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