Diagnostic accuracy of sperm chromatin dispersion test to evaluate sperm deoxyribonucleic acid damage in men with unexplained infertility

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Objective: To compare the sperm chromatin dispersion (SCD) test and the terminal uridine nick-end labeling (TUNEL) assay for assessment of sperm DNA damage.
Design: Prospective comparative experimental study.
Setting: Andrology laboratory.
Patient(s): Twenty subfertile men with unexplained infertility.
Intervention(s): Sperm DNA damage was determined in the same semen samples using the TUNEL assay with fluorescence microscopy and the SCD test with bright-field microscopy.
Main Outcome Measure(s): Correlation coefficient and receiver operating characteristic analysis outcomes. The TUNEL assay was used as the reference standard to identify optimal cutoff points for assessing DNA damage by SCD.
Result(s): The SCD test detected a significantly higher proportion of sperm with damaged DNA (20.6% $\pm$ 14.0%) than the TUNEL assay (11.5% $\pm$ 7.3%). Spearman's rank correlation showed that the methods were not comparable ($r = 0.29$). Receiver operating characteristic analysis revealed that 15% was the best SCD cutoff point to classify patients within the same levels of DNA fragmentation, normal or abnormal, as determined by the TUNEL assay, with an accuracy of 69%.
Conclusion(s): The SCD test is more sensitive than the TUNEL assay for the assessment of DNA damage in men with unexplained infertility. Although the methods are poorly correlated, SCD may discriminate men with normal and abnormal sperm DNA damage with moderate accuracy when compared with TUNEL. It is important to distinguish between the methods because they differently evaluate sperm DNA damage. (Fertil Steril® 2014;101: 58–63. ©2014 by American Society for Reproductive Medicine.)
Key Words: Sperm DNA damage, in situ nick-end labeling, sperm chromatin dispersion test, diagnosis, ROC curve

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The assessment of sperm chromatin integrity has emerged as an important biomarker for male infertility. Sperm DNA damage has been associated with several infertility phenotypes, including unexplained infertility, idiopathic infertility, repeated intrauterine and IVF failure, and recurrent miscarriage (1–4). Because ejaculates of infertile men harbor higher proportions of sperm with DNA damage compared with fertile counterparts (5, 6), different assays have been developed to evaluate DNA damage in sperm.

Among several tests, terminal uridine nick-end labeling (TUNEL) assay and sperm chromatin structure assay (SCSA) remain the gold standards for the identification of clinically significant sperm DNA damage (7–10). Although these methods have been implemented by many andrology laboratories, they cannot be performed routinely in the routine workup of male infertility because they are complex, difficult to implement, time-consuming, and expensive since they require fluorescent microscopy and flow cytometry, respectively (11). A less complex test would be desirable, and the sperm chromatin dispersion (SCD) test has reached technical

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maturity to allow its widespread application with a standardized protocol using conventional bright-field microscopy (12–14). Whereas testing thresholds have been extensively reported for TUNEL and SCSA (15–19), there are few studies focusing on the diagnostic accuracy of SCD (20) and none in specific patient subsets, such as in unexplained male infertility. This condition accounts for 6%–27% of the male cases (21), and therefore a detailed evaluation of the diagnostic accuracy of SCD in such cases is warranted before shifting from the more complex and validated methods to SCD.

Therefore, we conducted a study to determine the accuracy of the SCD test using conventional bright-field microscopy in the evaluation of DNA damage in sperm. For this, we used the TUNEL assay carried out with fluorescence microscopy as the gold standard method for sperm DNA damage assessment.

MATERIALS AND METHODS

Patient Inclusion Criteria

A total of 20 consecutive patients, aged 18–43 years, presenting at the study center for infertility evaluation and who met the study criteria, was included. The inclusion criteria comprised the following: [1] presence of normal semen parameters, in accordance with the 2010 World Health Organization (WHO) guidelines (22), in a minimum of two separate previous semen analyses performed in our laboratory; [2] patients should be nonsmokers and not taking any medication with potential gonadotoxic effects for at least 3 months before the study; [3] all subjects should have completed an initial evaluation by the consulting urologists, and no obvious infertility problems noted in the medical history, physical examination, and endocrine profiles. As such, all subjects enrolled in the study were classified as having unexplained male infertility (23). In addition, data collection was planned before the tests were performed. The recruitment period ranged from March to July 2012. The study complied with the standards for the reporting of diagnostic accuracy studies (START statement). Institutional review board approval was obtained before the investigation.

Initial Assessment of Semen Parameters

Subjects were asked to abstain from ejaculation for a fixed period of 3 days before collection. Semen specimens were collected by masturbation into sterile cups. All subjects used a collection room located in the same facility as the andrology laboratory. Semen was allowed to liquefy for 30 minutes, and an aliquot was taken for macroscopic and microscopic assessments. Specimens were assessed for volume, count, motility, vitality, morphology, and leukocytes, in accordance with the fifth edition of the WHO manual (22). We used the strict criterion (Tygerberg) for morphology evaluation. We assessed all specimens for the presence of rounded cells and used the Endtz test to determine the presence of polymorphonuclear leukocytes. The semen parameters of study subjects were above the fifth percentile, proposed as the lower reference limit by the WHO (22), and are presented in Supplemental Table 1 (available online). All tests were carried out in an International Organization for Standardization (ISO 9001:2008) certified andrology laboratory enrolled in both external and internal quality control programs (24, 25).

Sperm DNA Damage Assessment

After initial evaluation, semen specimens were split into two aliquots of equal volumes; one was tested by the TUNEL assay, set as the reference standard in the present study, and the other by the SCD test. Procedures were carried out in parallel.

TUNEL assay. The assay was performed using the Apo-Direct kit (Pharmingen) as described by Sharma et al. (15). A sperm aliquot containing 1 to 2 $\times$ 10^6 spermatozoa was washed in phosphate-buffered saline and resuspended in 3.7% paraformaldehyde. Thereafter, the suspension was placed on ice for 30–60 minutes at 4°C, washed again in phosphate-buffered saline to remove the paraformaldehyde, and then resuspended in 50 μL of freshly prepared staining solution for 60 minutes at 37°C. The staining solution was composed of terminal deoxytransferase (TdT) enzyme, TdT reaction buffer, fluorescein isothiocynate–tagged deoxyuridine triphosphate nucleotides (FITC-dUTP), and distilled water. All specimens were further washed in rinse buffer and counterstained with 4,6 diamidoino-2-phenylindome (DAPI, 2 micrograms/mL in vecta shield) followed by analysis using fluorescent microscopy. A fluorescence microscope (Eclipse E600; Nikon) equipped with an epi-illumination module and a mercury ultraviolet source was used to examine the slides at $\times$1,000 magnification. The B2A filter cube was used for FITC-dUTP, which fluoresces apple-green. Sperm showing bright apple-green fluorescence represented damaged cells (TUNEL positive), in which dUTP was incorporated to DNA breaks, in contrast to nonstained cells representing nondamaged sperm (Supplemental Fig. 1). The percentage of TUNEL-positive sperm was calculated and reported as the percentage of cells exhibiting DNA damage. A minimum of 400 sperm was assessed per specimen.

SCD test. We used the Halosperm kit (Halotech DNA) according to the protocol described by Fernandez et al. (13). In brief, a tube containing agarose was first heated at 100°C for 5 minutes to allow the agarose to melt. After stabilization at 37°C, 25-μL semen aliquots were added to the tube, and a 15-μL aliquot of the mixture was placed onto a pretreated microscope slide. A coverslip was placed and the slide was kept in the refrigerator for 5 minutes in order for the agarose to solidify. Meanwhile, 80 μL of a denaturing solution was added to 10 mL of distilled water to produce a fresh working solution. The slide was then taken from the refrigerator and the coverslip removed. Thereafter, the slide was immersed in the denaturation solution and incubated for 7 minutes. The slide was then transferred to the lysis solution and incubated for 25 minutes. Finally, the slide was washed by incubation in a Coplin jar containing distilled water for 5 minutes, followed by incubation in ethanol solutions of 70%, 90%, and 100%, each for 2 minutes. After air drying at room temperature, slides were stained with Wright's stain, and analysis was carried out using bright-field microscopy. Sperm containing nondamaged DNA were scored as the sperm showing large- or medium-sized haloes of dispersed chromatin

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