

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 μ L 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 μ L 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 μ g/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 $\times 100$ objective of the microscope. Sperm samples for 9 fertile men, 46 normozoospermic patients, 23 oligoasthenoteratozoospermic patients, and a sub-

ject with varicocele were analyzed with the SCD protocol. The semen samples were evaluated according to the World Health Organization criteria (12). Institutional Review Board approval was not available for the private clinics. Patients provided informed consent to use the specimen that otherwise would have been discarded.

Analysis of Variation in Scoring

To determine the intraobserver variability in scoring, the same technician scored the DNA fragmentation yield six times from each sample (i.e., three times a day for 2 different days). This procedure was performed by four different technicians. The interobserver variability in scoring was determined by comparing the mean DNA fragmentation level obtained from the same sample by the four different technicians.

Comparison With the SCSA

A comparison between SCD and SCSA was performed in a group of 45 semen samples from patients attending the Reproductive Medicine Center at the University of Minnesota after providing informed consent. The SCSA procedure has been previously described (13).

Analysis of DNA Damage Induced by Sodium Nitroprusside

Aliquots of fresh sperm samples from three different subjects were exposed to increasing concentrations of the nitric oxide donor sodium nitroprusside (SNP) (0, 60, 125, 250, and 500 μM) for 1 hour at room temperature and processed by the SCD technique, as described. The different doses were prepared in the same microgel slide for each individual.

Statistical Analysis

The interobserver variation in scoring by four different technicians was evaluated with the Pearson's χ^2 test. The concordance between SCD and SCSA was analyzed using the Altman methodology from the SPSS software (SPSS Inc., Chicago, IL) and two-tailed Student's *t* test. The comparison between the fragmentation level in a group of 9 fertile subjects, 46 normozoospermic patients, and 23 oligoasthenoteratozoospermic patients was performed using the Mann-Whitney *U* test ($P < .05$).

RESULTS

Technical Improvements With the New SCD Protocol

The SCD protocol, described previously (10), was good for assessment of halos using fluorescence microscopy but was inadequate for bright-field microscopy after Diff-Quik staining. The new SCD protocol, developed as the Halosperm[®] kit, is much less aggressive, resulting in very good preservation of the chromatin, keeping a higher density of material, therefore the halos can be accurately stained and assessed using conventional bright-field microscopy with Wright

staining. Moreover, unlike the previous SCD procedure, with the new SCD procedure the sperm tails remain intact. Therefore, discrimination from other possible cell types can be easily accomplished. Based on the results of simultaneous DBD-FISH on the same sperm cell, the scoring criteria by eye was established analyzing the relative halo size in simple linear measures (i.e., comparing the halo width with the minor diameter of the core from the same nucleoid) (Fig. 1). The relative parameter avoids the possible errors in estimation of the halo size due to differences in the absolute size of the sperm heads. Thus, although a bigger than normal sperm nucleus might have a high-to-medium absolute halo size, it really might be a small halo in relation to its core.

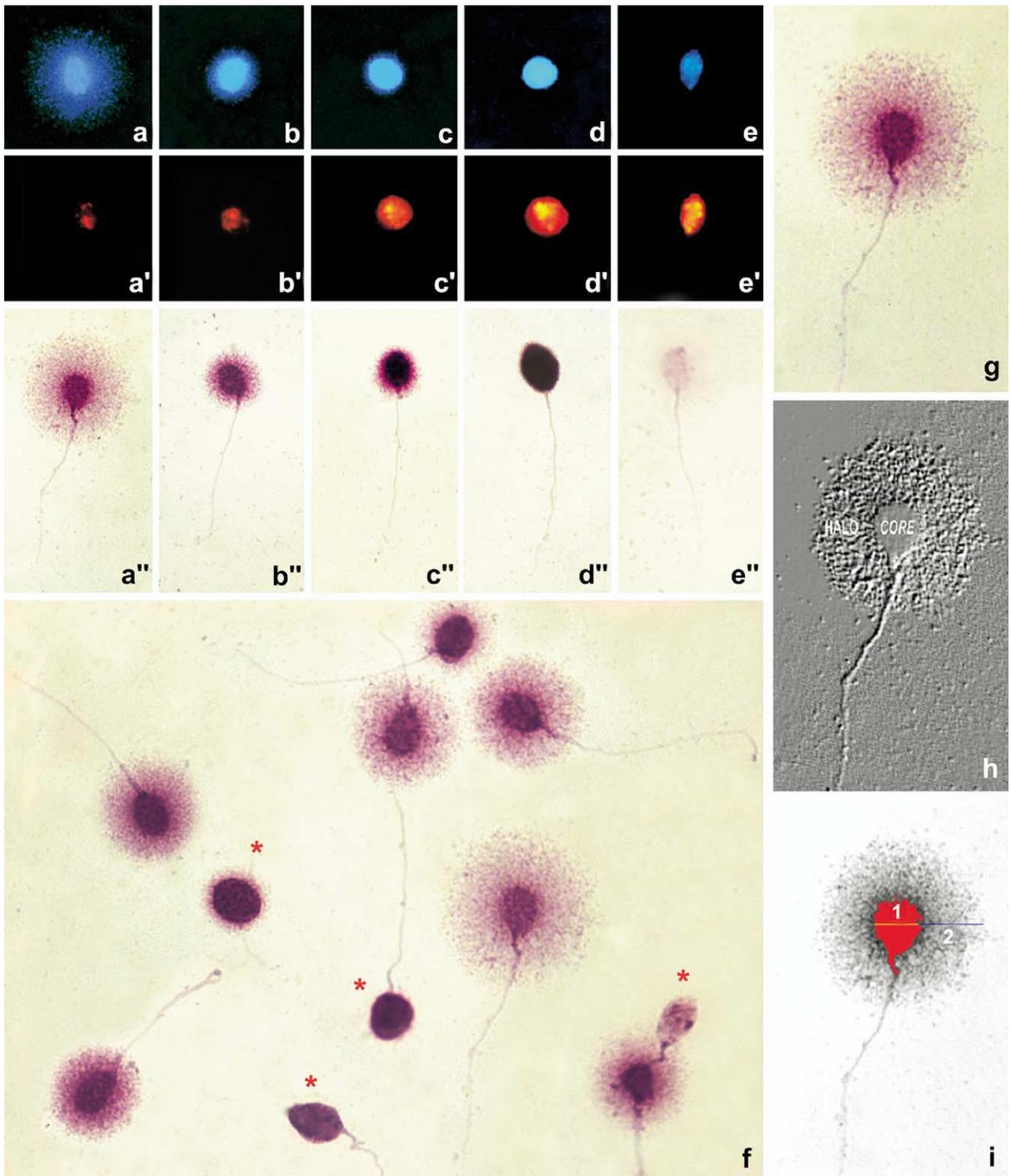
Five SCD patterns were established (Fig. 1): [1] sperm cells with large halos: those whose halo width is similar or higher than the minor diameter of the core; [2] sperm cells with medium-sized halos: their halo size is between those with high and with very small halo; [3] sperm cells with very small-sized halo: the halo width is similar or smaller than one-third of the minor diameter of the core; [4] sperm cells without a halo; and [5] sperm cells without a halo and degraded: similar to [4] but weakly or irregularly stained. The latter nucleoid appearance was rarely observed using the old SCD protocol. These nucleoids tend to spread in small fragments, and are sometimes difficult to detect and relate to the spermatozoa. They probably represent a greater level of DNA and nuclear degradation, and compromise of the nuclear matrix. Finally, nucleoids that do not correspond to sperm cells are separately scored. They are usually big nucleoids, without a tail, and that may correspond either to spermatids, epithelial cells, leukocytes, or other nongerm cell. Comparison of halo size using fluorescence microscopy with DAPI staining and simultaneous DBD-FISH signal demonstrates that sperm cells with very small halos, without halos, and without a halo and degraded, contain fragmented DNA (Fig. 1) (10).

Variations in Scoring

To determine an estimation of the interobserver variability in scoring of sperm cells with fragmented DNA, an aliquot of a single frozen semen specimen was distributed to three different laboratories and processed using the new SCD. Four technicians analyzed 500 sperm cells three times a day on 2 different days. Analysis of 500 sperm cells is usually performed in 3–5 minutes by experienced individuals and in no more than 10 minutes for less experienced technicians. Although the time could be reduced by increasing the cell number, analyzing 500 cells supposes an error of 4.4%, which may be acceptable. The results are presented in Table 1.

The χ^2 test did not detect significant differences ($P > .05$) between the mean of sperm cells with fragmented DNA observed among the four technicians. The intraobserver variability in scoring was estimated from the six scores for each technician. The coefficient of variation for the estimated

FIGURE 1



Fernández. Improved sperm chromatin dispersion (SCD) test. *Fertil Steril* 2005.

FIGURE 1 Continued

Nucleoids from human sperm cells obtained with the improved SCD procedure. (a to e) DAPI staining for fluorescence microscopy. (a' to e') Sequential DBD-FISH with a whole genome probe, to demonstrate DNA breakage. (a'' to e'') Wright staining for bright-field microscopy. (a, a', a'') Nucleoids with big halo of DNA dispersion. (b, b', b'') Nucleoids with medium-sized halo. (c, c', c'') Nucleoids with small halo size. (d, d', d'') Nucleoids without halo. (e, e', e'') Nucleoids without halo and degraded. According to the DBD-FISH signal, those nucleoids with small halo, without halo, and without halo and degraded, contain fragmented DNA. (f) Microscopic field visualized after Wright staining. Those sperm cells with fragmented DNA are indicated by an asterisk. (g to i) Besides the preservation of the tails, the improved SCD protocol allows for a better chromatin staining, obtaining highly contrasting images for bright-field microscopy (g), where the core and the periphery of the halo are well delimited (h). (i) The estimation of the halo size is established by comparison of the halo width (2) with the minor diameter of the core (1), as described in the text. See text for abbreviations.

percentage of spermatozoa with fragmented DNA ranged from 6% to 12% for the different technicians.

Comparison With the SCSA

The SCSA is considered as perhaps the gold standard procedure to analyze sperm DNA fragmentation. A com-

parison between SCD and SCSA was performed in a group of 45 semen samples from patients attending the Reproductive Medicine Center at the University of Minnesota (Fig. 2). Semen samples were simultaneously processed for SCD and SCSA. Concordance between the two techniques was very high (intraclass correlation coefficient R: 0.85), as determined using Altman analysis from

TABLE 1

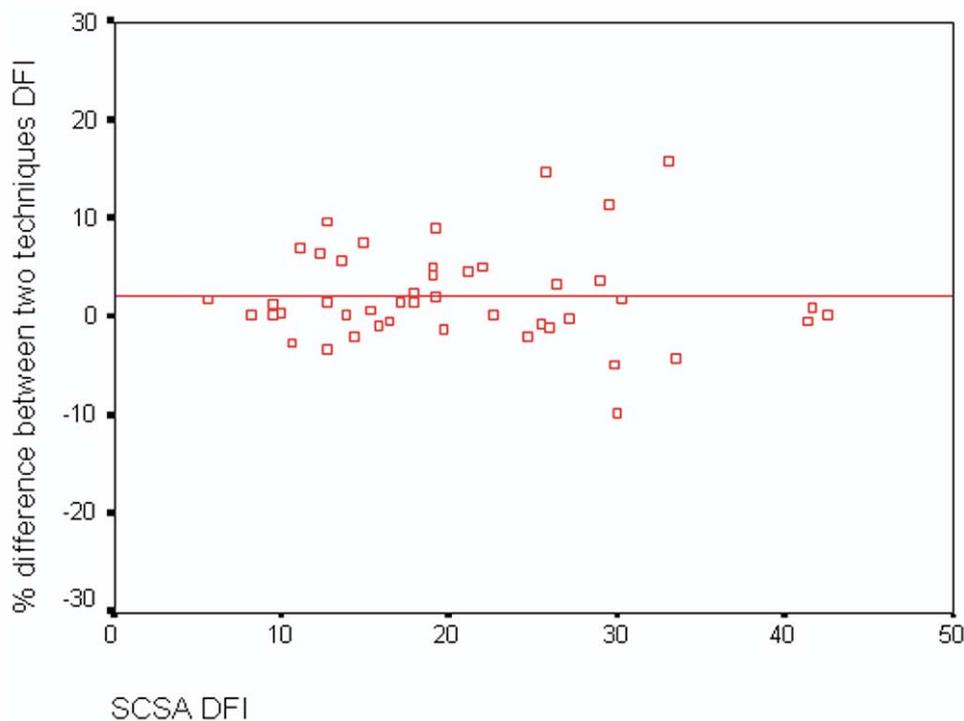
Recorded data scored by four different observers, from aliquots from the same sperm sample processed with the SCD kit.

Subject	Scoring day	% Big halo	% Medium halo	% Small halo	% Without halo	% Degraded	% Fragmented
1	1	64.2	21.0	8.6	2.6	2.2	13.4
		66.2	18.6	9.0	2.8	2.2	14.0
		68.6	18.2	7.0	3.0	2.2	12.2
	2	68.0	16.8	9.4	2.6	3.2	15.2
		70.8	13.4	9.4	5.0	1.4	15.8
		67.6	15.0	10.6	4.6	2.2	15.8
2	1	76.8	7.8	8.8	5.3	0.5	14.5
		72.6	10.8	8.0	7.0	1.2	16.2
		75.0	8.8	8.0	6.2	2.0	16.2
	2	74.5	9.0	7.8	5.5	1.3	14.5
		73.6	9.2	8.6	7.2	0.8	16.6
		72.0	12.6	8.8	4.0	2.0	14.8
3	1	74.8	9.2	9.2	6.4	0.4	16.0
		75.4	10.8	8.0	5.4	0.2	13.6
		76.4	8.4	9.2	3.4	2.2	14.8
	2	76.8	8.4	6.4	6.4	1.8	14.6
		76.4	8.0	8.6	4.8	2.0	15.4
		77.2	8.6	7.8	5.6	0.6	14.0
4	1	76.4	9.2	6.6	7.0	0.8	14.4
		82.0	3.2	4.4	7.4	3.0	14.8
		84.6	2.8	4.4	6.6	1.6	12.6
	2	72.9	8.8	8.6	7.6	1.3	17.5
		81.2	4.0	9.4	5.0	0.4	14.8
		84.0	3.0	6.6	5.2	1.2	13.0

Fernández. Improved sperm chromatin dispersion (SCD) test. *Fertil Steril* 2005.

FIGURE 2

Altman plot comparing DNA fragmentation obtained using the SCD test and SCSA. The x axis represents the DNA fragmentation obtained with the SCSA (range 0–50), whereas the y axis represents the percentage of the difference in the DNA fragmentation between both techniques. Each point represents a sample analyzed by both procedures.



Fernández. Improved sperm chromatin dispersion (SCD) test. *Fertil Steril* 2005.

the SPSS software (Fig. 2). The SCD test tended to estimate a slightly higher DNA fragmentation index level, the percent DNA fragmentation index mean difference being 2.16 (95% confidence interval [CI]: 0.69–3.63, two-tailed Student's *t* test, $P < .005$).

Detection of Radical-Induced DNA Damage

To demonstrate the sensitivity of the new SCD test, aliquots of sperm samples from three different subjects were exposed to increasing concentrations of the nitric oxide donor SNP, for 1 hour, at room temperature to induce DNA damage. A dose–response effect by SNP on DNA fragmentation was detected starting at a dose of 60 μM . Lower doses did not produce a detectable increase.

Two individuals (subjects 1 and 2), with relatively low spontaneous DNA fragmentation, showed a similar increase in DNA fragmentation in response to increasing doses of SNP, achieving nearly 75% with 500 μM (Table 2). In the case of subject 3, whose spontaneous background DNA fragmentation level was 52%, the 500 μM dose induced DNA fragmentation in almost the entire sperm population. In this subject, the increase was prac-

tically dependent on those sperm without halos, whereas in the other two individuals it was related to sperm with both small and no halos (Table 2). Because the DBD-FISH procedure detects a higher DNA breakage level in those sperm nuclei without halos compared to those with small halos (10), the degree of induced DNA damage should be greater in the subject with the higher spontaneous background DNA fragmentation level. In all three cases, no increase in the percentage of sperm without halo and degraded was detected after SNP treatment.

Clinical Application to Semen Samples

In addition to the microgel for the subject to be studied (test), a microgel for a semen sample with known DNA fragmentation frequency was simultaneously processed on the same slide (control). A group of men with proven fertility ($n = 9$) was compared to a group of normozoospermic ($n = 46$) and oligoasthenoteratozoospermic ($n = 23$) men. The percentage of sperm cells with fragmented DNA in the fertile group ranged from 5.2% to 23.0% (mean = 16.3 ± 6.0) (Table 3). This percentage was significantly ($P = .008$) increased in the normozoospermic patients (mean: 27.3 ± 11.7 ; range: 8.6–51.8), and even more significantly ($P < .000001$) in the oli-

TABLE 2

Induction of DNA damage with increasing doses of the no-halos donor (SNP) in sperm samples from three individuals, analyzed by the SCD test.

Subject	SNP (μ M)	% Big halo	% Medium halo	% Small halo	% Without halo	% Degraded	% Fragmented
1	0	61.8	14.1	12.0	12.1	0.0	24.1
	60	38.0	23.2	19.6	19.2	0.0	38.8
	125	21.8	24.2	32.6	23.1	0.0	55.7
	250	14.9	19.0	40.3	30.1	0.0	70.4
	500	3.8	22.5	39.6	34.1	0.0	73.7
2	0	46.8	17.4	17.2	13.4	5.2	35.8
	60	40.6	15.4	26.0	11.6	6.4	44.0
	125	21.6	24.5	21.6	21.5	10.7	53.8
	250	18.3	20.4	28.1	24.6	8.7	61.3
	500	7.0	16.2	32.8	30.4	13.6	76.8
3	0	37.2	11.0	14.4	22.2	15.2	51.8
	60	23.5	13.0	22.6	27.4	13.5	63.5
	125	7.6	14.4	33.6	32.2	12.2	78.0
	250	3.0	7.6	21.8	52.8	14.8	89.4
	500	2.2	3.8	12.8	68.0	13.2	94.0

Fernández. Improved sperm chromatin dispersion (SCD) test. Fertil Steril 2005.

goasthenoteratozoospermic group (mean: 47.3 ± 17.3 ; range: 23.2–85.8) (Table 3).

An infertile individual with varicocele was assessed. The semen sample from the individual with varicocele contained 63.8% DNA fragmentation and 40.8% of cells that were not spermatozoa. Moreover, 59.2% of the sperm cells with fragmented DNA corresponded to those without halo and degraded (Fig. 3). In contrast, this same category of nuclear damage was very low in the fertile control group (it constituted 0–9.5% of the total of fragmented cells), and even in the normozoospermic and oligoasthenoteratozoospermic patients (mean 10.4 ± 9.6 and 12.1 ± 10.5 ; range: 0.0–37.0 and 0.0–42.6, respectively).

DISCUSSION

Any technique to analyze sperm DNA fragmentation in any clinical andrology or assisted reproductive technology

(ART) laboratory should be simple, reproducible, and preferably without the need for new, complex, or expensive instrumentation (3). Thus, the possibility for DNA assessment using conventional bright-field microscopy should be globally applicable. To this purpose, the SCD test has been improved and developed as the Halosperm[®] kit.

The previously published SCD protocol was adequate for fluorescence microscopy, which is more sensitive than bright-field microscopy for DNA visualization (10). Although halos could be seen using the Diff-Quik dye and bright-field microscopy, the staining results are very weak and with poorly contrasting images. As a consequence, due to an inadequate delimitation of the peripheral border of the halo, mistakes can easily be made when measuring the halo size. For example, small halos observed with Diff-Quik may really be medium-sized halos. Therefore, the DNA fragmentation level could be overestimated. With the new SCD protocol, chromatin

TABLE 3

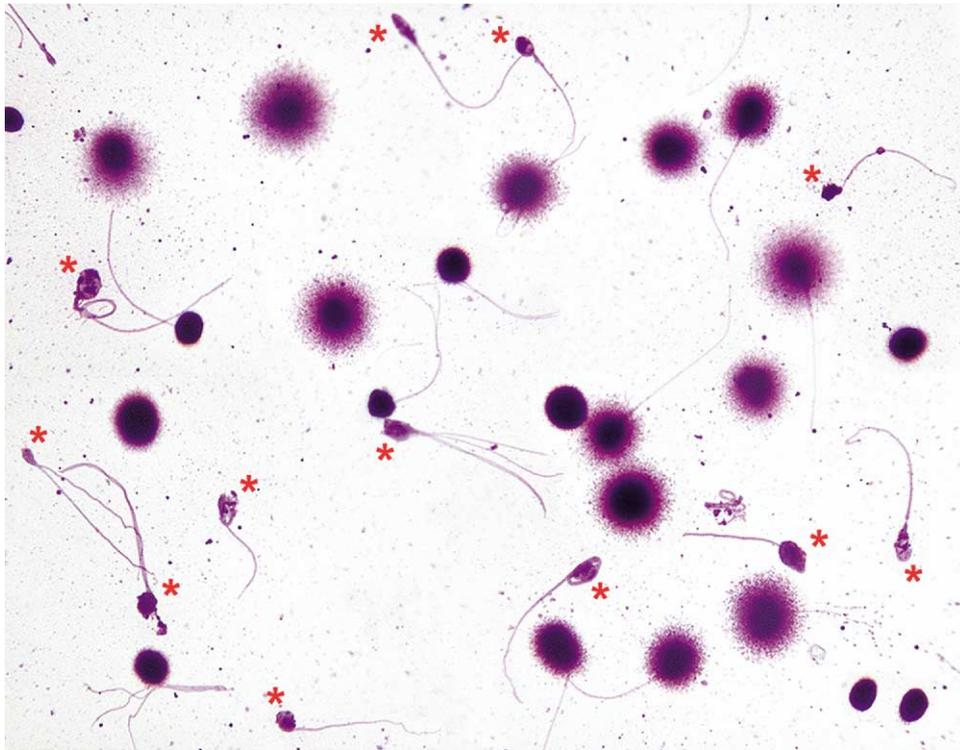
SCD data (mean \pm standard deviation) from semen samples from fertile subjects (F), normozoospermic patients (N), and oligoasthenoteratozoospermic patients (OAT).

Semen samples	% Big halo	% Medium halo	% Small halo	% Without halo	% Degraded	% Fragmented
F (n = 9)	73.8 ± 11.6	9.7 ± 8.3	6.9 ± 2.9	8.7 ± 5.0	0.6 ± 0.5	16.3 ± 6.0
N (n = 46)	65.1 ± 12.9	7.6 ± 5.7	11.1 ± 5.3	13.1 ± 8.2	3.1 ± 3.6	27.3 ± 11.7
OAT (n = 23)	45.0 ± 16.7	7.7 ± 4.2	17.1 ± 8.3	24.4 ± 15.5	5.8 ± 6.2	47.3 ± 17.3

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FIGURE 3

The SCD test in an individual with varicocele. The frequency of sperm cells without halo and degraded (asterisks) is high, reflective of a very high degree of nuclear damage.



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is very well preserved, remaining a higher density material. Therefore, the halos can be accurately stained and assessed using conventional bright-field microscopy after Wright staining. Moreover, unlike in the previous SCD procedure, with the new SCD procedure the sperm tails remain intact; discrimination from other possible cell types can be easily accomplished.

The sequential DBD-FISH procedure to label DNA breaks shows that sperm cells with very small halos, without halos, and without halos and degraded, that contain fragmented DNA (Fig. 1) (10). The categorization of the different halo sizes is simple: use the minor diameter of the core from the nucleoid as a reference to which the halo width is compared. Nevertheless, the use of this relative parameter is generally not necessary and needs only to be applied when doubts exist. Therefore, scoring of 500 sperm samples can usually be accomplished in 3–10 minutes, depending on the sperm cell concentration and experience of the technician.

Moreover, the intraindividual and interindividual variability in scoring of DNA fragmentation with the improved SCD was quite low, reflecting the ease and reliability of the test end points as assessed by the technicians (Table 1). There was some individual observer trends in scoring sperm with medium-sized halos. For example, observer 1 tended to score medium-sized halos with higher frequency, whereas

observer 4 scored them with lower frequency. Moreover, observer 1 also tended to estimate slightly higher levels of sperm with degraded nucleoids, decreasing those with small halo. Importantly, these small discrepancies do not affect the overall fragmentation index and may be easily reconciled with training and proficiency testing. Although visual scoring is clearly precise, the small interindividual variations may be overcome using image analysis software integrated with a microscope with an automated stage and focus and coupled with an image capture system. This configuration is actually in progress and should allow the accurate scoring of thousands of sperm cells in only a few minutes.

A comparison of the DNA fragmentation index estimated by the SCSA and the percentage of sperm cells with fragmented DNA detected by the SCD test was performed in 45 samples. The results showed that the SCD test is in good concordance with the gold standard procedure to determine sperm DNA fragmentation (intraclass correlation coefficient $R: 0.85$). Interestingly, the SCD test seems to have a slight but significantly higher sensitivity for detecting sperm DNA fragmentation (2.16% mean difference) than the SCSA (Fig. 2). The results suggest that the SCD test could be a good and cost-effective alternative to the SCSA.

Incubation of sperm cells in vitro with different sources of reactive oxygen species results in an increase in DNA fragmentation (14). Of the reactive oxygen species, nitric oxide has been shown to be an important regulator of several physiological processes. In sperm, nitric oxide may be produced during capacitation and the acrosome reaction (15). Alternatively, in some cases (e.g., inflammatory and infectious diseases) the concentration of this radical may be increased, producing a cytotoxic effect. Nitric oxide may react with the superoxide radical yielding peroxynitrite, a potent oxidant that induces DNA damage (16). Thus, to demonstrate the sensitivity of the SCD test to detect induced DNA damage, aliquots of sperm samples from three different subjects were exposed to increasing concentrations of the nitric oxide donor SNP. A dose-response in the frequency of sperm cells with fragmented DNA was evident (Table 2).

In addition to the application to detect in vitro-induced DNA damage, a preliminary trial to assess clinical semen samples was performed with the improved SCD procedure. It was found that infertile patients had higher percentages of sperm cells with fragmented DNA than fertile controls (Table 3), as also reported in several articles (17–19). Furthermore, sperm quality seemed related to the DNA fragmentation levels (20–22). Therefore, oligoasthenoteratozoospermic samples contain a higher level of DNA fragmentation than those from normozoospermic patients.

The discrimination of different degrees of DNA fragmentation is an interesting ability of the SCD test. The increase in DNA fragmentation after SNP treatment may be at expense of different nucleoids containing fragmented DNA, and depends on the individual subject (Table 2). In all three individuals, the increase of sperm cells with fragmented DNA was at the expense of those with small halos and those without halos. Nevertheless, the other category of sperm nucleoid with fragmented DNA (i.e., those sperm cells without halos and degraded) did not increase with the different SNP doses. Perhaps this yield of extreme DNA damage could not be produced by this radical attack, at least initially, and may probably be associated to nuclear matrix degradation. Discrimination of this kind of damage is a clear advantage of the improved SCD procedure, because this damage cannot be detected using the other DNA fragmentation techniques, such as the TUNEL, comet, and SCSA. Of interest clinically is the subject with varicocele from whom more than half (59.2%) of the sperm cells contained fragmented DNA; he displayed this extreme degree of DNA nuclear damage (Fig. 3). A group of varicocele patients is actually being assessed with the Halosperm® kit; preliminary data confirm the high frequency of degraded cells. Thus, the improved SCD test allows for the detection of an extreme degree of DNA damage that possibly affects nuclear structure and that could not be detected using the other DNA damage techniques (23).

In conclusion, any technique to analyze sperm DNA fragmentation in clinical andrology or ART laboratories should be

simple, reproducible, and preferably without the need for new, complex, or expensive instrumentation (3). Thus, the possibility for DNA assessment using conventional bright-field microscopy should be universally applicable. The results of this study show that the new improved SCD test, developed as the Halosperm® kit, is a simple, fast, accurate, and highly reproducible method for the analysis of sperm DNA fragmentation.

This new version results in a better preservation of the chromatin; the halo size can be confidently estimated with conventional bright-field microscopes using the Wright stain. Moreover, different degrees of DNA nuclear damage can be detected as well as the discrimination of nucleoids from other cell types. There is a very good correlation between the results from the Halosperm® kit and SCSA. Unlike the SCSA, the Halosperm® kit can be used without the requirement of complex or expensive instrumentation. However, if desired, the Halosperm® kit can be used with automation. Finally, laboratory technicians can easily, quickly, and reliably assess the test end points. Therefore, the improved SCD test could allow for the routine screening of sperm DNA fragmentation in the basic andrology laboratories.

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