Concordance among sperm deoxyribonucleic acid integrity assays and semen parameters

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Objective: To assess the concordance of sperm chromatin structure assay (SCSA) results, epifluorescence TUNEL assay results, and standard semen parameters.
Design: Prospective, observational study.
Setting: Tertiary referral andrology clinic.
Patient(s): A total of 212 men evaluated for subfertility by a single physician.
Intervention(s): Clinical history, physical examination, semen analysis, SCSA, and TUNEL assay.
Main Outcome Measure(s): Spearman's rank correlation coefficients (r) between SCSA DNA fragmentation index (DFI), percentage TUNEL-positive sperm, and semen analysis parameters.
Result(s): There was a positive correlation between SCSA DFI and TUNEL ($r = 0.31$), but the strength of this correlation was weaker than has previously been reported. The discordance rate between SCSA and TUNEL in classifying patients as normal or abnormal was 86 of 212 (40.6%). The SCSA DFI was moderately negatively correlated with sperm concentration and motility. The TUNEL results were unrelated to standard semen parameters.
Conclusion(s): The SCSA DFI and percentage TUNEL-positive sperm are moderately correlated measures of sperm DNA integrity but yield different results in a large percentage of patients. The DFI is well-correlated with semen analysis parameters, whereas TUNEL is not. These data indicate that the SCSA and TUNEL assay measure different aspects of sperm DNA integrity and should not be used interchangeably. (Fertil Steril® 2015;104:56–61. ©2015 by American Society for Reproductive Medicine.)
Key Words: DNA fragmentation, in situ nick-end labeling, infertility (male), semen analysis, spermatozoa

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Diagnosis and classification of male subfertility depends in large part on quantitative assessment of semen quality. Standard semen analysis (SA) performed according to protocols published by the World Health Organization (WHO) (1) is by far the most commonly utilized such test. However, SA has several significant limitations, including poor prognostic performance in predicting outcomes of natural and assisted reproductive cycles (2) and high levels of intraindividual variability (1). The limited clinical value of standard SA underscores the need for tests that enhance the ability to diagnose male factor infertility.

The critical importance of sperm DNA integrity for human fertility has been increasingly recognized over the past 15 years (3); and tests for the detection of sperm DNA damage have emerged as additional measures of semen quality. Sperm DNA damage is more prevalent among subfertile couples (4), and higher levels of sperm DNA damage are associated with impaired spermatogenesis (5). A growing body of literature has linked results of sperm DNA integrity assays with rates of natural conception (6), conception after IUI (7), pregnancy loss after assisted reproductive cycles (8), and rates of conception after varicocele repair (9).

The most commonly used of several available tests of sperm DNA integrity are the sperm chromatin structure assay (SCSA) and the TUNEL

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assay. The SCSA uses flow cytometry to measure the stability of double-stranded sperm chromatin when exposed to a denaturant (10). Test results are given as the percentage of sperm with denatured (single-stranded) DNA after denaturant exposure, which is termed the DNA fragmentation index (DFI). In the TUNEL assay, individual sperm with native DNA strand breaks are stained or labeled with a fluorochrome and detected by either fluorescent microscopy or flow cytometry (11). Results are given as the percentage of TUNEL-positive (or negative) sperm.

Though often used and discussed interchangeably as measures of sperm DNA damage, the SCSA and TUNEL assay measure different characteristics of sperm DNA. Furthermore, even flow cytometry and epiflourescence-based TUNEL assays may be measuring different aspects of sperm DNA damage. Flow cytometry does not discriminate sperm according to morphology, and the DFI reported by such assays indicates the percentage of all sperm with native DNA strand breaks, regardless of sperm morphology. In comparison, TUNEL assays using epifluorescence microscopy combined with contrast-phase or Nomarski optics, such as the assay used in this study, allow for direct visualization of sperm morphology and enable reporting of the percentage of morphologically normal sperm with native DNA strand breaks.

Previously published studies describing the concordance of the SCSA and TUNEL assay with each other and with standard semen parameters have been limited by low numbers of patients and inconsistent results (12–15). The present study is the largest to date evaluating the relationships between SCSA DFI, percentage TUNEL-positive sperm, and standard semen parameters.

MATERIALS AND METHODS

Patient Selection and Evaluation

This research protocol was approved by the institutional review board at Weill Cornell Medical College of Cornell University. This was a prospective analysis of baseline semen quality for 212 subfertile men evaluated by a single physician from 2009 to 2014. Starting in 2009, sperm DNA integrity testing with both the TUNEL assay and SCSA was offered to all men consecutively evaluated for subfertility. Both tests were routinely ordered as part of the study design, but performance of testing was subject to patient compliance with the physician recommendation for testing. Only patients who underwent standard SA and sperm DNA integrity testing with both the SCSA and TUNEL assay were included.

The baseline clinical evaluation for each patient included a comprehensive history and complete physical examination performed in a warm room after placing a heating pad on the scrotum to relax the dartos muscle. Testicular volumes were measured with an orchidometer. Serum FSH and total early morning T levels were assessed by a peripheral venous serum sample taken between 8:00 AM and 10:30 AM. Semen analysis was performed manually using the 1999 WHO protocol. Semen was collected in a specially designated room in our embryology laboratory, with the aid of audiovisual stimulation.

Sperm DNA Integrity Testing

The SCSA was performed by the proprietary SCSA diagnostics laboratory according to the original method described by Evenson et al (10). Patients used a prepackaged kit to collect, freeze, and mail semen samples produced at home to the SCSA diagnostics laboratory. Semen samples for the SCSA were collected within a range of 1–6 weeks from the time of semen collection for standard SA and TUNEL analysis. Frozen samples were thawed, diluted, exposed to acid detergent, and then stained with acridine orange. The fluorescence patterns of 5,000 sperm cells were sorted using flow cytometry and analyzed using proprietary software to determine the DFI of each sample. Values for SCSA DFI $\geq$25% were considered abnormal.

The TUNEL assay was performed as previously described (16), and TUNEL was performed on the same semen sample provided for standard SA. Four smears from each semen sample were prepared on glass slides and air-dried. The In Situ Cell Death Detection Kit with Fluorescein isothiocyanate (FITC; Roche Diagnostics) was used with modifications. Each slide was fixed with 4% paraformaldehyde (1 mL) in phosphate-buffered saline (PBS) solution and incubated at room temperature for 1 hour. Slides were washed with ice-cold PBS, then permeabilized with Triton X in 0.1% sodium citrate for 5 minutes. Slides were again washed with PBS, then incubated with a mixture of the TUNEL enzyme solution containing terminal deoxynucleotidyl transferase plus TUNEL labeling solution containing deoxyuridine triphosphate. A Parafilm M strip (Alcan Packaging) was applied to each slide, and the slides were incubated in a dark, moist chamber at 37°C for 1 hour. After labeling, slides were taken out of the chamber, the Parafilm M was removed, and the cells were washed with PBS. Vectashield (Vector Laboratories) with 4′,6-diamidino-2-phenylindole (DAPI) was applied to each slide for DNA counterstaining, and a cover slip was applied. Cells were allowed to stain overnight. Two negative and two positive controls were tested with each batch.

Slides were analyzed using an epifluorescent microscope at 400× magnification. The number of DAPI-positive cells was counted, then, in the same field, the number of FITC-positive cells was recorded. At least 100 DAPI-positive cells were counted for a single tally. The number of FITC-positive cells detected was divided by DAPI-positive cells × 100 to produce the percentage of TUNEL-positive cells (containing fragmented DNA), and at least four separate fields were analyzed. Only sperm with presence of normal midpiece, tail, and normal-appearing head were counted for TUNEL assay because such sperm would be normally chosen during IVF. In this respect the TUNEL assay performed in our laboratory uses "strict" criteria (17). The TUNEL tests were considered abnormal when the percentage of TUNEL-positive sperm was $\geq$7%.

Statistical Analysis

Correlations between SCSA DFI, percentage TUNEL-positive sperm, and WHO semen parameters were analyzed by nonparametric Spearman's rank correlation coefficients (r) using GraphPad Prism 5 software. The discordance rate

TABLE 1

Spearman's correlation coefficients (*r*) between SCSA DFI, percentage TUNEL-positive sperm, and WHO semen parameters.

Variable	Semen volume	Sperm concentration	% Motility	% Normal morphology (WHO 1999)
SCSA DFI	−0.06 (*P*= .4)	−0.34 (*P*< .000001)	−0.45 (*P*< .000001)	−0.1 (*P*= .19)
% TUNEL-positive sperm	−0.1 (*P*= .15)	−0.01 (*P*= .85)	−0.15 (*P*= .03)	0.06 (*P*= .43)

Stahl. Concordance of sperm DNA assays. Fertil Steril 2015.

between the SCSA and TUNEL assay in classifying sperm DNA integrity as normal or abnormal was determined using laboratory-recommended cutoff values for each assay.

RESULTS

The mean age of the patient cohort was 39.3 years (SD 6.2, range 25–58 years). Unilateral or bilateral varicoceles were present in 166 of 212 patients, with the size of the largest varicocele being grade 1 in 48 men, grade 2 in 87, and grade 3 in 31. No patients had been exposed to prior chemotherapy or radiation, and none had current or prior testicular malignancies.

Mean semen volume was 2.7 mL (SD 1.3, range 0.2–8.1 mL). Mean sperm concentration was 46.5 $\times 10^6$/mL (SD 38.2, range 0.01–184 $\times$ 10^6/mL). Mean overall motility was 51.2% (SD 16.7%, range 5%–91%). Mean percentage normal morphology according to WHO 1999 criteria was 9.4% (SD 9.3%, range 0–52%). Mean serum FSH was 6.1 IU/L (SD 4.8, range 0.1–29.9 IU/L). The mean SCSA DFI was 20.1% (SD 15.1%, range 2%–81%). The mean percentage TUNEL-positive sperm was 12.1% (SD 8.0%, range 2%–50.8%).

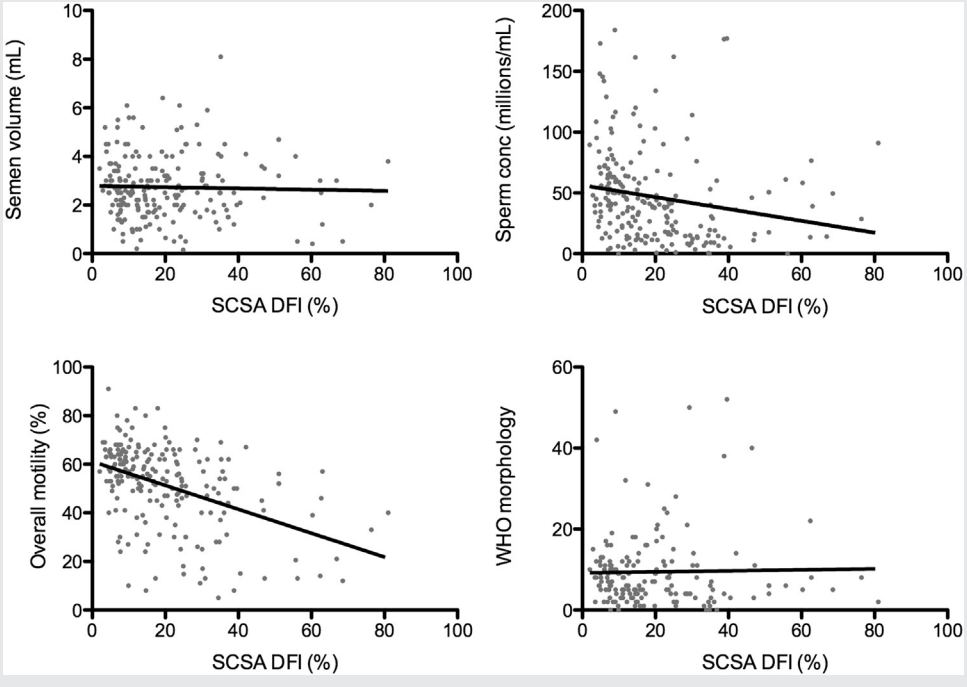
Spearman's correlation coefficients indicating correlations between SCSA DFI and percentage of TUNEL-positive sperm with WHO SA parameters are given in Table 1 and are visually displayed in Figures 1 and 2. The SCSA DFI exhibited moderate and strong negative relationships with sperm concentration and motility, respectively. Correlations between TUNEL assay results and WHO semen parameters were not observed or were negligible.

There was a moderate positive correlation between SCSA DFI and the percentage TUNEL-positive sperm (r = 0.314, P< .00001), but this correlation was weaker than has previously been reported. The discordance rate between SCSA DFI and the percentage of TUNEL-positive sperm in classifying patients as normal or abnormal was 86 of 212 (40.6%) (Fig. 3).

DISCUSSION

Assays evaluating native sperm DNA strand breaks or the susceptibility of sperm DNA to denaturation have been developed as measures of sperm DNA integrity. The SCSA and TUNEL assay are the most commonly utilized such tests. Other

FIGURE 1



Relationships between SCSA DFI and standard semen parameters. Solid lines represent the best-fit linear regression lines.

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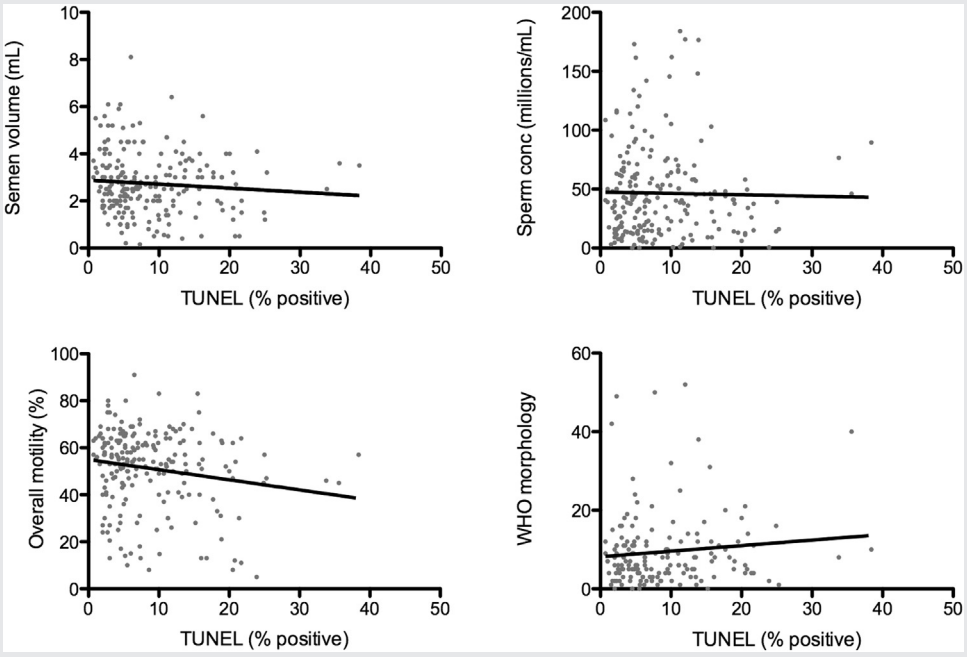
FIGURE 2



Relationships between the percentage of TUNEL-positive sperm and standard semen parameters. Solid lines represent the best-fit linear regression lines.

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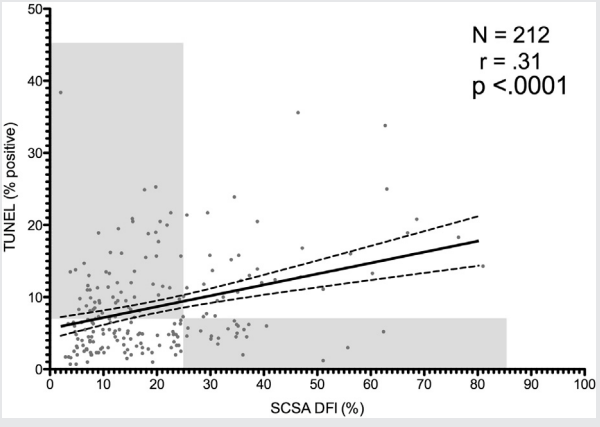
well-described assays include the single-cell gel electrophoresis assay (COMET) (18) and the sperm chromatin dispersion test (19). These four tests and others have been espoused as alternative assessments of semen quality that are less variable than standard semen parameters and can detect occult male factors not readily identifiable with conventional semen testing (20). Utilization of sperm DNA integrity testing in clinical practice has become common, though at present the currently available evidence is insufficient to support routine clinical use (21).

FIGURE 3



Concordance between SCSA DFI and TUNEL assay results. Grey shaded areas indicate patients who would be labeled as normal by one assay and abnormal by the other. Solid and dashed lines represent the best-fit linear regression line and its 95% confidence interval, respectively. Grey shaded boxes indicate patients with abnormal results from one assay but normal results from the other.

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The enthusiasm for integration of sperm DNA integrity assays into clinical practice is based on studies that have demonstrated associations between sperm DNA integrity and reproductive outcomes. A recent systematic review of sperm DNA integrity testing concluded that such tests predict pregnancy after natural conception, IUI, and IVF but do not impact pregnancy rates after IVF/intracytoplasmic sperm injection (22). However, a recently presented study from our institution revealed no live births after IVF/intracytoplasmic sperm injection when sperm TUNEL testing exceeded 25% (17). These data suggest that testing may be useful in counseling of subfertile couples about the likelihood of achieving pregnancy and in selection of assisted reproductive techniques.

Selection of a particular sperm DNA integrity assay for use in the clinical evaluation of a subfertile couple is driven by clinician preference and institutional assay availability. Guidelines for assay selection do not exist. Patients who undergo sperm DNA integrity testing are classified according to reference values provided by the laboratory performing each test as having either normal or abnormal sperm DNA integrity, and clinical decisions are made based on these results in the context of the existing literature. Although the available assays are methodologically distinct, they are often used and discussed interchangeably as nonspecific measures of sperm DNA integrity.

The notion that sperm DNA integrity assays may be used interchangeably in clinical decision making is based on several studies that have examined interassay concordance. These prior studies have consistently demonstrated strong correlations between SCSA DFI and TUNEL results, with correlation coefficients ranging from 0.56 to 0.99 (Supplemental Table 1, available online) (12, 13, 15, 23, 24). However, most of these studies are limited by small numbers of patients (range 25–60 patients). In the present study, which includes more patients than all of the previously reported studies combined, the concordance between SCSA and TUNEL results was far weaker ($r = 0.31$) than has previously been reported. Moreover, there was high likelihood of a patient being categorized as having normal sperm DNA by one assay and damaged sperm DNA by the other (41%).

One explanation for the observed discordance between SCSA and TUNEL assay results is the significant conceptual difference between these two tests. The SCSA is a test of sperm DNA stability upon exposure to a denaturant, whereas the TUNEL assay detects in situ pre-existing DNA strand breaks under neutral conditions. It has been said that the SCSA is therefore a measure of "potential" sperm DNA damage, and that in contrast the TUNEL assay detects "real" sperm DNA damage (15). Furthermore, the addition of contrast microscopy to exclude morphologically abnormal sperm from DFI calculations may also have altered the performance characteristics of the TUNEL assay and its correlation with the SCSA, which does not account for sperm morphology at all.

Another explanation, which may also contribute to the differences between our results and prior studies that have shown stronger correlations between SCSA and TUNEL, is that neither the SCSA nor the TUNEL assay have been methodologically standardized to provide optimal clinical information and predictive value. Results on SCSA have been shown to vary according to several protocol and laboratory factors, including use of ice for postthaw semen sample incubation and even by the laboratory technician performing the assay (25). Furthermore, several different protocols are available and used for labeling DNA strand breaks during the TUNEL assay, and detection of DNA-damaged sperm may be performed using flow cytometry or fluorescent microscopy.

The choice of sperm DNA integrity tests should depend on local familiarity with the protocols and the clinical scenario. In academic centers like ours, which deal with high numbers of severely oligospermic men, flow cytometry–based assays are not feasible because they require 10,000 to 100,000 sperm per run. In contrast, the TUNEL assay can be performed on samples containing fewer than 1,000 sperm after centrifugation. In addition, the epiflourescence-based TUNEL assay is the only assay that allows for use of the same methodology for analysis of ejaculated and surgically retrieved sperm, which may prove to be useful in selection of sperm to use for assisted reproduction.

Although our study is the largest study of sperm DNA integrity assay concordance published to date, there are several methodologic limitations that may have influenced our results. As a tertiary referral center for patients with varicocele, our study population was composed of a higher percentage of patients with varicoceles (78%) than would be expected in the general population of subfertile men. Varicoceles may exert discriminate adverse effects on TUNEL and SCSA results, and it is possible that a less-biased patient population would have resulted in a stronger observed concordance between SCSA and TUNEL results. Another important methodologic limitation of our study is that the SCSA and TUNEL assay were performed on different semen samples. Intraindividual variation in semen quality may have influenced the observed concordance between TUNEL and SCSA results.

In conclusion, the observed concordance between the SCSA DFI and the percentage of TUNEL-positive sperm was weaker in this study than has been previously described. These assays are moderately correlated measures of sperm DNA integrity but yield conflicting results in a large percentage of patients. The SCSA DFI is well-correlated with SA parameters, whereas TUNEL is not. These data indicate that the SCSA and TUNEL assays measure different aspects of sperm DNA integrity and should not be used or discussed interchangeably. At this time there are insufficient available data to guide selection of specific sperm DNA integrity assays for use in clinical practice. Prospective studies investigating the correlations of specific sperm DNA assay results with clinical and reproductive outcomes are needed to help clinicians select specific assays that will guide patient counseling or change clinical care.

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SUPPLEMENTAL TABLE 1

Previously reported studies evaluating the concordance of SCSA DFI and TUNEL.

Study (reference)[a]	No. of patients	Correlation (*r*)	*P* value
Gorczyca 1993 (13)	25	0.87	< .05
Sailer 1995 (23)	25	0.56	.004
Zini 2001 (12)	40	0.71	< .0001
Chohan 2006 (24)	60	0.90	< .001
Henkel 2010 (15)	52	0.99	< .0001

[a] Full reference citations can be found in main text.

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