Comparison of Chromatin Assays for DNA Fragmentation Evaluation in Human Sperm

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ABSTRACT: Sperm chromatin integrity is vital for successful pregnancy and transmission of genetic material to the offspring. We evaluated chromatin integrity in sperm from 60 infertile men and 7 fertile donors comparing the sperm chromatin structure assay (SCSA), TdT-mediated-dUTP nick end labeling (TUNEL), the sperm chromatin dispersion (SCD) test, and acridine orange staining technique (AOT). The TUNEL and SCD assays showed a strong relationship with the SCSA ($r > .866$; $P < .001$) for sperm DNA fragmentation, both in infertile men and donors of known fertility. AOT did not show any relationship with SCSA. The breakdown of the DNA fragmentation index (DFI) into 3 categories ($\leq15\%$, $>15\%–<30\%$, and $\geq30\%$) showed that the SCSA, TUNEL, and SCD test predict the same levels of DNA fragmentation. AOT consistently showed higher levels of DNA fragmentation for each DFI category. DNA fragmentation in sperm between infertile men and donor sperm was significantly different ($P < .05$) under SCSA (22.0 ± 1.6 vs 11.8 ± 1.4), TUNEL (19.5 ± 1.3 vs 11.1 ± 0.9) and SCD (20.4 ± 1.3 vs 10.8 ± 1.1), respectively. DNA fragmentation in sperm evaluated by AOT did not differ ($P > .05$) between infertile men (31.3 ± 2.4) and donors (32.7 ± 4.8). AOT showed extreme variations for sperm DNA fragmentation in semen from both infertile men and donors. The problems of indistinct colors, rapid fading, and the heterogeneous staining were also faced. In conclusion, SCSA, TUNEL, and SCD show similar predictive values for DNA fragmentation, and AOT shows variable and increased levels of DNA fragmentation, which makes it of questionable value in clinical practice.

Key words: SCSA, TUNEL, SCD, AOT.

J Androl 2006;27:53–59

The origin and impact of sperm DNA fragmentation has been the subject of numerous studies. Infertile men with poor sperm motility and morphology have increased DNA fragmentation compared with individuals with normal semen parameters (Lopes et al, 1998b; Irvine et al, 2000; Zini et al, 2001a,b). Men with normal semen analysis may also have a high degree of DNA fragmentation, which can be a major cause of undiagnosed/unexplained infertility. Sperm DNA fragmentation may result from aberrant chromatin packaging during spermatogenesis (Gorczyca et al, 1993; Manicardi et al, 1995; Sailer et al, 1995), defective apoptosis before ejaculation (Sakkas et al, 1999, 2002), or excessive production of reactive oxygen species (ROS) in the ejaculate (Kodama et al, 1997; Sun et al, 1997; Aitken et al, 1998; Lopes et al, 1998a; Irvine et al, 2000; Aitken and Krausz, 2001; Moustafa et al, 2004). Exposure to environmental or industrial toxins, genetics, oxidative stress, smoking, etc, are known to cause sperm DNA fragmentation and infertility (Potts et al, 1999; Oliva et al, 2001; Saleh et al, 2002; Wang et al; 2003; Bain et al, 2004). Fertilization by sperm containing fragmented DNA may lead to fetal mutations and may also increase the risk of cancer in offspring (Hansen et al, 2002; Aitken et al, 2004). Moreover, fertilization by sperm with fragmented DNA results in poor embryonic development, decreased implantation, lower pregnancy rates, and recurrent pregnancy losses (Hoshi et al, 1996; Hammadeh et al, 1998; Lopes et al, 1998b; Evenson et al, 1999; Host et al, 2000; Larson et al, 2000; Spano et al, 2000; Virant-Klun et al, 2002; Benchaib et al, 2003; Bonde et al, 2003; Carrell et al, 2003; Henkel et al, 2003, 2004; Larson-Cook et al, 2003; Saleh et al, 2003; Seli et al, 2004; Virro et al, 2004). Therefore, it seems logical to evaluate the chromatin integrity of sperm in infertile men before assisted reproduction.

Sperm DNA fragmentation can be evaluated in a variety of ways. The sperm chromatin structure assay (SCSA®) measures the intensity of acridine orange (AO) fluorescence using flow cytometry (Evenson and Jost, 1994). AO fluoresces green when binding to native DNA and red when it binds to the fragmented DNA. The ratio of red/red+green yields the percentage of DNA fragmentation, referred to as DFI. While the SCSA® is a statistically robust test (Evenson et al, 1999), not all labora-

tories have access to a flow cytometer or the technical expertise to perform this assay.

Other methods for DNA fragmentation assessment include TdT-mediated-dUTP nick end labeling (TUNEL) assay, single cell gel electrophoresis (COMET) assay, and acridine orange staining technique (AOT). The TUNEL assay detects both single- and double-stranded DNA breaks by labeling the free 3′-OH terminus with modified nucleotides in an enzymatic reaction with terminal deoxynucleotidyl transferase (TdT) and can be analyzed microscopically or using flow cytometry. The COMET assay involves embedding spermatozoa in agarose on a glass slide, applying electrophoresis, and evaluating DNA migration in comet tails with a software program. The AOT is a simple microscopic procedure based on the same principle as the SCSA® but indistinct colors, rapid fading of fluorescence, and heterogeneous staining of slides makes AOT a test of questionable value in clinical practice (Duran et al, 1998).

Recently, a new method, the sperm chromatin dispersion test (SCD), was introduced for evaluating sperm DNA fragmentation (Fernández et al, 2003). The SCD test is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo of dispersed DNA loops that is observed in sperm with nonfragmented DNA following acid denaturation and removal of nuclear proteins. The TUNEL, AOT, and SCD are simple, less expensive procedures and can be performed in a short period of time. The objective of this study was to compare SCSA, TUNEL, SCD, and AOT to assess the levels of DNA fragmentation in sperm from infertile men and donors of known fertility.

Materials and Methods

Following Institutional Review Board approval, semen samples were evaluated from 60 men attending the Andrology Laboratory at the University of Utah. Semen samples from 7 fertile donors were also used in the study. Semen samples were collected by masturbation after a minimum sexual abstinence of 2 days and were evaluated according to World Health Organization (WHO) criteria (1999). An aliquot of the neat semen from each patient and donor was snap frozen in LN_2 for SCSA®. The remaining semen sample was washed with modified human tubal fluid (mHTF: Irvine Scientific, Santa Ana, Calif) to remove the seminal plasma because smears with seminal plasma are mostly lost during washing and labeling processes under TUNEL and AOT. Briefly, 5–6 mL mHTF medium was added to the centrifuge tube to achieve a ratio of at least 2 parts media to 1 part semen. The semen was mixed with media by gently inverting 3–5 times and then was centrifuged for 10 minutes at $300 \times g$. The supernatant was decanted and the sperm pellet was diluted in mHTF and aliquoted for DNA damage evaluation following TUNEL, SCD, and AOT. All the assays were performed following the established protocols under dim light. Unless otherwise

stated, the chemicals were purchased from Sigma Chemicals (St Louis, Mo).

The SCSA® was done at the Reproductive Medicine Center, University of Minnesota, following exactly the established protocol of Evenson and Jost (1994). More than 5000 sperm were evaluated for each semen sample and the results were expressed as percent DNA fragmentation index (%DFI) using SCSA Software (SCSA Diagnostics Inc, Brookings, SD). The semen samples with SCSA value of less than or equal 15% DFI represent low levels, greater than 15% to less than or equal 30% DFI values represent moderate, and more than or equal 30% DFI values represent high levels of DNA fragmentation.

The TUNEL assay was performed using the In-Situ Cell Death Detection Kit: Fluorescein following the manufacturer's guidelines (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, the air-dried smeared slides were fixed in 4% paraformaldehyde at room temperature and rinsed in phosphate buffer (PBS), pH 7.4, and then permeabilized with 2% Triton X-100. The TdT-labeled nucleotide mixture was added to each slide and incubated in a humidified chamber at 37°C for 60 minutes in the dark. Later, slides were rinsed twice in PBS and counterstained with 10 mg/mL 4′,6 diamidoino-2-phenylindole (DAPI). Negative controls without TdT enzyme were run in each replicate. A total of 500 sperm per individual were evaluated using fluorescence microscopy by the same examiner. The number of sperm per field stained with DAPI (blue) was first counted; the number of cells with green fluorescence (TUNEL positive) was expressed as a percentage of the total sample.

The SCD test was performed following the procedure of Fernández et al (2003), with a minor change of using 0.4 M DTT instead of 0.8 M DTT in the lysing solution 1 (Fernández; personal communication; 0.4 M dithiothreitol (DDT) gives similar results if solution is not stored for a long period). Briefly, an aliquot of the washed neat semen sample was mixed with 1% low melting-point agarose (to obtain a 0.7% final agarose concentration) at 37°C. Aliquots of 50 μL of the mixture were pipetted onto the precoated glass slides with 0.65% standard agarose dried at 80°C, covered with a cover slip (24 × 60 mm), and left to solidify at 4°C for 5 minutes. Cover slips were carefully removed, and the slides were immediately immersed horizontally in a tray with freshly prepared acid denaturation solution (0.08 N HCl) for 7 minutes at 22°C in the dark to generate restricted single stranded DNA (ssDNA) motifs from DNA breaks. The denaturation was then stopped and proteins were removed by transfer of the slides to a tray with neutralizing and lysing solution 1 (0.4 M Tris, 0.4 M DTT, 1% SDS, and 50 mM EDTA, pH 7.5) for 10 minutes at room temperature, which was followed by incubation in neutralizing and lysing solution 2 (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) for 5 minutes at room temperature. Slides were thoroughly washed in Tris-borate-EDTA buffer (0.09 M Tris borate and 0.002 M EDTA, pH 7.5) for 2 minutes; dehydrated in sequential 70%, 90%, and 100% ethanol baths (2 minutes each); and air dried. Cells were stained with 2 μg/mL DAPI for fluorescence microscopy. A total of 500 sperm were evaluated manually on each slide for halo size and dispersion pattern as described by Fernández et al (2003): 1) nuclei with large DNA dispersion halos, 2) nuclei with medium-sized halos, 3) nuclei with small sized halos, and 4) nuclei with
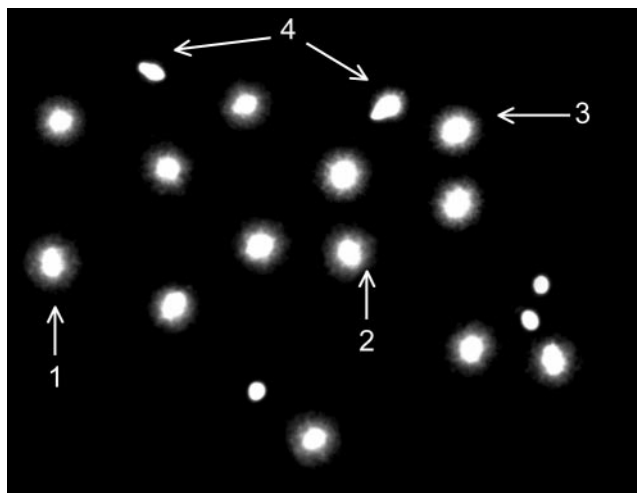
Figure 1. Sperm chromatin dispersion test: sperm with different size halos. The nuclei with large- to small-size halos represent sperm with nonfragmented DNA, whereas nuclei with no halo represent sperm with fragmented DNA. The arrows represent 1) a large halo, 2) a medium halo, 3) a small halo, and 4) no halo. The original picture is in color with DAPI staining and printed in gray scale.

no halo (Figure 1). The nuclei with large to small size halo were considered sperm with nonfragmented DNA, whereas nuclei with no halo were considered sperm with fragmented DNA.

The AOT assay was performed following the protocol of Tejada et al (1984). Briefly, 20 μL of washed sperm suspension for each patient was smeared on a precleaned glass slide. The smeared slides were air dried and later fixed in Carnoy's solution (1 part glacial acetic acid:3 parts methanol) for 2 hours. After fixation, slides were air dried and stained with freshly prepared 0.19 mg/mL AO stain (Polysciences, Warrington, Pa) for 5 minutes in the dark as follows: 10 mL of 1% AO in distilled water added to a mixture of 40 mL of 0.1 M citric acid and 2.5 mL of 0.2 M Na_2 $HPO_4$0.7H_2O and pH adjusted to 2.5. After staining, slides were washed with distilled water, covered with glass cover slips, and immediately evaluated using a Nikon (Eclipse E400) fluorescence microscope at the excitation wavelength of 450–490 nm. A total of 500 sperm cells were evaluated on each slide by the same examiner with no more than 40 seconds duration of observation per field. Spermatozoa displaying green fluorescence were scored as having normal DNA content, whereas sperm displaying a spectrum of yellow-orange to red fluorescence were considered to have damaged DNA.

Statistical Evaluation

Data were analyzed with Student's *t* test, analysis of variance (ANOVA) and linear regression, where appropriate, using Sigma Stat program (SPSS, Chicago, Ill). The *P* value of less than .05 was considered statistically significant.

Results

The SCSA, TUNEL, and SCD showed similar levels of DNA fragmentation in sperm from infertile men and fertile donors. A significant correlation (Figure 2: $r > .866$; $P < .001$) was observed between SCSA, TUNEL, and SCD for sperm DNA fragmentation in semen for both infertile men and donors. The TUNEL and SCD also correlated significantly for both infertile men ($r = .94$; $P < .001$) and donors ($r = .93$; $P < .002$). No correlation was observed between SCSA and AOT for DNA fragmentation in sperm for both infertile men ($r = .184$; $P > .157$) and donors ($r = .037$; $P > .937$). DNA fragmentation in sperm from infertile men and donors differed significantly ($P < .05$) using SCSA, TUNEL, and SCD, but no difference was observed using AOT ($P > .05$; Table). The breakdown of sperm DNA fragmentation data for infertile men into 3 SCSA DFI categories, that is, less than or equal 15% DFI, greater than 15% to less than 30% DFI, and more than or equal 30% DFI (Figure 3) exhibited the same levels of DNA fragmentation under SCSA, TUNEL, and SCD in each DFI category except AOT, which showed higher levels of DNA fragmentation in each category. The technical problems of indistinct colors, rapid fading, and the heterogeneous staining of slides as reported in other studies were also encountered in the present study. The SCD dispersion patterns revealed a high percentage of large size halos for donor sperm compared with infertile men (Figure 4).

Discussion

We compared 4 different techniques for the assessment of DNA fragmentation in human sperm. The SCSA and AOT are based on the same principle, which is acid-induced denaturation of DNA followed by staining with AO. The SCSA is a flow-cytometric method whereas AOT is a microscopic technique, and both measure the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA). The TUNEL assay quantifies the incorporation of fluorescinated dUTP at strand breaks by labeling the free 3′-OH terminus with TdT. The SCSA and TUNEL are established techniques and have been extensively used to assess sperm DNA fragmentation in human and other mammalian species. The SCD test is a relatively new technique reported to assess DNA fragmentation in human sperm (Fernández et al, 2003). The SCD is based on the principle that sperm with fragmented DNA fail to produce the halo of dispersed DNA loops, which is a characteristic of sperm with nonfragmented DNA.

Similar to Sailer et al (1995) and Aravindan et al (1997), we observed a strong relationship between SCSA and TUNEL results for sperm DNA fragmentation (Figure 2A). Our study is the first to report a strong relationship between SCSA, TUNEL, and SCD for the assessment of DNA fragmentation in human sperm. The per-
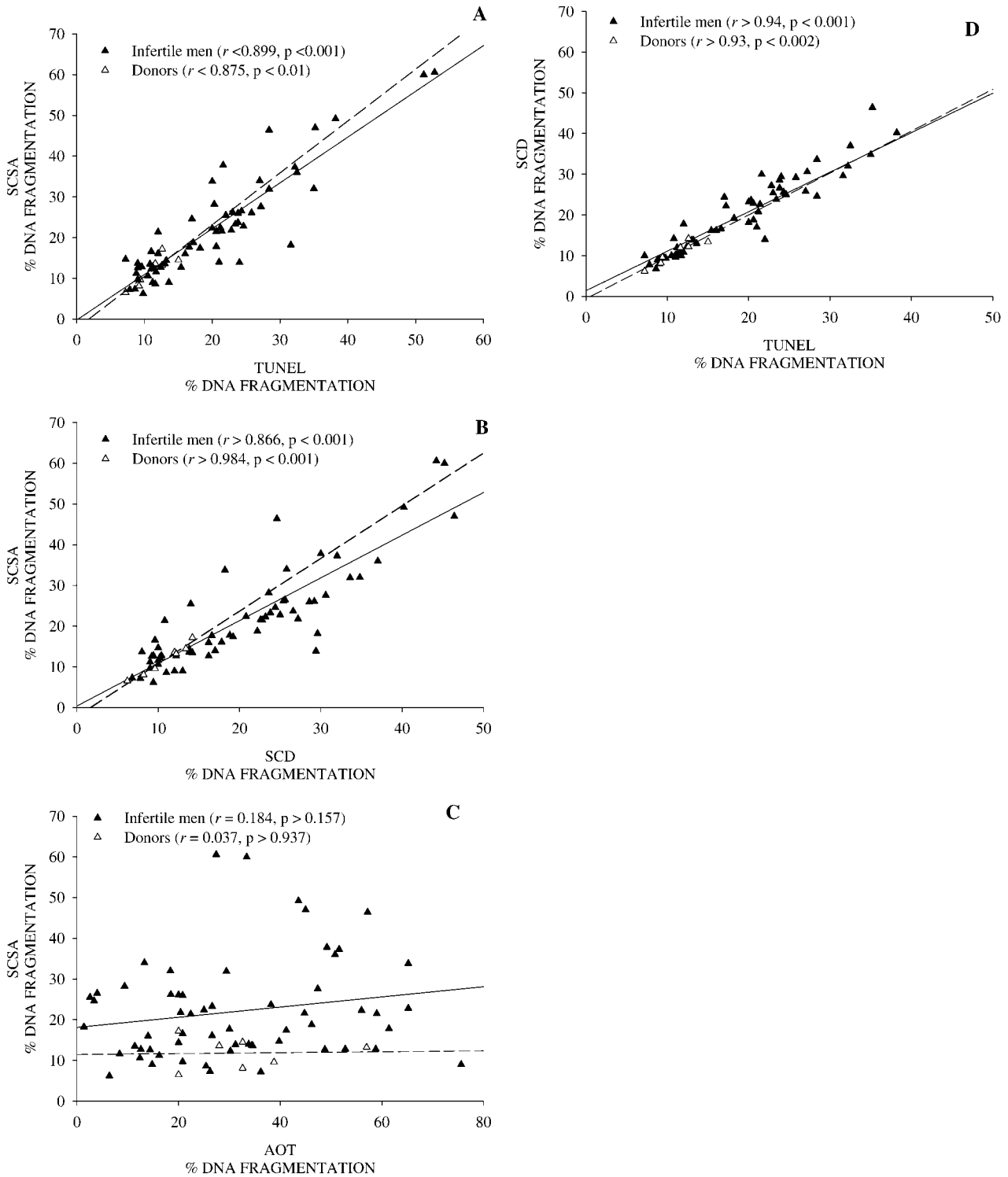
Figure 2. Relationship between different chromatin assays for DNA fragmentation in sperm. The straight curve represents infertile men and the dotted curve represents donors. **(A)** Shows relationship between SCSA and TUNEL, **(B)** shows relationship between SCSA and SCD, **(C)** shows relationship between SCSA and AOT, and **(D)** shows relationship between TUNEL and SCD.

Comparison of sperm DNA fragmentation in infertile men and fertile donors; values are mean ± SEM; different superscript lowercase letters show statistical difference (P < .05) within rows; different superscript capital letters show statistical difference within columns (P < .05)*

	SCSA	TUNEL	SCD	AOT
Infertile men (n = 60)	22.0 ± 1.6[aA]	19.5 ± 1.3[aA]	20.4 ± 1.3[aA]	31.3 ± 2.4[bA]
Donors (n = 7)	11.8 ± 1.4[aB]	11.1 ± 0.9[aB]	10.8 ± 1.1[aB]	32.7 ± 4.8[bA]

* SCSA indicates sperm chromatin structure assay; TUNEL, TdT-mediated-dUTP nick and labeling; SCD, sperm chromatin dispersion; and AOT, acridine orange staining technique.

centage of sperm that failed to show the characteristic halo of dispersed DNA loops under SCD correlated well with SCSA %DFI values and the percent of TUNEL-positive cells. Fernández et al (2003) confirmed SCD test results for sperm DNA fragmentation using breakage detection-fluorescence in situ hybridization (DBD-FISH). The sperm with a very small or absent halo showed extensive DNA fragmentation by DBD-FISH. Present results of SCSA values and percentage of TUNEL-positive cells also demonstrate that sperm without DNA dispersion halos have fragmented DNA.

Donors were found to have a significantly higher percentage of sperm with large halos as compared with infertile men. Men with DFI values of 15% or less also showed an increased number of large- and medium-size sperm DNA halos compared with individuals with DFI values greater than 15% (Figure 4). The different size halos shown in Figure 2 may be due to the difference in the morphology of sperm. This argument has been supported by the findings of Fernández et al (2003) that sperm harvested from the 90% pellet of density gradient columns show large halos of DNA dispersion. The density gradient centrifugation not only improves the motility

but also results in the isolation of sperm with normal morphology and chromatin integrity (Sakkas et al, 2000; Hammadeh et al, 2001). Ankem et al (2002) observed uniform sperm DNA halos in fertile men with normal semen parameters, which coincide with our findings for donor sperm.

Considering SCSA as a gold standard, when DNA fragmentation data were categorized according to %DFI values (Figure 3), the SCSA, TUNEL, and SCD again showed the same levels of DNA fragmentation under each DFI category. These results demonstrate that SCSA, TUNEL, and SCD show similar levels of sperm DNA fragmentation. AOT consistently exhibited high levels of DNA fragmentation in infertile men as well as in donors. Present results show that assessment of DNA fragmentation in sperm using AOT is questionable. Previous studies have also shown that AOT cannot be recommended as a screening test for sperm quality and functional capacity and that AOT has a very low clinical significance for infertility testing (Claassens et al, 1992; Eggert-Kruse et al, 1996). Another explanation for the lack of a rela-
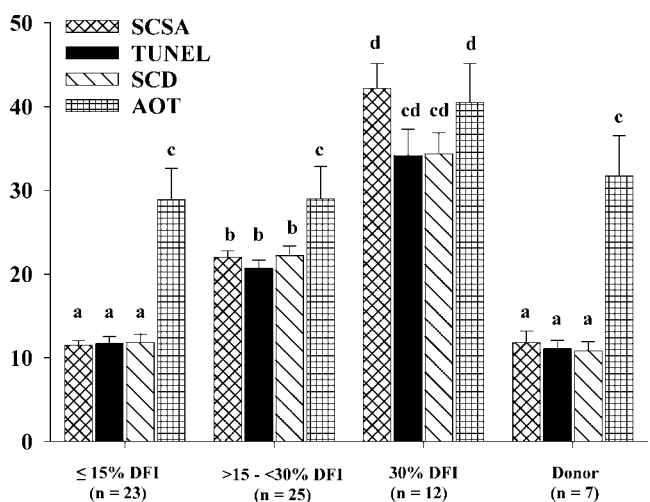

Figure 3. Comparison of DNA fragmentation in sperm of infertile men and donors according to SCSA percent DNA fragmentation index (%DFI) values. Values are mean ± SEM. Different letters (a, b, c, d) show statistical difference (*P* < .05) within groups. The numbers in parentheses are number of cases in each DFI category.
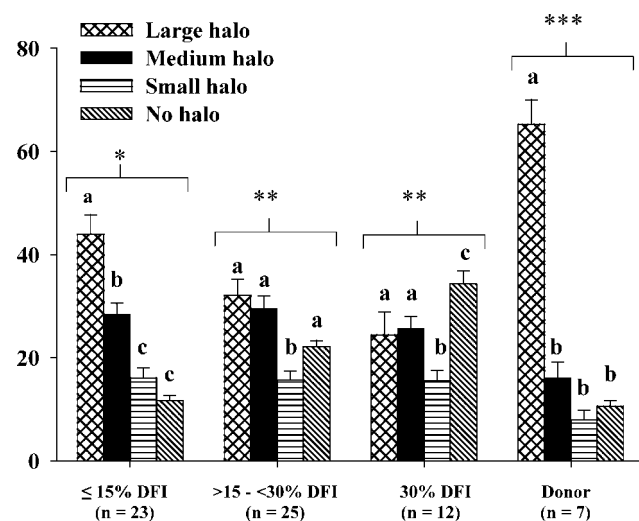

Figure 4. Sperm chromatin dispersion test: percentage of different size sperm DNA halos according to DFI categories in infertile men and donors. Values are mean ± SEM. The numbers in parentheses are number of cases under each DFI category. Different letters (a, b, c) represent statistical differences (*P* < .05) for halo size within each DFI category. Different asterisk (*) signs show statistical difference (*P* < .05) for the large-size halo among DFI categories and donors.

tionship between AOT and SCSA, TUNEL, or SCD may be the cut-off values for these assays. The only point where AOT results coincided with SCSA values was when DFI was greater than 30% (Figure 3). Gopalkrishnan et al (1999) observed greater than 50% green fluorescence in samples from fertile donors and used this as a normal cut-off value for AOT. Hoshi et al (1996) also reported that in vitro fertilization (IVF) was successful when sperm exhibited more than or equal 50% green AO fluorescence and no pregnancies were obtained when green-fluorescing sperm were less than 50% even though an average 26% of oocytes were able to be fertilized using intracytoplasmic sperm injection (ICSI). Although SCSA and AOT both use acid conditions to denature DNA followed by staining with acridine orange, the reason they have no correlation for results might be the different evaluation procedure. Evenson et al (1999) suggested that fluorescence microscopy under AOT provides a general picture of the status of DNA denaturation. AOT is limited to only 2 to 3 classifications (green, red, yellow) compared with SCSA, which evaluates 1024 discrete channels of red and green fluorescence using a flow cytometer. We hypothesize that the microscopic evaluation under AOT only provides reliable results when there is a high degree of DNA fragmentation, and this might be the reason that 50% was set as a cut-off value in the above-mentioned studies. Preparation of the same semen samples by SCSA and AOT protocols followed by evaluation using a flow cytometer may answer the question of whether the difference lies in the technique or in the evaluation method.

A possible explanation for the same outcomes of SCSA, TUNEL, and SCD test lies in the fact that sperm from men with abnormal semen parameters are characterized by higher levels of DNA strand breaks, which can be indicative of apoptosis (Irvine et al, 2000). The TUNEL assay quantifies the percentage of sperm with fragmented DNA by labeling the strand breaks with TdT, whereas in SCSA, the sperm chromatin is exposed to acid denaturation, which exposes the single-stranded DNA to acridine orange binding as an aggregate and differentiates them from the unfragmented double-stranded DNA. The SCD test also involves acid denaturation, which generates single-stranded DNA motifs from DNA breaks and further deproteinization of nuclear proteins suppresses the formation of a halo. The mechanism for this halo suppression is unknown but the suppression of halo formation is not observed in sperm with unfragmented DNA. Though these assays use different principles and protocols, they successfully detect DNA fragmentation with similar results, accuracy, and sensitivity.

In this study, sperm from infertile men showed increased DNA fragmentation compared with sperm from donors of known fertility under SCSA, TUNEL, and SCD test. These results are in agreement with previous findings (Irvine et al, 2000; Erenpreiss et al, 2001; Zini et al, 2001a,b). No difference was observed for overall DNA fragmentation using AOT between infertile men and donors in the present study, which strengthens the fact that AOT results are not reliable.

In conclusion, our data show that SCSA, TUNEL, and SCD are sensitive diagnostic tools for detecting DNA fragmentation in sperm. The AOT on the other hand is associated with more variability.

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