion of M540 bodies from sperm DNA fragmentation analysis (Muratori et al, 2008a). M540 bodies are round elements that may be present at high levels in semen of sub-fertile and infertile patients (Muratori et al, 2004; Marchiani et al, 2007a) and show size and internal complexity similar to sperm. Hence, they represent a real interference in flow cytometric analysis of sperm (Marchiani et al, 2007b). In addition, TUNEL/PI coupled to flow cytometry identifies two sperm populations with different extent of DNA fragmentation, whose separated analysis improves the accuracy of the measures as well (Muratori et al, 2008a and b).

The aim of the present study was to investigate the precision of TUNEL/PI, by calculating intra-assay coefficients of variation (CVs) of the techniques. In addition, we studied the effects of several of the many variants by which TUNEL assay is performed on measures of sperm DNA fragmentation.

MATERIAL AND METHODS

Chemicals

Diff-Quick kit was purchased from CGA, Diasint (Florence, Italy). Human tubal fluid (HTF) medium was purchased from Celbio (Milan, Italy). In Situ Cell Death Detection Kit, fluorescein and In Situ Cell Death Detection Kit, TMR were purchased from Roche Molecular Biochemicals, Milan, Italy. YO-PRO1 (Y1) and Propidium Iodide (PI) were from Invitrogen (San Giuliano Milanese, Milan, Italy) and Calbiochem (Nottingham, UK), respectively. Bovine serum albumin (BSA) was from ICN Biomedicals, Ohio, USA. Paraformaldehyde (PFA) was obtained by Merck Chemicals, Milan Italy. Formaldehyde (FA) solution (37%) and the other chemicals were from Sigma Aldrich (Milan, Italy)

Semen Samples Collection and Preparation

Semen samples were collected [according to WHO criteria (WHO, 1999)] from 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 Diff-Quik staining (WHO, 1999), were excluded from the study. Sperm morphology and motility were assessed by optical microscopy, according to WHO criteria (WHO, 1999). Sperm morphology was evaluated by

determining the 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 (i), non progressive motile (ii) (i+ii=total motility) and immotile spermatozoa by scoring at least of 100 sperm/slide. Overall, 71 semen samples were collected from normozoospermic (n=21), asthenozoospermic (n=9), teratozoospermic (n=18), asthenoteratozoospermic (n=19) and oligoasthenoteratozoospermic (n=4) subjects (WHO, 1999). The average values (\pm SD) of semen parameters and age of the patients included in this study were: normal morphology: 13.8 \pm 8.2%; progressive motility: 49.1 \pm 15.2%; total motility: 62.3 \pm 12.8%; sperm count: 241.0 \pm 176.1 spermx10⁶/ejaculate; sperm concentration: 73.1 \pm 51.4 x10⁶/ml; age: 34.8 \pm 7.8 years.

All experiments were performed in raw semen. Semen samples were washed twice with HTF medium and then fixed with 3.7% PFA (200 μ L, in phosphate-buffered saline [PBS] pH 7.4) for 30 minutes at room temperature. PFA solution was prepared by solving 1g of PFA powder in 17.5 ml of dH₂O at 58-60°C under stirring. One drop 10N NaOH was added to clarify the solution. The solution was filtered in a tube containing 6 ml of phosphate buffer (0.5M, pH 7.4) and dH₂O was added up to a final volume of 27 ml. pH was routinely checked. The solution was kept at 4°C up to one week. In some experiments, semen samples were fixed with 1% PFA or with 3.7% (FA). The latter was prepared by diluting in PBS a commercial 37% formaldehyde solution. Unless otherwise indicated, experiments were performed by fixing samples with 3.7% PFA for 30' at room temperature.

TUNEL/PI

Semen Samples were processed by terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick end labeling (TUNEL) as described elsewhere (Muratori et al, 2000). Briefly, fixed spermatozoa (10x10⁶) were centrifuged at 500xg for 10 minutes and washed twice with 200 μ L of PBS with 1% BSA. Then, spermatozoa were permeabilized with 0.1% Triton X-100 in 100 μ L of 0.1% sodium citrate for 2 minutes in ice. After washing 2 times, the labelling reaction was performed by incubating sperm in 50 μ L of labelling solution (supplied with the In Situ Cell Death Detection Kit, fluorescein) containing the TdT enzyme for 1 hour at 37°C in the dark. Finally, samples were washed twice, resuspended in 500 μ l of PBS, stained with 10 μ l of PI (30 μ g/ml in PBS) and incubated in the dark for 10 minutes at room temperature. For each test sample, a negative control (omitting TdT) and a sample for fluorescence compensation (labelled only with TUNEL) were prepared.

To compare the measures obtained using different kits to label sperm DNA breaks, 12 semen samples were labelled both by "In Situ Cell Death Detection Kit, fluorescein" and "In Situ Cell Death Detection Kit, TMR". The two kits are from the same manufacturer and differ only for the conjugated fluorochrome to dUTP [i.e fluorescein (F) or Tetramethylrhodamine (TMR)]. Since nuclear staining is

necessary to distinguish sperm from M540 bodies, we used 25nM Y1 (instead of PI), when the TMR kit was employed to avoid the overlapping between emission spectra of TMR and PI. We have demonstrated previously that Y1 is suitable to distinguish between sperm and bodies (Muratori et al, 2004; Marchiani et al, 2007a). From herein, we refer to these experiments of double staining with TMR and Y1 as TUNEL/Y1 sperm labelling. For each test sample, a negative control (omitting TdT) and a sample for fluorescence compensation (labelled only with Y1) were prepared.

Flow Cytometry and data analysis

For each sample, 10.000 events were recorded within the flame shaped region characteristic of spermatozoa (Muratori et al, 2000; 2003 and 2004) in the Forward light Scatter/Side light Scatter (FSC/SSC) dot plot. Sperm DNA fragmentation was determined within the nucleated events (i.e the events labelled with PI or Y1) of such region (Muratori et al, 2008a). This strategy guarantees that fluorescence is analysed in a population formed only by spermatozoa. It is possible that some sperm with large head are excluded from the flame shaped region, but avoiding to select nucleated cells and to gate FSC/SSC events would, on the other hand, include debris and other non sperm cells, such as M540 bodies in the fluorescence analysis (Muratori et al, 2003; 2004 and 2008a). Green fluorescence (of Y1 and nucleotides conjugated with F) and red fluorescence (of PI and nucleotides conjugated with TMR) were revealed, respectively, by the FL-1 (515-555 nm wavelength band) and the FL-2 (563-607 nm wavelength band) detectors of a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 15-mW argon-ion laser for excitation.

Analysis of flow cytometric data was performed by both of the two main method used to quantify the percentage of sperm with DNA fragmentation. The most popular method consists in determining the amount of sperm DNA fragmentation as the percentage of sperm having fluorescence intensities above a threshold established in the negative control histogram. In the present study, such threshold was fixed by giving a preset background $\leq 1\%$ in the negative control (Figure 1A). From herein this method is indicated as threshold setting (TS) method. The second method of flow cytometric data analysis (Sergerie et al, 2005) consists in the subtraction of the negative control histogram from the corresponding test sample fluorescence histogram (Figure 1B). The obtained result is expressed as percentage of the total events in the test sample. From herein this method is indicated as subtraction of the blank (SB) method. In both cases, sperm DNA fragmentation was calculated by adding together the values obtained in the two sperm populations differing for nuclear staining (Muratori et al, 2008a). Except for these experiments comparing the two methods to analyze the data, all the measures were obtained by TS method.

Extraction, labelling and detection of sperm DNA.

DNA was extracted by using the standardized phenol–chloroform technique from fresh or fixed semen samples. Accurate assessment of DNA quantity and quality was performed using the Nanodrop ND-1000 Spectrophotometer (NanoDrop, DE, USA). Two μg of DNA from each tested sample were labelled with 100 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. After precipitation with ethanol, DNA was resuspended in 20 μl of distilled H₂O and kept at 37° for 2 hours in the dark. Labelled DNA was loaded into 2% agarose gel. To visualize agarose gels, the Typhoon Trio image acquisition station (Amersham Biosciences, Sydney, Australia) was set to scan fluorescence due to DNA fragmentation, using a blue 488 nm excitation laser, and a 526 SP fluorescein emission filter. The photo-multiplier tube was tuned to 600 V with normal sensitivity. The scanning resolution was adjusted to 200 μm , with a focal point of +3 mm. Images were analyzed using the ImageQuant TL software (Amersham Biosciences, Sydney, Australia).

Intra-assay coefficients of variation (CVs) and effect of storage time.

For calculation of intra-assay coefficients of variation, the same fixed sample was split into three aliquots that were washed twice in PBS and simultaneously processed by TUNEL/PI: i) immediately (n=12) or after 1 day (n=8) and after 1 week (n=4) of storage in PBS at 4°C. To investigate the effects of storage time on the measures of TUNEL/PI, the same fixed sample (n=9) was split into three aliquots that were washed twice with PBS. One aliquot was immediately processed by TUNEL/PI, the other two were stored at 4°C in PBS and processed by TUNEL/PI after one and two weeks.

Statistical analysis

Data were analysed with Microcal Origin software, 6.1 version (MicroCal Software Inc., Northampton, MA, USA). Results are shown as mean \pm SD. CVs for sperm DNA fragmentation were calculated using the formula $(\text{SD}/\text{mean})\times 100$. Analysis of variance and the Student's *t* test (paired data) were used to assess statistically significant differences between percentages of i) sperm DNA fragmentation, as calculated by TUNEL/PI and TUNEL/Y1 and by TS and SB methods; ii) sperm population within flame shaped region in FSC/SSC dot plot, as detected by Y1 and PI; iii) dimmer and brighter sperm, as detected by Y1 and PI within total sperm population. Correlation tests were performed by linear regression analysis.

RESULTS

Intra-assay CVs

We determined intra-assay CVs of TUNEL/PI, by following the procedure indicated in Figure 2. To this aim, three aliquots of the same fixed samples were washed twice and simultaneously processed by TUNEL/PI immediately (n=12) and after one day (n=8) and one week (n=4) of storage at 4°C in PBS. The resulting mean and range of intra-assay CVs are reported in Table I. As shown, intra-assay CVs were quite low, indicating that TUNEL/PI is a precise technique when samples are processed immediately after fixation or after an established time of storage.

Effect of PFA

TUNEL assay is largely performed by using semen samples fixed by PFA. However, different concentrations of PFA are used in different protocols, being 1% and 3.7% the most popular ones. To assess the effect of different concentrations of PFA on the measures of sperm DNA fragmentation, we fixed two aliquots of the same semen samples (n=13) with 1 and 3.7% of PFA and then evaluated sperm DNA damage by TUNEL/PI. The results of these experiments are shown in Figure 3A. As shown, the amount of sperm DNA damage did not change with PFA concentration only in two semen samples. In 3 out of 13 samples the amount of sperm DNA damage in the aliquots fixed with 3.7% PFA resulted lower respect to those fixed with 1% PFA, likely due to dissolving of a fraction of DNA fragmented sperm in the higher concentration of the fixative. In most cases (8 out 13), the amount of sperm DNA damage increased by PFA concentration, suggesting an insulting action of PFA on sperm chromatin.

To test this hypothesis, we investigated the effect of a prolonged incubation with PFA. Eleven semen samples were fixed by 3.7% PFA and processed for detecting DNA breakage both immediately after fixation and after 24 h incubation with the fixative. In 9 out 11 samples, DNA fragmentation was detected by TUNEL/PI (Figure 3B) whereas in two samples we used a different strategy: sperm DNA was extracted and fragments were labelled in 3'OH ends, separated by gel electrophoresis and fluorescence was detected by a Typhoon scanner (Figure 3C and D). As shown in Figure 3B, the amount of sperm DNA fragmentation, as detected by TUNEL/PI method, resulted unchanged in 1 sample, decreased in 3 samples (again likely due to disappearance of a fraction of fragmented cells) and increased in 5. Consistently, prolonged incubation in PFA 3.7% induced an increase of the smear due to labelled DNA fragments, respect to basal (i.e. the sample processed immediately after fixation) in two out of two samples, (Figure 3C). Similarly, an increase of the amount of DNA fragmentation was seen by comparing fresh and fixed samples incubated in 3.7% PFA (Figure 3D).

To assess whether the effects of PFA on sperm chromatin was caused by some unknown bias introduced during preparation of the fixative (see material and methods section), 6 semen samples were

fixed with both 3.7% PFA and a solution containing the same concentration (3.7%) of formaldehyde (FA), prepared by diluting a 37% ready to use solution. After sperm DNA fragmentation determination (by TUNEL/PI method), we found that the measures obtained with the two fixatives were in close agreement. Indeed the mean CV ($4.6 \pm 4.1\%$, range: 0.3-11.0%) value was similar to that of intra-assay CVs.

Effect of sample storage

To assess the reproducibility of the measures of TUNEL/PI after sample storage, three aliquots of 9 semen samples were fixed in 3.7% PFA, washed twice in PBS and assayed at three different time points: immediately and after one and two weeks of storage at 4°C in PBS. The agreement between the three measures obtained at different days (as expressed by CVs, Table I) was very poor, thus suggesting that the time of storage of fixed samples deeply affects the reproducibility of the technique.

Effect of the fluorochrome conjugated to nucleotides

Many kits are commercially available to label sperm DNA breakage. In this study we compared two of these kits which differ only for the fluorochrome (F or TMR) conjugated to the nucleotides to be added by TdT enzyme to DNA breaks. By using nucleotides conjugated with TMR to reveal DNA fragmentation, PI had to be substituted by Y1 for nuclear staining (see material and method section for details). We have previously shown that Y1 is able to discriminate sperm from M540 bodies (Muratori et al, 2004; Marchiani et al, 2007a), similarly to PI (Muratori et al, 2008a). We also showed that labelling with PI revealed the presence of two sperm populations as one population stains more (brighter population) and the other less (dimmer population) with such nuclear probe (Muratori et al, 2008a). As shown in Figure 4A, also Y1 is able to distinguish the two sperm populations previously identified by PI staining (Figure 4B and Muratori et al, 2008a). In addition, we found that the average percentage of sperm within the flame shaped region (R1, FSC/SSC dot plots of Figure 4, upper panels) was not different in semen samples stained by Y1 and PI (respectively $79.4 \pm 17.3\%$ vs $79.9 \pm 16.8\%$, $n=12$, $p>0.05$). Further, in the same samples we found that the average percentage of the dimmer population on total sperm detected by Y1 ($14.5 \pm 6.5\%$) was similar ($p>0.05$) to that detected by PI (13.5 ± 5.5). In a group of 12 semen samples, sperm DNA fragmentation was labelled by both TUNEL(F)/PI and TUNEL(TMR)/Y1 coupled to flow cytometry. In Figure 4 (middle and lower panels), typical dot plots of TUNEL (TMR)/Y1 (A) and TUNEL(F)/PI (B) fluorescences are reported. In Figure 4C, the corresponding fluorescence histograms after M540 bodies subtraction are shown. The measures of sperm DNA fragmentation obtained by TUNEL(F)/PI ($36.7 \pm 12.4\%$) were statistically greater ($p<0.0001$) than those obtained by

TUNEL(TMR)/Y1 ($26.6 \pm 10.1\%$), even if a strict correlation was found between the two groups of data ($r=0.9$, $SD=5.9$, $p<0.001$, $n=12$).

Effect of the method of analysing flow cytometric data.

Among the available approaches for analysing flow cytometric data on sperm DNA fragmentation, the most popular are the TS and the SB methods (for description see Material and Methods section and Figure 1). A cohort of 32 semen samples was processed by TUNEL/PI coupled to flow cytometry and sperm DNA fragmentation was calculated by both the TS and the SB method and the resulting values were compared. We found that the values of sperm DNA damage obtained by using the SB method were significantly greater than those obtained with the TS method (respectively, $66.0 \pm 16.2\%$ and $51.0 \pm 17.3\%$, $p<0.0001$), even if the two types of measures were correlated (Figure 5). In line with previous results (Muratori et al, 2008a), the amount of sperm DNA fragmentation calculated by the TS method was inversely related to sperm concentration, motility and morphology (Table II) but, interestingly, such associations were lost when sperm DNA fragmentation was calculated by the SB method (Table II).

Effect of the storage time of labelled semen samples

To investigate whether the storage of TUNEL/PI labelled semen samples affects the measures of sperm DNA fragmentation, we processed semen samples by TUNEL/PI and acquired them by FACScan immediately and after storage of 1 day and of 1 week in the dark at 4°C . We found that acquisition of labelled samples acquired at different times did not affect the measures of sperm DNA fragmentation, implying that the fluorescent signals are stable at least for 1 week. Indeed, the calculated mean value of CVs were: $2.2 \pm 2.8\%$, $n=8$ (1 day storage) and $4.9 \pm 2.5\%$, $n=6$ (1 week storage).

DISCUSSION

TUNEL assay was developed in the 90's (Ausubel et al, 1992) and the first application on sperm cells was reported in 1993 (Gorczyca et al, 1993). Since then, the assay has been widely employed in studies on the origin, mechanism and clinical implication of sperm DNA fragmentation. However, many variants of TUNEL assay have been utilized, consisting in different procedures of sperm preparation, fixation and permeabilization, as well as different protocols of labelling DNA breaks, different technologies to detect fluorescent signals and different methods to analyse resulting data. Given that, the heterogeneity of the values for sperm DNA fragmentation reported in studies using TUNEL assay is not surprising. As an example, the reported amounts for sperm DNA fragmentation in raw semen

in similar groups of infertile subjects may vary from $2.36 \pm 1.37\%$ (n=29, Younglai et al, 2001) to $39.82 \pm 23.75\%$ (n=66, Domínguez-Fandos D et al, 2007).

Keeping in mind that the goal is to find a semen parameter with the ability to predict male fertility status, whatever is the technique to reveal sperm DNA damage, the lack of standardisation of the used assays makes difficult the comparison of data from different laboratories and, consequently, the assessment of the clinical value of sperm DNA integrity. Further, in this scenario, referring to threshold values for clinical purpose appears inappropriate and poorly reliable.

In this study, we analytically evaluated the effect of some of the many variants by which TUNEL assay is run to evaluate sperm DNA fragmentation in different laboratories. Overall, our results indicate that a minimal modification in one of the crucial steps of TUNEL assay may produce a great variation of the measures. We show here that the concentration of the fixative, the time of storage of fixed samples, the fluorochrome used to label the 3'-OH ends of DNA and the method to analyse flow cytometric data, all greatly affect the measures of sperm DNA fragmentation. Treatments of sperm with PFA showed unexpected results, suggesting that PFA produces an insult to sperm chromatin. Indeed, we show here, with two independent techniques, that treatment with PFA may increase sperm DNA fragmentation. Indeed, we found that an increase of DNA fragments occurred both after treatment of fresh samples with PFA and by concentration and incubation time in the fixative.

However, such an increase of DNA fragmentation was not observed in all the studied samples. The fact that in few samples the amount of DNA fragmentation does not change after fixation, suggests that the effect of the fixative also depends on the chromatin status of the spermatozoon. This finding indicates that DNA fragmentation revealed by TUNEL (as run in PFA fixed samples) in one subject is not only the “real” (Alvarez, 2006) damage, but may include the results of a balance between an exogenously induced damage (for instance by PFA) and the susceptibility of sperm chromatin in that subject. Hence, also TUNEL assay can be considered an indirect assay when using PFA fixed samples. Further, the fact that PFA induces an additional damage on sperm chromatin depending on the concentration points out the importance of standardizing this step of the procedure. Time of storage of fixed samples needs to be standardized as well. Indeed we showed here that the agreement between measures obtained in the same fixed samples processed after different times from collection day was very poor.

Another crucial step of TUNEL assay is the labelling reaction of DNA breaks. Many commercial kits are available, using differently labelled nucleotides added by TdT enzyme which may have different enzymatic activity (not tested here). In the present study we compared two similar kits which use nucleotides conjugated to two different fluorochromes: F and TMR. We found that the percentages of sperm DNA fragmentation obtained by labelling with F well correlated to those obtained by labelling with TMR, but the average value of the former group of measures was statistically greater

than that of the latter. This result is not surprising and may be related to the technique used to detect sperm DNA fragmentation, i.e. flow cytometry. Indeed, the distinction between fragmented and not fragmented DNA spermatozoa by flow cytometry is not sharp and the distribution of fluorescence intensities in test sample very often overlaps that of negative control (omitted TdT, Figure 4C). Hence, when sperm DNA fragmentation is labelled with a fluorochrome (such as TMR) emitting with a lower fluorescence intensity than F, the histogram of test sample is shifted towards left in the x-axis thus producing a decrease in resulting labelled cells (Figure 4).

As mentioned above, there is a great variability, in the literature, in the measures of sperm DNA fragmentation by using TUNEL assay. An important source of variability in detecting sperm DNA damage comes from the different technologies used to reveal fluorescence, i.e. fluorescence microscopy and flow cytometry (Domínguez-Fandos et al, 2007, Cohen-Bacrie et al, 2009, Muratori et al, 2008b). Moreover, even when detection is carried out by the same technique, for instance flow cytometry, it should be pointed out that the different methods that can be used to analyse data could produce different results. In our study, semen samples acquired by FACScan were analysed by the two main methods to elaborate flow cytometric data of sperm DNA fragmentation, the TS and SB method, and results from both methods were compared. We demonstrated here that the two types of measures, albeit correlating, are not equivalent. Interestingly, at variance with sperm DNA fragmentation calculated by TS analysis, the values obtained by SB analysis in the same cohort of subjects showed no correlation with semen quality, suggesting that a possible clinical meaning of sperm DNA damage may be masked by using this strategy to elaborate flow cytometric data. We can extend the importance of this finding by speculating that, in general, two techniques yielding correlating measures may lead to different clinical implications.

Finally, the experiments aimed to assess whether the TUNEL fluorescence signals were stable by time indicated that the measures of sperm DNA fragmentation were reproducible in labelled samples kept at 4°C at the dark at least for 1 week.

Overall, the present investigation shows that small variations in the crucial steps of TUNEL assay are sufficient to produce important changes in the measures of sperm DNA fragmentation. Indeed, among the tested variables, only the time of storage of labelled samples did not affect the measures of sperm DNA fragmentation by TUNEL assay. However, once the procedure is rigorously established, the assay results quite precise as demonstrated by the low values of intra-assay CVs obtained both in aliquots processed immediately after fixation and in those processed after storage.

In addition, although the measures obtained by two different procedures are correlated, they may not have the same clinical meaning. These findings point out the need to standardize the procedure to perform TUNEL assay (see Fig. 2 for the one we propose) in order to make possible the comparison between different laboratories and thus to better investigate the clinical meaning of this type of sperm

damage. In case that a scientific agreement will be reached about the clinical value of sperm DNA fragmentation tests in predicting male fertility status and/or ART outcomes, the results of this study point out the need for reference labs as well as for the development of internal and external quality assessment.

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FIGURE LEGENDS

Figure 1. Methods of analysis flow cytometric data of sperm DNA fragmentation. In upper panel the histogram of negative control (empty) is overlapped to that of the corresponding test sample (solid) **A.** TS method. In the fluorescence histogram of the negative control, a threshold is set excluding <1% of the events. The threshold is translated into the histogram of the corresponding test sample, where the events showing fluorescent intensities higher than the threshold are considered positive for TUNEL. **B.** SB method. The histogram of negative control is subtracted from that of the corresponding test sample. The resulting events of the subtraction (solid) are considered positive for TUNEL and are expressed as percentage of total events in histogram of test sample (empty).

Figure 2. A proposed scheme of the procedure for TUNEL/PI.

Figure 3. Effect of PFA on sperm DNA **A.** Sperm DNA fragmentation in two aliquots of each of 13 semen samples fixed by 1 and 3.7% PFA. **B.** Sperm DNA fragmentation, in aliquots of the same fixed samples labelled before and after 24 h incubation in 3.7% PFA at 4°C. **C** and **D**, Scanning of TUNEL-labelled DNA, after electrophoresis in agarose gel. A representative experiment out of two similar is shown. In **C**, DNA was extracted from samples immediately after fixation (lane 1) and after 24 h incubation in 3.7% PFA at 4°C (lane 2). In **D**, DNA was extracted from fresh samples (lanes 1) and after sperm incubation (24 h) in 3.7% PFA at 4°C (lanes 2).

Figure 4. Typical dot plots showing sperm DNA fragmentation in the two sperm populations with different avidity to nuclear probes previously identified by our group (Muratori et al, 2008). In **A**, DNA breaks and sperm nuclei were labelled, respectively, by TUNEL(TMR) and Y1. In **B**, DNA breaks and sperm nuclei were labelled, respectively, by TUNEL(F) and PI. Upper panels: FSC/SSC dot plots showing the flame shaped region, in which fluorescence signals are analysed. Middle panels: negative samples (TdT omitted) prepared by labelling only with Y1 (**A**) or PI (**B**). Lower panels: test samples. Note that the two sperm populations were revealed with similar efficiency by Y1 (**A**), and PI (**B**). **C.** Histograms of fluorescence distribution of the same sperm sample labelled by TUNEL(TMR) (left panel) and TUNEL(F) (right panel). Note that the test sample is shifted towards low fluorescence intensities in the test sample histogram corresponding to TUNEL(TMR).

Figure 5. Linear regression analysis between the measures of sperm DNA fragmentation as calculated by Threshold Setting (TS) and Subtraction Blank (SB) methods.

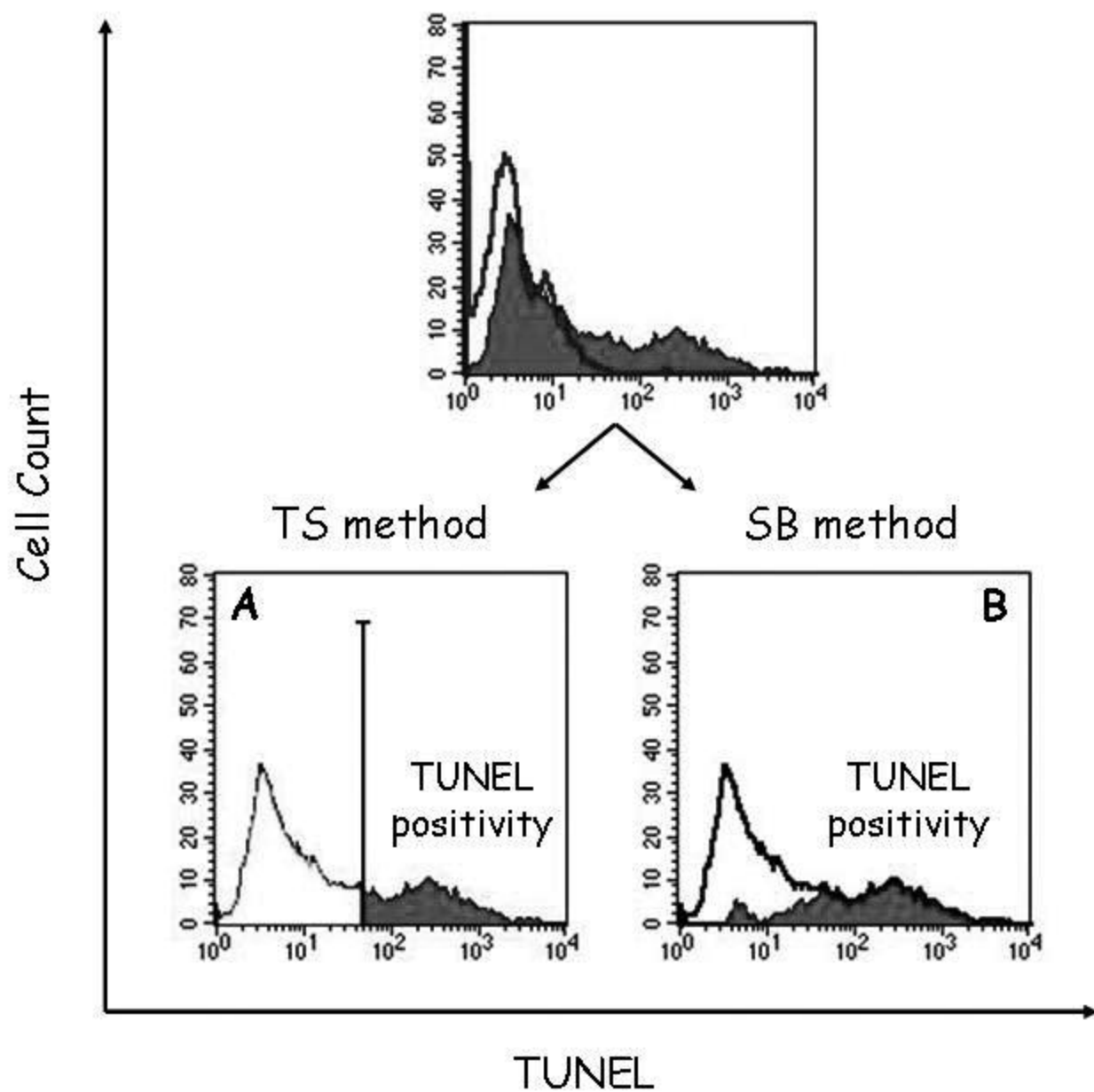
Table I. Intra-assay CVs and effect of sample storage on the measures of TUNEL/PI

	Intra-assay CV (%)			Effect of sample storage, CV (%)
	Immediately after fixation	After 1 day of storage	After 1 week of storage	
mean	5.9	3.7	3.3	28.3
SD	3.4	2.4	1.9	17.9
n	12	8	4	9
range	0.18 -12.9	0.5-7.9	2.0-5.5	10.8- 67.0

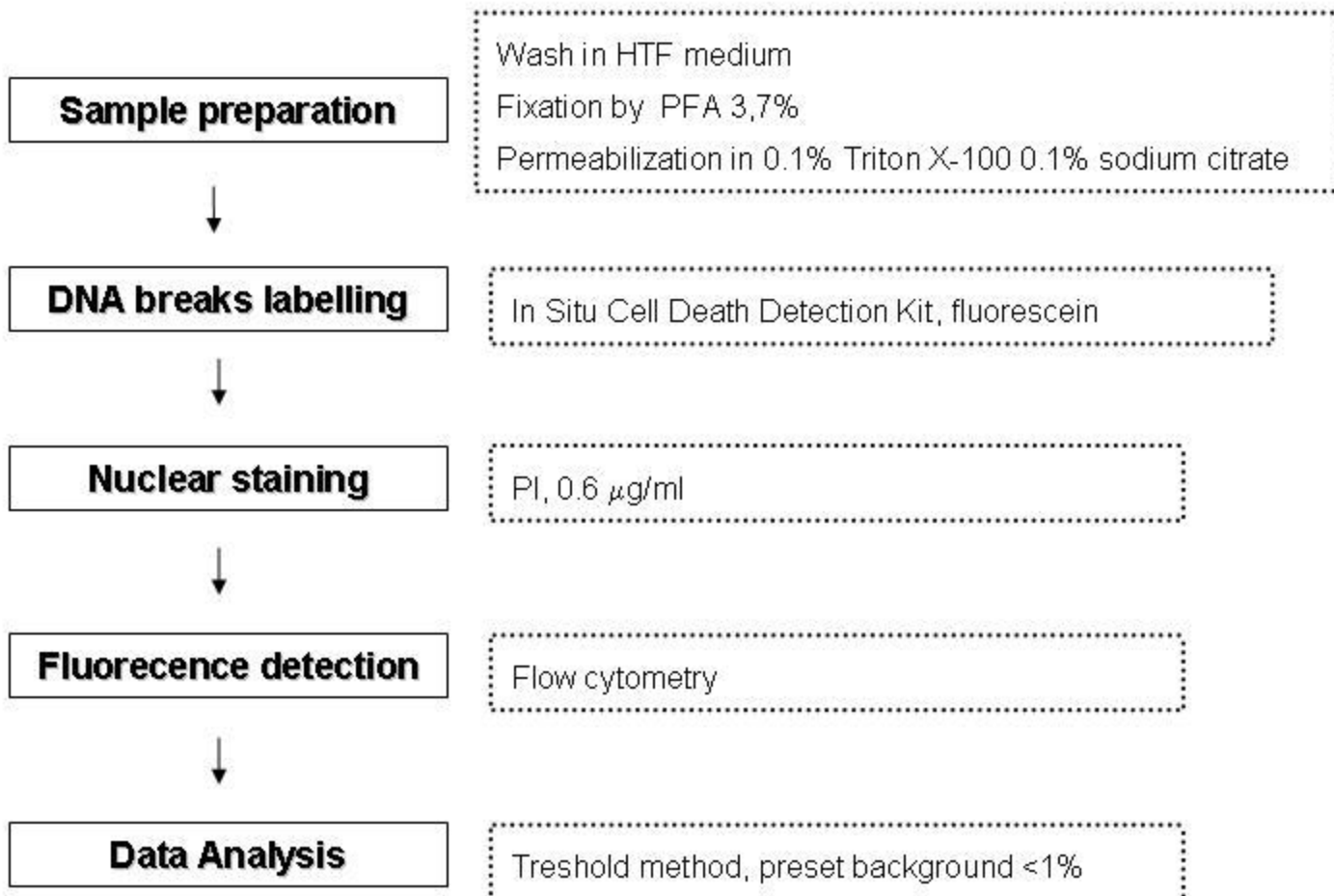
Table II. Correlation analysis between standard semen parameters and the amount of sperm DNA Fragmentation as calculated by TS and SB methods

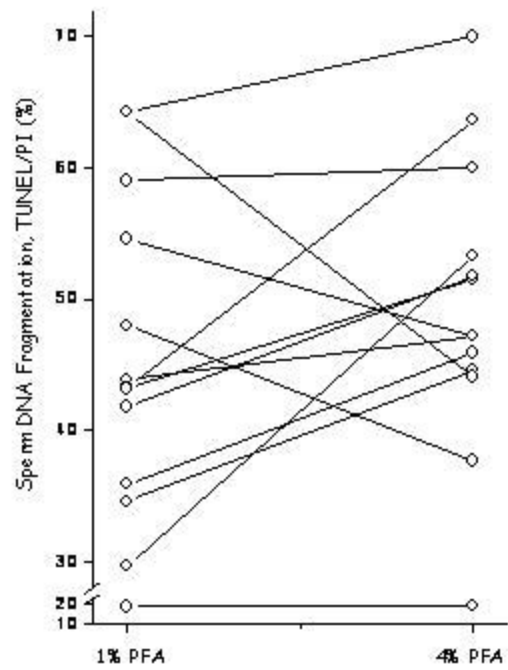
SEMINAL PARAMETERS	TS Method			SB Method	
	n	r	p	r	p
Sperm count (sperm/ejaculate)	32	-0.28	0.12	0.07	0.68
Sperm concentration (sperm/ml)	32	-0.38	0.03 ^a	0.02	0.92
Total motility (%)	32	-0.37	0.04 ^a	0.02	0.90
Progressive motility (%)	32	-0.42	0.02 ^a	-0.12	0.49
Normal morphology (%)	32	-0.63	0.00 ^a	-0.17	0.34

a=statistical significance (p<0.05)



TUNEL/PI assay





A

