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Sperm DNA fragmentation: threshold value in male fertility

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BACKGROUND: The extent of sperm DNA fragmentation, which can be measured by the TUNEL assay, is one of the determinants of male fertility. However, the clinical application of this test to in-vivo situations is difficult owing to the absence of a statistically validated threshold value. METHODS: The aim of this study was to compare the results of TUNEL assay applied to semen samples from men of proven fertility (n = 47) and patients from an infertile population (n = 66), in order to establish a discriminating threshold value. RESULTS: Infertile patients had a higher mean level of DNA fragmentation than men of proven fertility (40.9 ± 14.3% versus 13.1 ± 7.3%, respectively; P < 0.001). The area under the receiver operating characteristics curve was 0.93 for 20% sperm DNA fragmentation. The calculated threshold value for TUNEL assay to distinguish between fertile controls and infertile men was 20%. At this threshold, specificity was 89.4 [95% confidence interval (CI) 83.7–95.1] and sensitivity was 96.9% (95% CI 93.8–100). The positive and negative predictive values of the 20% sperm DNA fragmentation threshold were high: 92.8% (95% CI 87.9–97.5) and 95.5% (95% CI 91.6–99.3), respectively. CONCLUSION: This study demonstrates that sperm DNA fragmentation, as measured by TUNEL assay, is a highly valuable indicator of male fertility.

Key words: DNA integrity/male infertility/spermatozoa/threshold value/TUNEL

Introduction

Disorders of the male reproductive system have become an important public health issue as they can cause infertility, miscarriages and abnormal outcomes in the offspring. It is well recognized that spermatozoa from infertile men often have multiple structural and functional defects (Mortimer *et al.*, 1986; Aitken *et al.*, 1991; Liu and Baker, 1994). In 20% of infertile couples, the problem is predominantly male, and in up to 40% of men with sperm abnormalities, no specific aetiological factor is found (de Kretser, 1997). In some cases, these disorders may be consequences of environmental or occupational exposure to chemical and/or physical agents (Friedler, 1996).

The standard semen analysis, which includes sperm concentration, motility and morphology, can be considered a 'sensitive' biological marker of exposure to toxicants in the work place (Wyrobek, 1993; Bigelow *et al.*, 1998). However, the predictive value of these measurements is difficult to ascertain owing to their subjective nature and high intra- and interobserver variability (Keel and Webster, 1990). Although these analyses may describe important aspects of the functions of the testis and sperm, they do not address the integrity of the genetic material of the male gamete. In this regard, occupational and environmental exposure can lead to abnormal reproductive outcomes by altering the male genome at the chromosome or DNA level (Wyrobek, 1993).

Over the last two decades, rapid advances in reproductive molecular biology have resulted in numerous techniques to assess sperm chromatin quality and DNA fragmentation (for reviews see Evenson *et al.*, 2002; Agarwal and Said, 2003). The chromatin of mature spermatozoa is highly condensed as a result of the replacement of nucleosomal histones by intermediate proteins and protamines during spermiogenesis; final nuclear maturation during transit in the epididymis, through the formation of disulfide bonds, ensures further stabilization (reviewed in Fuentes-Mascorro *et al.*, 2000; Dadoune, 2003). Packaging of sperm chromatin may also serve to reprogram the paternal genome and set the appropriate genes to be expressed in the early stages of embryo development (Braun, 2001). Thus, a correct chromatin packaging level seems essential to fully express the fertilizing capacity of sperm. Defects in sperm chromatin structure can be associated with abnormal nucleoprotein content and/or DNA strand breaks, and the former can be evaluated using dye techniques such as Acridine Orange or Chromomycin A_3.

The degree of sperm DNA fragmentation reflects the integrity of the genetic material of the gamete; this parameter is important since many types of DNA lesions induce mutations commonly observed in mutated oncogenes and tumour suppressor genes (Marnett, 2000). Transmission of damaged DNA to the offspring, particularly at levels that exceed the DNA repair capacity of the oocyte, could have serious consequences

(Ahmadi and Ng, 1999). The most commonly used techniques to assess sperm DNA integrity are the TUNEL, Comet and sperm chromatin structure (SCSA) assays. For these tests to be clinically useful, threshold values for DNA fragmentation must be set above which normal expression of the paternal genome, and thus pregnancy, are compromised. In the context of assisted reproductive technologies, two studies provided information on the discriminating power of the TUNEL assay to predict outcome. In IVF patients, Henkel *et al.* (2004) reported a lower pregnancy rate of 19% when the percentage of TUNEL-positive sperm was above the cut-off value of 36.5%, calculated from receiver operating characteristics (ROC) curve analysis, compared with 35% at or below this cut-off value. For ICSI couples, a threshold value of 24.3% TUNEL-positive sperm was calculated, cases with results above this value tended to have a lower chance of pregnancy (Henkel *et al*., 2003). Using a similar approach, Benchaib *et al.* (2003) found that the results of TUNEL assay (high or low degree of fragmentation) were not related to IVF outcome in terms of pregnancy. In ICSI, the percentage of TUNEL-positive sperm in cases of couples who obtained a pregnancy was lower than in those who did not; however, no threshold value could be arrived at, the area under the ROC curve being 0.5. It was noted that no pregnancy occurred above 20% sperm DNA fragmentation. In these two studies, no statistically significant correlation was found between TUNEL results and fertilization rates, in both IVF and ICSI. Discordant views were reported in the literature concerning such correlation with the TUNEL assay, particularly in IVF programs (Sun *et al*., 1997; Lopes *et al*., 1998; Host *et al*., 2000).

In comparison, a threshold value of 27% for the DNA fragmentation index (DFI) was initially established for the SCSA in the context of IVF/ICSI (Larson-Cook *et al.*, 2003). While a revised value of 30% was later proposed (Virro *et al*., 2004), the latter authors as well as others observed that IVF and ICSI term pregnancies can often be obtained even with semen samples presenting much higher DFI values (Bungum *et al*., 2004; Gandini *et al*., 2004). As for the Comet assay, indices of sperm DNA damage were found to be higher in couples with poorer embryo quality in IVF (Tomsu *et al*., 2002) and ICSI (Morris *et al*., 2002), but no discriminating cut-off value was obtained.

In the context of natural conception, TUNEL assay with microscopic evaluation indicates higher percentages of sperm DNA fragmentation in infertility patients compared with normozoospermic controls (Gandini *et al*., 2000). Also, with microscopic evaluation, a study on intrauterine insemination showed that none of the semen samples with >12% sperm DNA fragmentation resulted in pregnancy (Duran *et al*., 2002). With the SCSA, a percentage of spermatozoa with fragmented DNA of ≥30% was proposed as a threshold not compatible with good fertility (Evenson *et al*., 1999).

With respect to in-vivo situations, there is a lack of information on the clinical value of the TUNEL assay in predicting outcome in terms of pregnancy. The aim of this study was therefore to set a discriminating threshold value by comparing data on sperm DNA fragmentation, as measured by TUNEL assay with flow cytometry assessment, in men of proven fertility and men from couples consulting for infertility.

Materials and methods

Study participants

Semen samples (one per subject) were obtained from 66 men of an unselected group of couples attending the Andrology Laboratory of the Département d'Obstétrique-Gynécologie, CHUM – Hôpital Saint-Luc, for the investigation of infertility. Their age ranged from 24 to 45 years, and they had a normal physical evaluation. All the couples of this infertile group had a minimum of 2 years of regular unprotected intercourse.

Men of proven fertility (n = 47; had recently fathered a child) were either volunteers (n = 40) who supplied one semen samples for the study, or regular semen donors (n = 7) in our donor insemination program. Their age varied between 22 and 41 years. Informed consent for participation was obtained, and the project was approved by the ethics committee of the CHUM.

Sample collection and storage

Semen samples were collected by masturbation, after a period of 48–72 h of sexual abstinence, into sterile polypropylene containers (Sarstedt, Montréal, Canada) at the Andrology Laboratory. Standard clinical semen analysis was performed according to World Health Organization (1999) criteria by a technician who was blinded to the identity of the study subjects; all the semen samples used for analysis had to contain motile sperm, with no significant leukocytospermia, as per World Health Organization (1999) guidelines.

Spermatozoa from fresh semen (5×10^6 sperm) were separated from seminal plasma by centrifugation (10 min at 600 *g*) at room temperature. The pellets were then resuspended in 1 ml of Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL, Life Technologies, Grand Island, NY, USA) and fixed with 8 ml of 1% formaldehyde (BDH Inc., Toronto, Canada) in PBS (pH 7.4) for at least 30 min at room temperature. After centrifugation, the pellets were washed twice and then stored in PBS at 4°C.

Measurement of DNA fragmentation by TUNEL assay

A detailed protocol for the TUNEL assay of human sperm has been described previously (Sergerie *et al*., 2000). Briefly, after centrifugation (4 min at 5000 *g*), permeabilized, fixed sperm cells were resuspended in 100 µl of terminal deoxynucleotidyl transferase (TdT) buffer containing single-strength 1 mol/l Na-cacodylate, 150 mmol/l Tris (pH 7.4), 25 mmol/l $CoCl_2$ and 0.1% Triton X-100 (Sigma Chemical Co., St Louis, MO, USA). After re-centrifugation (4 min at 5000 *g*), the TdT buffer was removed, and 50 µl of TdT buffer containing 3 µmol/l of biotin 16-deoxyuridine triphosphate (Roche Diagnostics, Laval, Canada), 10 U of TdT (Roche Diagnostics) and 0.1% Triton X-100 were added. Negative control sperm cells from each sample were treated identically except for the omission of TdT. The sperm suspension was then incubated at 37°C for 60 min. After two washes with TN buffer (30 mmol/l Tris, pH 7.4, 300 mmol/l NaCl), the permeabilized, fixed spermatozoa were treated with 100 µl of staining AP buffer (50 mmol/l Tris, pH 7.4, 150 mmol/l NaCl) containing 2% streptavidin–fluorescein (FITC), followed by incubation at room temperature in the dark for 45 min. The stained spermatozoa were washed twice with AP buffer and resuspended in 1 ml of PBS containing 10 µg/ml propidium iodide (PI) (Sigma Chemical Co.) before analysis. The samples were stored at 4°C in the dark until analysis.

Flow cytometry measurements

Sperm DNA fragmentation and PI labelling were measured with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 15 mW argon-ion laser for excitation. Flow during analysis was controlled at ~300 spermatozoa/s. Each analysis included a minimum of 10 000 stained spermatozoa quantified simultaneously by green and red fluorescence. Light scattering and fluorescence data were obtained at a fixed gain setting in logarithmic mode. Green fluorescence (from FITC) was detected in the FL1 sensor through a 550-nm dichroic long-pass filter and a 525-nm band-pass filter, while red fluorescence (from PI) was collected in the FL3 sensor through a 645-nm dichroic long-pass filter and a 620-nm band-pass filter. Debris were gated out by establishing a region around the population of interest on the basis of PI characteristics of the selected sperm. These characteristics were assessed in several assay-selected sperm samples before starting the study (Sergerie M. and Bleau G., unpublished data). The percentage of labelled sperm was determined by selecting a region that excluded debris. Hence, all events out of this region were considered positive; 10 000 events were collected. The spermatozoa gate was set to permit slight sample-to-sample variations, taking into account the light scattering properties of the asymmetrical sperm head.

TUNEL data analysis

TUNEL analysis was performed as described by Sailer *et al.* (1995). TUNEL assay list mode files were converted to histogram files with the 2150 Data Handler, transferred to a PC-compatible computer by Multilink (Phoenix Flow Systems, San Diego, CA, USA), and analysed by Multi2D software (Phoenix Flow Systems). TUNEL assay analysis consisted of subtracting control (no TdT enzyme) green fluorescence histograms from TdT-positive green fluorescence histograms, yielding the percentage of cells showing DNA strand breaks (Figure 1). The number of cells analysed in both control and TdT-positive green fluorescence histograms were equal for each sample pair. The subtraction routine employed by Multi2D was that of Overton (1988).

Statistical analyses

Group comparisons (men of proven fertility and infertility patients) were performed using Mann–Whitney rank sum tests for continuous variables. The results are expressed as means ± SD. All hypothesis testing was two-sided, with a *P*-value of 0.05 deemed as significant. The sample size of infertility patients ($n = 66$) provided a 90% power to detect a 10% difference in percent sperm DNA fragmentation with the 47 semen samples from men of proven fertility. The likelihood ratio χ^2-test served to determine whether TUNEL assay provided statistically significant thresholds between groups. The predictive capacity of sperm DNA fragmentation, as measured by TUNEL assay, to differentiate between men of proven fertility and infertility patients was studied by ROC curve analysis and logistic regression with the group's status as the outcome variable (Altman and Bland, 1994). Sperm concentration was not included in the ROC curve analysis as this sperm parameter was not a biomarker in our study. The area under the curve (AUC) and 95% confidence intervals (CIs) were estimated. To identify the clinical utility of TUNEL assay, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated. The analyses were conducted with STATA 6.0 (Stata Corporation, College Station, TX, USA).

Results

There were no statistically significant differences between the groups of infertile patients and fertile men with respect to age, semen volume and days of sexual abstinence (data not shown). Since sperm motility and morphology were evaluated as subjective criteria, we only compared sperm DNA fragmentation with sperm concentration, even though the latter parameter was not a marker in our study. As expected, sperm concentration for the fertile men was significantly higher than that of infertile patients: $102.4 \pm 66.4 \times 10^6$/ml versus $62.9 \pm 33.2 \times 10^6$/ml, respectively ($P < 0.001$). As determined by TUNEL assay, infertile patients had a higher mean level of DNA fragmentation than fertile men: 40.9 ± 14.3% versus 13.1 ± 7.3%, respectively ($P < 0.001$) (Table I). In the group of fertile men, the highest value for sperm DNA fragmentation was 43.8%. In the group of infertile patients, the lowest percentage of sperm DNA fragmentation was 14.6%.

The discriminating power of sperm DNA fragmentation, measured by TUNEL assay, to identify a threshold value

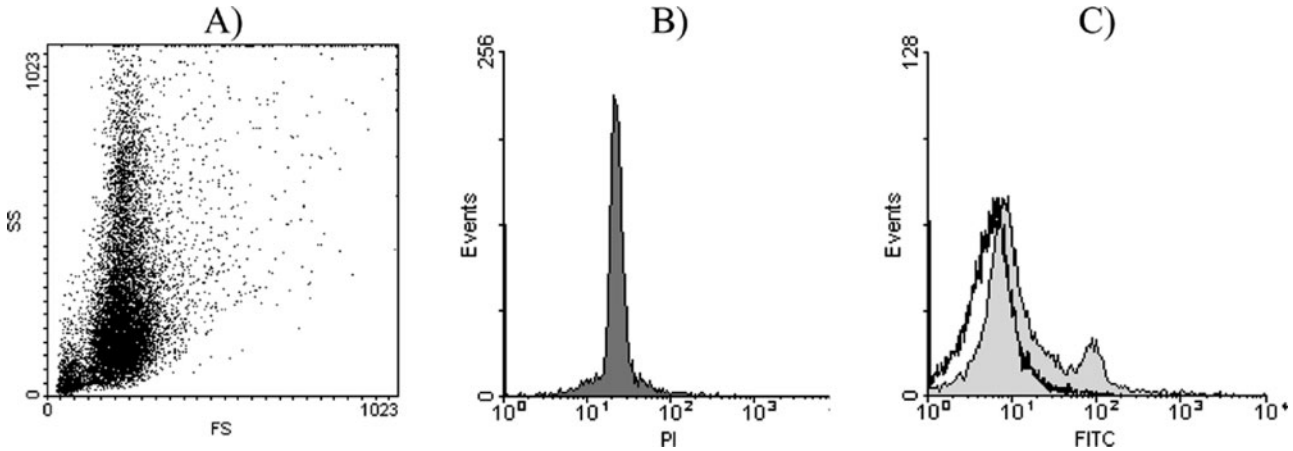

Figure 1. Flow cytometry data from TUNEL assay of spermatozoa. (**A**) Forward-angle light scatter/side-angle light scatter dot plotting. (**B**) A red fluorescence frequency histogram obtained by gating red (PI stain) fluorescence to separate cells from debris for the creation of green fluorescence (FITC–streptavidin) frequency histograms. (**C**) The grey histogram represents sperm exposed to TdT enzyme, and the clear histogram with heavy tracing the control sperm (no TdT enzyme); the area of the grey histogram above the heavy black tracing corresponds to the subtraction (with TdT minus without TdT) used to calculate the percentage of cells showing DNA strand breaks.

Table I. Semen concentration and percentage of spermatozoa with DNA fragmentation in infertility patients and men of proven fertility

Subjects	*n*	Concentration ($\times 10^6$/ml)	Sperm DNA fragmentation (%)
Infertility patients	66	62.9 ± 33.2[a]	40.9 ± 14.3[a]
Men of proven fertility	47	102.4 ± 66.4[a]	13.1 ± 7.3[a]

Values are mean ± SD.
[a]Mann–Whitney rank sum test between groups $P < 0.001$.

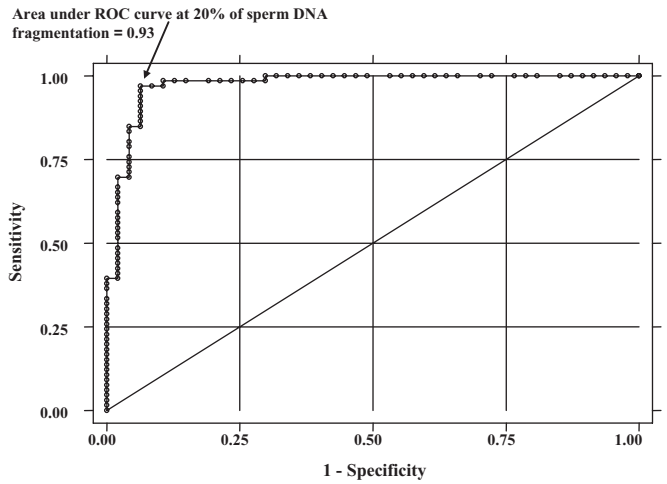

Figure 2. Receiver operating characteristics at 20% sperm DNA fragmentation.

between fertile men and infertile patients, was calculated by ROC curve analyses. During ROC analyses (Figure 2), varying percentages of sperm DNA fragmentation values were used to calculate optimum sensitivity and specificity values for TUNEL assay. The best area under the ROC curve was 0.93 for 20% of sperm DNA fragmentation. The calculated threshold value for TUNEL assay to distinguish between fertile men and infertile patients was 20%. Furthermore, this threshold had 89.4% (95% CI 83.7–95.1) specificity and 96.9% (95% CI 93.8–100) sensitivity for a discrimination level between infertile patients and fertile men. The PPV and NPV of the 20% sperm DNA fragmentation threshold were high: 92.8% (95% CI 87.9–97.5) and 95.5% (95% CI 91.6–99.3), respectively

Discussion

The prevalence of male infertility and the availability of new therapeutic options make it necessary to challenge the usefulness of tests to measure sperm DNA integrity. New developments of these tests (Robaire and Hales, 2003) follow the recommendations of the 2nd International Conference on Male Mediated Developmental Toxicity, Montreal, Quebec, 2001. Classical semen analysis gives an approximate evaluation of the functional competence of spermatozoa, but improved predictive values could be obtained from validated sperm DNA fragmentation assays. A suitable sperm DNA integrity assay relies not only on its discriminative power to predict fertilization failure but also on its capacity to help clinicians in the choice of therapeutic procedures (Perreault *et al.*, 2003).

Sperm DNA fragmentation as a prognostic factor of a man's fertility potential is a matter of debate (see review by Alvarez, 2003). Particularly in the in-vivo situation, there is little information on the threshold values of sperm DNA fragmentation indicative of a man's fertility potential. Several criteria have been used to arrive at discriminating values or thresholds, including comparison of fertility status (Gandini *et al.*, 2000; Irvine *et al.*, 2000; Donnelly, *et al.*, 2001; Ollero *et al.*, 2001; Zini *et al.*, 2001; Saleh *et al.*, 2002; 2003).

In our study, the results of ROC curve analysis indicated that sperm DNA fragmentation, as measured by TUNEL assay, was a good predictive parameter to distinguish between fertile and infertile populations on the basis of the largest AUC (0.93; Figure 2). The cut-off point was at 20% sperm DNA fragmentation, giving 96.5% sensitivity and 89.4% specificity. This threshold value for sperm DNA fragmentation in our study is similar to the 24% and 25% values obtained by others (Lopes *et al.*, 1998; Saleh *et al.*, 2002; 2003).

In assisted reproductive clinics, the objective of a good sperm DNA integrity test is to identify the proportion of men who will contribute to the infertility problem of couples (and not to classify them as possibly fertile or subfertile).

Sensitivity and specificity are associated with the intrinsic performance of TUNEL assay, and do not depend upon sample prevalence. However, PPV and NPV are strongly associated with prevalence. In practice, when we obtain, by TUNEL assay, 20% or more of sperm DNA fragmentation in a sample, with what confidence can we be sure that a patient is infertile? For instance, using a threshold value of 20% sperm DNA fragmentation with a prevalence of 58.4%, the PPV obtained was 92.8%. This value indicates that for a positive test (20% or more), the subject would be falsely pronounced as infertile in 7.2% of cases (false-positive). Assuming a 50% prevalence of male subfertility in an assisted reproductive setting (Ombelet *et al.*, 1997), under similar TUNEL assay conditions and similar sensitivity and specificity, the PPV obtained would remain above 90%. In this case, the subject would be falsely pronounced infertile in less than 10% of cases (false-positive). It seems more acceptable though to diagnose subfertile males falsely as fertile than to diagnose fertile males as subfertile. This approach would prevent over-treatment of potentially fertile males, for instance, referring couples for ICSI treatment when IVF could have been employed.

The cause of human sperm DNA fragmentation and its impact on fertilization and pregnancy remain unclear. Gorczyca *et al.* (1993) proposed that the presence of endogenous nicks in ejaculated human sperm is characteristic of programmed cell death, as seen in apoptosis of somatic cells. Furthermore, active caspase-3, an enzyme associated with apoptosis, has been detected in human spermatozoa (Weng *et al.*, 2002). In addition, oxidative stress has been shown to affect the integrity of sperm chromatin and to cause high frequencies of DNA single- and double-strand breaks. Exposing spermatozoa to artificially produced reactive oxygen species (ROS) significantly increases DNA damage by modifying all bases and producing base-free sites, deletions, frame shifts and DNA cross-links

(Irvine *et al.*, 2000). Sperm DNA fragmentation can also be caused by ROS generated by elevated number of leukocytes in semen, particularly during genital tract inflammation. A third possibility is based on the fact that histone replacement and chromatin rearrangement are related to the presence of endogenous DNA nicks in elongating spermatids (McPherson and Longo, 1993); ejaculated sperm with DNA fragmentation might result from failure of spermatozoa to mature normally. The predictive values of the TUNEL assay appear rather high; in this regard, further prospective studies on larger cohorts are needed to refine these figures. This also gives evidence that whatever the cause of sperm DNA fragmentation in most infertile men, the damages are likely to extend to other sperm structures and functions, as opposed to, for example, the more discrete effects of gamma radiation on the DNA of mature epididymal mouse sperm. Our results suggest that the TUNEL assay is not a strictly independent measure of sperm quality. It could complement other tests aimed at evaluating sperm functions in the context of assisted reproductive technologies or natural reproduction. Sperm DNA fragmentation was suggested as an independent measure of sperm quality that may have better diagnostic and prognostic capabilities than standard semen parameters (Sun *et al.*, 1997).

In summary, our data indicate that the cut-off of sperm DNA fragmentation value to differentiate between fertile and infertile men, as applicable to the in-vivo situation, is slightly lower than those for other methods (e.g. SCSA) or under in-vitro situation as proposed in the literature. More importantly, our current data, which are based on ROC curve analysis, allow a classification of 'normality', i.e. fertile or infertile. This approach, using a predictive threshold value for sperm DNA fragmentation, can now more readily identify false-positive rates. However, if a consensus can be reached on the lower normal limits for sperm DNA integrity values, more studies based on standardized protocols will be necessary to achieve the ultimate goal of correctly predicting male fertility potential.

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