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% ± 14.0%) than the TUNEL assay (11.5% ± 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

surrounding a compact and well-defined core. Sperm with damaged DNA (SCD-positive cells) showed small haloes of dispersed chromatin or no haloes (Supplemental Fig. 1). The percentage of SCD-positive sperm was calculated and reported as the percentage of cells exhibiting damaged DNA. A minimum of 400 sperm was assessed per specimen.

Sperm DNA evaluation using the studied methods was carried out by a single experienced senior technician, with more than 4 years' expertise in performing sperm DNA damage using the TUNEL assay. This same technician has undergone prior training with the SCD test, as provided by Halotech DNA. Each run was carried out in duplicate with an appropriate experimental positive and negative control using patient specimens. For SCD, positive controls were made by treating specimens with 50 μL of H_2O_2 before immersing slides into the denaturation solution. Negative controls were created by omitting the lysis step from the protocol. For TUNEL, positive control slides were made by adding 1 μL of DNase I, which generates DNA breaks in most cells, to the staining solution. In TUNEL-negative controls, the TdT enzyme was omitted from the labeling solution. In addition, frozen–thawed specimens from patients with negative and positive DNA damage were included as internal controls in each run. Categorization of SCD and TUNEL-positive sperm was performed on the fixed specimens in a blind manner (ie, a technician who was not involved in conducting the experiment masked the slides, to withhold information from the reader).

Statistical Analysis

Sperm DNA damage data were assessed for homogeneity and normal distribution by the Kolgomorov-Smirnov test, and results were expressed as mean ± standard deviation, median, and minimum and maximum values. Comparison of means was performed by the paired *t* test. The TUNEL assay and SCD test were compared for the detection of DNA damage using Spearman's rank correlation. For these tests, the significance level was .05. Receiver operating characteristic (ROC) analysis was performed using four different TUNEL thresholds (4%, 10%, 12%, and 19%) to select the optimal cutoff points of SCD to discriminate between patients with normal and abnormal levels of sperm DNA damage, as determined by the TUNEL assay. Accuracy of all possible SCD cutoff points was determined by calculating the area under the curve (AUC). Sensitivity, specificity, and positive (PPV) and negative (NPV) predictive values were also calculated for all possible SCD cutoff points using the TUNEL thresholds. The 95% confidence intervals (CIs) of AUC, sensitivity, and specificity were used to quantify uncertainty. Statistical analysis of the data was performed by using the Statistical Analysis System (SAS Institute), version 9.2.

RESULTS

The proportion of sperm with damaged DNA, as assessed by SCD and TUNEL, is shown in Table 1. The SCD test detected a significantly higher proportion of sperm with damaged DNA (20.6% ± 14.0%) than the TUNEL assay (11.5% ± 7.3%; P=.001). The relationship between the SCD test and the TUNEL assay, as assessed by Spearman's rank correlation, was 0.29 (P=.25; Fig. 1).

TABLE 1

Proportion of spermatozoa with DNA damage, as assessed by SCD and TUNEL, in 20 ejaculates obtained from men with unexplained infertility.

Method of DNA damage assessment	Mean[a]	Standard deviation	Median	Minimum	Maximum
SCD	20.6	14.0	16.5	7.0	66.0
TUNEL	11.5	7.3	10.5	2.0	29.0

[a] Paired *t* test = 0.001 for comparison of means.

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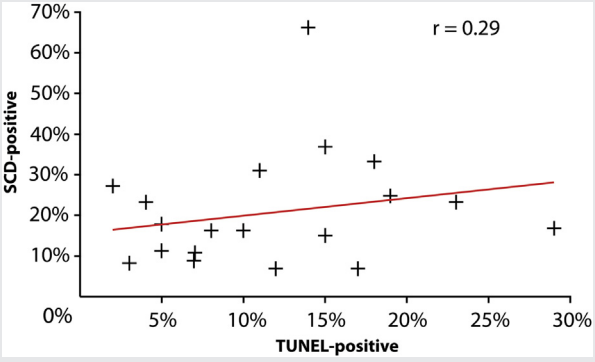
Receiver operating characteristic analysis showed that the SCD cutoff point of 15% yielded the largest AUC (0.687; 95% CI 0.522–0.753) when the TUNEL threshold was set to 10%, with a sensitivity, specificity, PPV, and NPV of 82%, 56%, 69%, and 71%, respectively (Fig. 2). Sensitivity, specificity, PPV, NPV, and accuracy of the optimal SCD cutoff points, as determined by ROC analysis, are presented in Table 2.

Using the cutoff points of 15% and 10% for SCD and TUNEL, respectively, 60% (12 of 20) and 45% (9 of 20) of the patients had ejaculates with an elevated proportion of sperm with DNA damage. In six patients, the results of TUNEL and SCD differed with regard to the diagnostic category assessed, normal or abnormal. In four patients, the results of TUNEL were below the cutoff point, whereas those of SCD were above it. In two patients, contrary results were observed. None of the patients were excluded because of inadequate or failed staining and undetermined or missing results. The tabulation of the results of the SCD test and the TUNEL assay is shown in Supplemental Table 2.

DISCUSSION

The SCD test and the TUNEL assay are methods to assess sperm DNA integrity. When the SCD test was used in our

FIGURE 1



Relationship between the SCD test and the TUNEL assay for assessing sperm DNA damage in ejaculates of 20 men with unexplained infertility.

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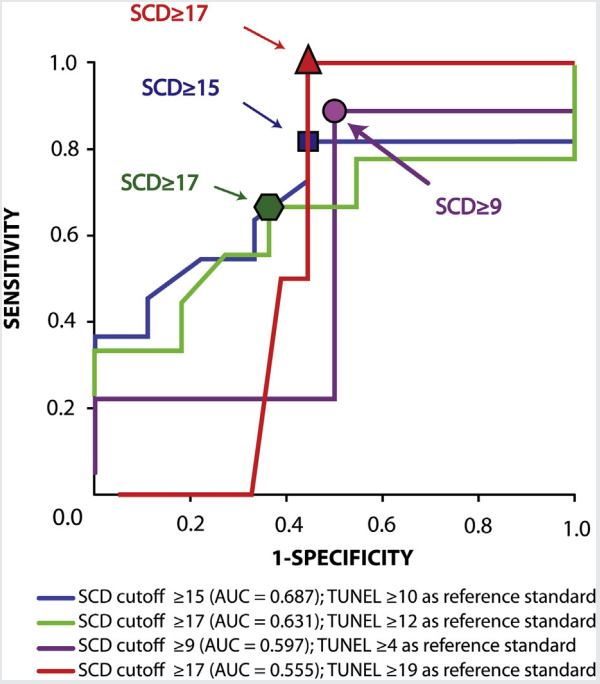
FIGURE 2



Receiver operator characteristic curves showing four different TUNEL thresholds (4%, 10%, 12%, and 19%) to select the optimal cutoff points of SCD to discriminate between patients with normal and abnormal levels of sperm DNA damage. The SCD cutoff points yielding the largest AUC for each TUNEL threshold are presented (*arrows*).

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group of men with unexplained infertility, significantly more sperm with DNA damage was detected compared with the TUNEL assay. In addition, the methods were not comparable in their ability to detect sperm DNA damage, as shown by an observed Spearman's correlation coefficient of 0.29. As a diagnostic method, SCD showed an accuracy of approximately 70% to detect sperm DNA damage within the same category established by TUNEL, normal and abnormal, when cutoff points of 15% and 10% were used for SCD and TUNEL, respectively.

Our results indicate that the SCD method is more sensitive than TUNEL in the evaluation of sperm DNA damage. Additionally, increases in the proportion of DNA-damaged sperm assessed by one method will not necessarily be confirmed by the other. It means that the results obtained by the methods are not interchangeable because they differently assess DNA damage. As a result, andrology laboratories willing to switch from one method to the other should exercise caution because the methods are not comparable. However, logistic regression analysis showed that SCD can be used with moderate accuracy to discriminate between men with abnormal and normal levels of sperm DNA damage, as established by the reference standard, the TUNEL assay. The clinical implication of our findings is that SCD, at a cutoff point of 15%, will correctly identify ejaculates with normal or abnormal levels of DNA damage in up to 69% of the cases, as established by TUNEL using a threshold of 10%. Nevertheless, lower accuracy would be expected if different TUNEL thresholds were adopted.

The TUNEL assay and SCD test are based on different principles and therefore differ in their ability to detect DNA damage (26). The TUNEL assay relies on a terminal transferase that catalyzes the incorporation of modified nucleotides at the site of damage (5′–3′ ends) in a fixed specimen, and it is therefore a method that directly measures DNA damage. In contrast, SCD measures the susceptibility of DNA to denaturation, that is, its principle is to first promote DNA denaturation and then measure DNA damage by detecting the formation of DNA strand breaks (11). The difference in the pattern of forming a loop around lysed and acid-treated nuclear membrane carcass reflects the overall chromatin structure and is used to indirectly measure DNA breaks in the SCD test. Given the fact that probe incorporation in TUNEL depends on the amount of chromatin that is partially freed from the proteins protecting the DNA (27), it is possible that existing breaks are not detected owing to chromatin compaction, thus explaining the observed different levels of sperm DNA damage by the method and the poor correlation between TUNEL and SCD. In fact, modified protocols for TUNEL have been proposed aiming to increase the accessibility of terminal transferase to the sites of DNA breaks (27, 28). In a recent study, Antonucci et al. (28) proposed a novel protocol to improve sperm chromatin accessibility, which involves the use of 0.5% paraformaldehyde, 5 mM 1,4-dithiothreitol, 100 U/mL heparin, and 0.1% Triton-X 100, that might be useful if used in conjunction with the TUNEL assay.

Whereas the denaturation step results in the production of single-strand DNA motifs from pre-existing single- or double-strand breaks, the lysis step differentially removes proteins linked to single- or double-strand DNA stretches,

TABLE 2

Sensitivity, specificity, PPV, and NPV of optimal SCD cutoff points, as determined by ROC analysis, using different TUNEL thresholds for discrimination between normal and abnormal levels of sperm DNA damage.

Optimal SCD cutoff (%)	Accuracy (AUC)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	TUNEL threshold (%)
≥9	0.597 (0.441–0.736)	88.9 (32.5–99.1)	50.0 (35.2–88.6)	94.1	33.3	≥4
≥15	0.687 (0.522–0.753)	81.8 (59.0–92.6)	55.6 (23.1–77.3)	69.2	71.4	≥10
≥17	0.631 (0.508–0.724)	66.7 (35.9–97.5)	63.6 (35.2–92.1)	60.0	70.0	≥12
≥17	0.555 (0.31–0.795)	100.0 (100.0–100.0)	55.6 (32.6–78.5)	20.0	100.0	≥19

Note: Values in parentheses are 95% CIs.

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thus facilitating the detection of DNA damage using the SCD assay (13, 26). These features also explain the higher sensitivity of SCD compared with TUNEL.

In our settings, we take approximately 7.3 hours to conduct a single test, from specimen collection to reporting results, using the TUNEL assay. A maximum of two assessments is scheduled per day, because this represents the limit of tests that can be run in parallel by a single technician. In contrast, we take only 1.7 hours to perform an integral evaluation of sperm DNA damage using the SCD test, and up to four tests can be run concomitantly. As such, it would be advantageous to use SCD as a surrogate for the more complex methods in the evaluation of sperm DNA damage. Nevertheless, conflicting data exist on the correlation among different tests in reporting abnormal levels of sperm DNA fragmentation. In one study, Chohan et al. (29) have shown that SCSA, TUNEL, and SCD techniques had similar predictive values for detecting DNA damage (29). In contrast, Henkel et al. (30) could not corroborate the aforesaid findings and concluded that the assays were not comparable. Our data are in agreement with the latter, which seem sound owing to the different nature of DNA damage assessed by each method.

Thresholds for the SCD test have not yet been determined in men with unexplained infertility. However, a cutoff point of 17% was shown to discriminate pregnant and nonpregnant couples in IVF treatment, with sensitivity and specificity close to 75% (20). To our best knowledge, this study is the first to use a logistic regression model and the TUNEL assay, as the reference standard method, to determine the diagnostic accuracy of SCD to assess sperm DNA damage in men with unexplained infertility. Because different threshold values of 4%, 10%, 12%, and 19% have been reported for the TUNEL assay (15–19), we included these cutoff points in our model to establish the best accuracy of SCD as a diagnostic method. Of note, we observed that 60% and 45% of our study group had elevated levels of sperm DNA damage using the optimal SCD and TUNEL cutoff points of 15% and 10%, respectively, despite normal semen analysis according to the WHO criteria. Men with unexplained infertility have no obvious history of fertility problems, and physical examination, endocrine laboratory testing, and semen analysis results are normal (21, 23). It has been shown that these men have significantly higher levels of DNA damage compared with fertile individuals, which may explain their inability to conceive (5, 6). Hence, assessment of sperm function is of utmost importance in this category of male infertility.

Sperm DNA damage is a broad term that accounts for many defects in the DNA structure, including [1] single or double DNA strand breaks, [2] base deletion or modification, [3] interstrand or intrastrand cross-linkage, and [4] DNA–protein cross-linkage (31). Sperm DNA damage can occur at any level during spermatogenesis, spermiogenesis, and epididymal transit (11). Postmeiotically initiated abortive apoptosis, unresolved strand breaks during spermiogenesis, and oxidative stress have all been implicated as potential sources of this damage (21). Sperm with damaged DNA are released in the semen, and despite the likely result of infertility, these defective cells may still retain the ability to fertilize. Elevated levels of sperm DNA damage have been associated with infertility and repeated intrauterine and IVF failure (1–4). Additionally, offspring generated from such defective sperm may harbor an increased risk of imprinting defects and cancer (32, 33). Notwithstanding, there seems to be an association with increased DNA fragmentation and pregnancy loss after IVF and intracytoplasmic sperm injection (risk ratio 2.16; 95% CI 1.54–3.03; $P<.00001$) (34). Altogether, these considerations argue in favor of using sperm DNA damage testing in the workup of infertile males. Yet at present no single test seems to be reliable enough to detect clinically significant DNA damage with high accuracy to predict the sperm reproductive profile in natural conception, IUI, IVF, or intracytoplasmic sperm injection, thus preventing recommendation of their routine use in the evaluation and treatment of infertile males. Hence there is an urgent need to refine the methods for assessing the sperm DNA integrity and validating their cutoff points in different subsets of patients, as well as in different interventions. Despite these limitations, the results provided by sperm DNA damage assays can be used as additional markers of sperm quality in men with unexplained infertility, because they have been associated with better diagnostic and prognostic value than routine semen analysis (7, 10, 21, 26).

In conclusion, our data indicate that 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 up to 70% accuracy, considering the TUNEL assay as the gold standard for sperm DNA evaluation. Whereas TUNEL depends on a terminal transferase to directly incorporate fluorescent UTP at single and double 3′-OH-free ends, SCD involves the combination of DNA denaturation and depletion of proteins protecting the DNA. Andrology laboratories should distinguish between the methods because they differently evaluate sperm DNA damage.

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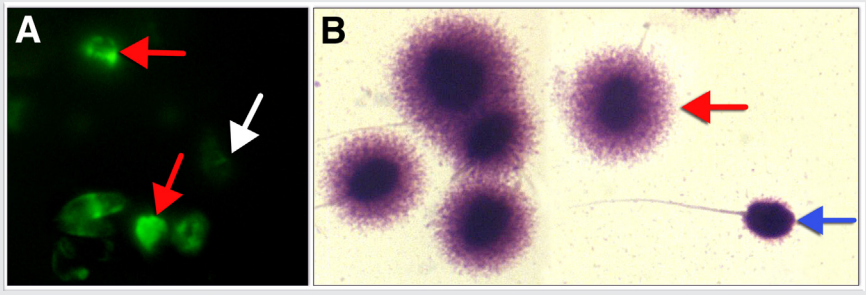
REFERENCES

1. Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil Steril 2000;73:43–50.
2. Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertil Steril 2003;80:895–902.
3. Saleh RA, Agarwal A, Nada ES, El-Tonsy MH, Sharma RK, Meyer A. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril 2003; 79:1597–605.
4. Check JH, Graziano V, Cohen R, Krotec J, Check ML. Effect of an abnormal sperm chromatin structural assay (SCSA) on pregnancy outcome following (IVF) with ICSI in previous IVF failures. Arch Androl 2005;51:121–4.
5. Host E, Lindenberg S, Ernst E, Christensen F. DNA strand breaks in human spermatozoa: a possible factor, to be considered in couples suffering from unexplained infertility. Acta Obstet Gynecol Scand 1999;78:622–5.

6. Saleh RA, Agarwal A, Nelson DE, Nada EA, El-Tonsy MH, Alvarez JG, et al. Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. Fertil Steril 2002;78:313–8.
7. Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil Steril 2010;93: 1027–36.
8. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, et al. Utility of the sperm chromatin assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 1999;14:1039–49.
9. Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, et al. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. Hum Reprod 2005;20:226–30.
10. Bungum M, Bungum L, Giwercman A. Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. Asian J Androl 2011;13:69–75.
11. Shamsi MB, Imam SN, Dada R. Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in management of infertility. J Assist Reprod Genet 2011;28:1073–85.
12. Fernández JL, Muriel L, Goyanes V, Segrelles E, Gosálvez J, Enciso M, et al. Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion (SCD) test. Fertil Steril 2005;84:833–42.
13. Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 2003;24:59–66.
14. Gosálvez J, Rodríguez-Predreira M, Mosquera A, López-Fernández C, Esteves SC, Agarwal A, et al. Characterisation of a subpopulation of sperm with massive nuclear damage, as recognised with the sperm chromatin dispersion test. Andrologia. 2013 May 26. http://dx.doi.org/10.1111/and.12118. [Epub ahead of print].
15. Sharma RK, Sabanegh E, Mahfouz R, Gupta S, Thiyagarajan A, Agarwal A. TUNEL as a test for sperm DNA damage in the evaluation of male infertility. Urology 2010;76:1380–6.
16. Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod 2006;21:2876–81.
17. Benchaib M, Lornage J, Mazoyer C, Lejeune H, Salle B, Francois Guerin J. Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. Fertil Steril 2007;87:93–100.
18. Huang CC, Lin DP, Tsao HM, Cheng TC, Liu CH, Lee MS. Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. Fertil Steril 2005;84:130–40.
19. Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertil Steril 2004;82:378–83.
20. Nuñez-Calonge R, Caballero P, López-Fernández C, Guijarro JA, Fernández JL, Johnston S, et al. An improved experimental model for understanding the impact of sperm DNA fragmentation on human pregnancy following ICSI. Reprod Sci 2012;19:1163–8.
21. Hamada A, Esteves SC, Nizza M, Agarwal A. Unexplained male infertility: diagnosis and management. Int Braz J Urol 2012;38:576–94.
22. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: WHO Press; 2010.
23. Sigman M, Lipshultz L, Howard S. Office evaluation of the subfertile male. In: Lipshultz LI, Howards SS, Niederberge CS, editors. Infertility in the male. 4th ed. Cambridge, UK: Cambridge University Press; 2009:153–76.
24. Esteves SC, Bento FC. Implementation of air quality control in reproductive laboratories in full compliance with the Brazilian Cells and Germinative Tissue Directive. Reprod Biomed Online 2013;26:9–21.
25. Esteves SC, Agarwal A. Ensuring that reproductive laboratories provide high-quality services. In: Bento FC, Esteves SC, Agarwal A, editors. Quality management in ART clinics. 1st ed. New York: Springer US; 2013:129–46.
26. Gosálvez J, López-Fernández C, Fernández JL. Sperm chromatin dispersion (SCD) test: technical aspects and clinical applications. In: Zini A, Agarwal A, editors. Sperm DNA damage: biological and clinical applications in male infertility and assisted reproduction. 1st ed. New York: Springer US; 2011:151–70.
27. Mitchell LA, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. Int J Androl 2011;34:2–13.
28. Antonucci N, Manes S, Corradetti B, Manicardi GC, Borini A, Bizzaro D. A novel in vitro sperm head decondensation protocol for rapid flow cytometric measurement of deoxyribonucleic acid content. Fertil Steril 2013;99:1857–61.
29. Chohan KR, Griffin JT, Lafromboise M, De Jonge CJ, Carrell DT. Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. J Androl 2006;27:53–9.
30. Henkel R, Hoogendijk CF, Bouic PJ, Kruger TF. TUNEL assay and SCSA determine different aspects of sperm DNA damage. Andrologia 2010;42: 305–13.
31. Aitken RJ, de Iullis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. Int J Androl 2009;32:46–56.
32. Aitken RJ, Koopman P, Lewis SE. Seeds of concern. Nature 2004;432:48–52.
33. Zini A, Meriano J, Kader K, Jarvi K, Laskin CA, Cadesky K. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. Hum Reprod 2005;20:3476–80.
34. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod 2012;27:2908–17.

SUPPLEMENTAL FIGURE 1



Assessment of DNA damage in sperm. (A) TUNEL assay. Sperm showing bright apple-green fluorescence represent cells with damaged DNA (*red arrows*), in which dUTP is incorporated to DNA breaks, in contrast to non–DNA-damaged sperm (*white arrows*) that have been first identified using DAPI staining (not shown). Sperm photomicrographs obtained at ×1,000 using a fluorescence microscope (Eclipse E600; Nikon) equipped with an epi-illumination module and a mercury ultraviolet source. Filter B2A was used for FITC-dUTP. (B) SCD test. Nucleoids obtained with the improved SCD procedure (Halosperm; Halotech DNA) under bright-field microscopy and Wright's stain. The *red arrow* indicates a spermatozoon containing a normal DNA molecule (a halo of dispersed chromatin is seen). The *blue arrow* indicates a spermatozoon with damaged DNA molecule (an absent halo of dispersed chromatin is seen). Microphotographs obtained at ×1,000 using bright-field microscopy (Alphaphot-2 YS; Nikon).

Feijó. SCD accuracy in unexplained male infertility. Fertil Steril 2014.

SUPPLEMENTAL TABLE 1

Distribution of semen characteristics of 20 men with unexplained infertility.

Semen characteristic	Mean	SD	Median	Minimum	Maximum
Volume	3.0	1.5	2.6	1.5	8.5
Sperm count ($\times 10^6$/mL)	49.5	26.8	41.8	15.5	109.0
Total sperm count ($\times 10^6$)	147.5	115.8	94.8	52.7	442.5
Total motility (%)	70.9	6.8	71.0	46.0	79.0
Progressive motility (%)	57.1	9.4	57.5	33.0	68.0
Vitality (% alive)	71.2	7.5	72.0	59.0	78.2
Morphology (% normal forms)	7.0	3.0	6.0	4.0	10.0
Leukocyte count ($\times 10^6$)	0.2	0.3	0.1	0.0	0.5

Feijó. SCD accuracy in unexplained male infertility. Fertil Steril 2014.

SUPPLEMENTAL TABLE 2

Tabulation of the results of the SCD test (index test) and the TUNEL assay (reference standard).

			Method of DNA damage assessment	
Specimen no.	Age (y)	Chart no.	TUNEL (% positive-sperm[a])	SCD (% positive-sperm[a])
1,125	39	2,602	17	7
1,165	27	External	23	23
1,223	38	1,525	7	9
1,224	41	958	5	18
1,225	26	2,806	15	15
1,271	40	280	4	23
1,272	18	2,877	7	11
081	39	3,156	15	37
079	35	2,884	2	27
108	34	2,888	11	31
109	31	2,941	8	16
134	33	2,790	12	7
271	43	2,943	3	8
298	41	3,281	14	66
609	35	3,076	10	16
607	29	2,213	29	17
652	43	1,464	18	33
710	41	2,972	5	11
738	42	2,385	19	25
739	35	2,557	5	11

[a] Positive sperm represent the cells with DNA damage.

Feijó. SCD accuracy in unexplained male infertility. Fertil Steril 2014.