Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test

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Objective: To improve the sperm chromatin dispersion (SCD) test and develop it as a simple kit (Halosperm® kit) for the accurate determination of sperm DNA fragmentation using conventional bright-field microscopy.
Design: Method development, comparison, and validation.
Setting: Medical genetics laboratory, academic biology center, and reproductive medicine centers.
Patient(s): Male infertility patients attending the Reproductive Medicine Center. A varicocele patient and a group of nine fertile subjects.
Intervention(s): None.
Main Outcome Measure(s): [1] The quality of chromatin staining in relaxed sperm nuclear halos and tail preservation; [2] SCD scoring reproducibility; [3] comparison with the sperm chromatin structure assay in 45 samples; [4] frequency of sperm with DNA fragmentation after incubation with increasing doses of the nitric oxide donor sodium nitroprusside and in sperm samples for 9 fertile men, 46 normozoospermic patients, 23 oligoasthenoteratozoospermic patients, and a subject with varicocele.
Result(s): The sperm nuclei with DNA fragmentation, either spontaneous or induced, do not produce or show very small halos of DNA loop dispersion after sequential incubation in acid and lysis solution. The improved SCD protocol (Halosperm® kit) results in better chromatin preservation, therefore highly contrasted halo images can be accurately assessed using conventional bright-field microscopy after Wright staining. Moreover, unlike in the original SCD procedure, the sperm tails are now preserved, making it possible to unequivocally discriminate sperm from other cell types. The χ^2 test did not detect significant differences in the mean number of sperm cells with fragmented DNA as scored by four different observers. The intraobserver coefficient of variation for the estimated percentage of spermatozoa with fragmented DNA ranged from 6% to 12%. There was good correlation between the SCD and the sperm chromatin structure assay DNA fragmentation index (intraclass correlation coefficient R: 0.85; percent DNA fragmentation index mean difference: 2.16 significantly higher for SCD). Using the Halosperm®® kit, a dose-dependent increase in sperm DNA damage after sodium nitroprusside incubation was detected. The percentage of sperm cells with fragmented DNA in the fertile group was 16.3 ± 6.0, in the normozoospermic group, 27.3 ± 11.7, and in the oligoasthenoteratozoospermic group, 47.3 ± 17.3. In the varicocele sample, an extremely high degree of nuclear disruption was detected in the population of sperm cells with fragmented DNA.
Conclusion(s): The improved SCD test, developed as the Halosperm® kit, is a simple, cost effective, rapid, reliable, and accurate procedure, for routinely assessing human sperm DNA fragmentation in the clinical andrology laboratory. (Fertil Steril® 2005;84:833–42. ©2005 by American Society for Reproductive Medicine.)

Key Words: Sperm chromatin dispersion test, DNA fragmentation, DNA damage, sperm chromatin structure assay, sperm chromatin

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The integrity of sperm DNA is being recognized as a new parameter of semen quality and a potential fertility predictor (1, 2). However, although DNA integrity assessment appears to be a logical biomarker of sperm quality, it is not being assessed as a routine part of semen analysis in the clinical laboratory (3). Several techniques exist to detect sperm DNA fragmentation, such as the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL), in situ nick translation, comet assay, and sperm chromatin structure assay (SCSA) (1, 4). For the latter, extensive basic and clinical research, mainly on human sperm samples, shows the SCSA as a very powerful technique (5–9). However, some DNA fragmentation techniques, as is the case for the SCSA, require expensive instrumentation for optimal and unbiased analysis, are labor intensive, or require the use of enzymes whose activity and accessibility to DNA breaks may be irregular. As a consequence, some of these procedures are still best suited for research purposes and not for routine diagnostic use in the clinical andrology laboratory.

Recently, we have developed a new procedure for the determination of DNA fragmentation in human sperm cells, called the sperm chromatin dispersion (SCD) test (10). Briefly, intact spermatozoa are immersed in an agarose matrix on a slide, treated with an acid solution to denature DNA that contains breaks, and then treated with lysis buffer to remove membranes and proteins. The agarose matrix allows working with unfixed sperm on a slide in a suspension-like environment. Removal of nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops. Using fluorescent staining, we found that those sperm nuclei with elevated DNA fragmentation produce very small or no halos of DNA dispersion, whereas those sperm with low levels of DNA fragmentation release their DNA loops forming large halos. These results were confirmed by DNA breakage detection-fluorescence in situ hybridization (DBD-FISH), a procedure in which the restricted single-stranded DNA motifs generated from DNA breaks can be detected and quantified (11). Thus, DNA fragmentation as reflected by halo size can be accurately determined using the SCD test, a simple, accurate, highly reproducible, and inexpensive technique.

In the SCD protocol, the sperm nucleoids may be visualized using fluorescence microscopy, after staining with a DNA specific fluorochrome (e.g., 6-diamino-2-phenylindole [DAPI]) or with bright-field microscopy after Diff-Quik (Dade Behring, Switzerland) staining. Fluorescence staining was determined to be much more sensitive for visualizing the DNA and detecting the peripheral limit of the halo. In contrast, Diff-Quik stains the low-density nucleoids more faintly, producing less contrasting images. Thus, the peripheral limit of the halo, where the chromatin is even less dense, may not be accurately discriminated from the background. Lack of contrast can cause mistakes when quantifying the halo size. Thus, it was concluded that the original SCD protocol, although adequate for fluorescence, was not so for bright-field microscopy. Moreover, sperm tails were not preserved, therefore discrimination from other cell types was problematic. The initial SCD protocol has been improved, therefore assessment of sperm cell nuclear halo size and distinction from nongerm cell types may be accurately determined and confidently performed in every basic laboratory of semen analysis using conventional bright-field microscopy.

MATERIALS AND METHODS

SCD Protocol

A new and improved SCD test has been developed, the Halosperm® kit (INDAS laboratories, Madrid, Spain). In brief, an aliquot of a semen sample was diluted to 10 million/mL in phosphate-buffered saline (PBS). Gelled aliquots of low-melting point agarose in eppendorf tubes were provided in the kit, each one to process a semen sample. Eppendorf tubes were placed in a water bath at 90°–100°C for 5 minutes to fuse the agarose, and then in a water bath at 37°C. After 5 minutes of incubation for temperature equilibration at 37°C, 60 mL of the diluted semen sample were added to the eppendorf tube and mixed with the fused agarose. Of the semen–agarose mix, 20 μL were pipetted onto slides precoated with agarose provided in the kit, and covered with a 22- by 22-mm coverslip. The slides were placed on a cold plate in the refrigerator (4°C) for 5 minutes to allow the agarose to produce a microgel with the sperm cells embedded within. The coverslips were gently removed and the slides immediately immersed horizontally in an acid solution, previously prepared by mixing 80 μL of HCl from an eppendorf tube in the kit with 10 mL of distilled water and incubated for 7 minutes. The slides were horizontally immersed in 10 mL of the lysing solution for 25 minutes. After washing 5 minutes in a tray with abundant distilled water, the slides were dehydrated in increasing concentrations of ethanol (70%, 90%, 100%) for 2 minutes each and then air-dried.

Slides may be stored at room temperature for several months in a tightly closed box in the dark, stained immediately for fluorescence microscopy using DAPI (2 μg/mL) (Roche Diagnostics, Barcelona, Spain) in Vectashield (Vector Laboratories, Burlingame, CA), stained for bright-field microscopy, or incubated with a whole genome probe for DBD-FISH, as previously described (10, 11). For bright-field microscopy in the improved SCD test (Halosperm® kit), slides were horizontally covered with a mix of Wright's staining solution (Merck, Darmstadt, Germany) and PBS (Merck) (1:1) for 5–10 minutes with continuous airflow. Slides were briefly washed in tap water and allowed to dry. Strong staining is preferred to easily visualize the periphery of the dispersed DNA loop halos. The distilled water, ethanol, Wright staining solution (Merck 1.01383.0500), and PBS (Merck 1.07294.1000) are not provided in the kit. However, these reagents are inexpensive and easy to obtain.

For this study, a minimum of 500 spermatozoa per sample were scored under the ×100 objective of the microscope. Sperm samples for 9 fertile men, 46 normozoospermic patients, 23 oligoasthenoteratozoospermic patients, and a sub-