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The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility

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ABSTRACT

Thirty-five years ago the pioneering paper in *Science* (240:1131) on the relationship between sperm DNA integrity and pregnancy outcome was featured as the cover issue showing a fluorescence photomicrograph of red and green stained sperm. The flow cytometry data showed a very significant difference in sperm DNA integrity between fertile and subfertile bulls and men. This study utilized heat (100 °C, 5 min) to denature DNA at sites of DNA strand breaks followed by staining with acridine orange (AO) and measurements of 5000 individual sperm of green double strand (ds) DNA and red single strand (ss) DNA fluorescence. Later, the heat protocol was changed to a low pH protocol to denature the DNA at sites of strand breaks; the heat and acid procedures produced the same results.

SCSA data are very advantageously dual parameter with 1024 channels (degrees) of both red and green fluorescence. Hundreds of publications on the use of the SCSA test in animals and humans have validated the SCSA as a highly useful test for determining male breeding soundness. The SCSA test is a rapid, non-biased flow cytometer machine measurement providing robust statistical data with exceptional precision and repeatability. Many genotoxic experiments showed excellent dose response data with very low coefficient of variation that further validated the SCSA as being a highly powerful assay for sperm DNA integrity.

Twelve years following the introduction of the SCSA test, the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling (TUNEL) test (1993) for sperm was introduced as the only other flow cytometric assay for sperm DNA fragmentation. However, the TUNEL test can also be done by light microscopy with much less statistical robustness.

The COMET (1998) and Sperm Chromatin Dispersion (SCD; HALO) (2003) tests were introduced as light microscope tests that don't require a flow cytometer. Since these tests measure only 50–200 sperm per sample, they suffer from the lack of the statistical robustness of flow cytometric measurements. Only the SCSA test has an exact standardization of a fixed protocol. The many variations of the other tests make it very difficult to compare data and thresholds for risk of male factor infertility. Data from these four sperm DNA fragmentation tests plus the light microscope acridine orange test (AOT) are correlated to various degrees.

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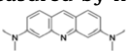
1. The SCSA test: origin of concept of sperm DNA fragmentation (sDF) compared to newer sDF tests

Classical semen analysis consisting of sperm count, motility and morphology has been the backbone of semen related male factor infertility for many decades of time. Yet there have been numerous cases where these parameters are all within the "normal" range but male factor persists for reduced and/or pregnancy failure.

In the late 1970s this author (DPE) received an NIH RO1 grant to study mammalian sperm chromatin structure. Using various techniques to open up the highly dense chromatin structure, transmission electron microscopy clearly showed, especially for human sperm (Evenson et al., 1978), that there was a great amount of chromatin heterogeneity.

This heterogeneity was later confirmed by a flow cytometry study comparing acridine orange (AO) staining of human and mouse chromatin over time of incubation with disulfide reducing agents and proteases (Evenson et al., 1980a). The mouse sperm population reacted very homogenously while the human sperm reacted very heterogeneously. These studies evolved into the question of the potential for sperm nuclear DNA being heterogeneous. In particular, sperm DNA was evaluated for DNA strand breaks, or "sperm DNA fragmentation" (sDF). sDF is defined as single or double strand breaks in nuclear DNA resulting in a potential loss/alteration of genetic information.

In our initial experiments (Evenson et al., 1980b), known sub-fertile bulls in an artificial insemination (AI) station and men attending an infertility clinic were compared with proven fertile bulls and men. Sonication freed sperm nuclei were purified in sucrose gradients. Neither sonication nor sucrose purification has any effect on SCSA data (Evenson et al., 1991). The nuclei were heated in buffer at 100 °C for 5 min, stained with AO and measured by flow cytometry (FCM). The small AO molecule  (MW 265), kept in equilibrium during measurement, easily penetrates the dense sperm chromatin and intercalates into double stranded DNA (ds DNA) that fluoresces green under blue laser light (488 nm). AO stacks on single stranded DNA (ss DNA) and this complex then collapses and under blue laser light has a metachromatic shift of green (515–530 nm) to red (>630 nm) fluorescence (Darzynkiewicz et al., 1975) (Fig. 1).

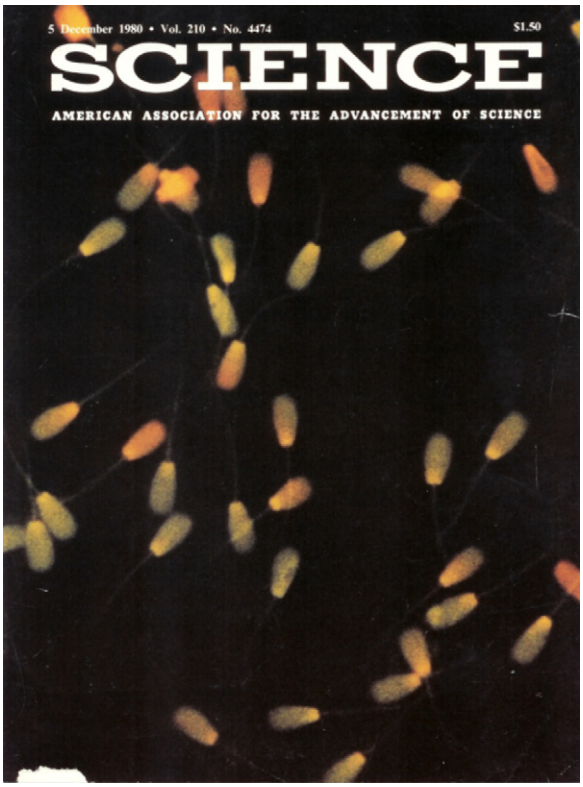


Fig. 1. Cover of *Science* (v. 240, p.1131, 1980) (Evenson et al., 1980a,b).

This shift was expressed as the function alpha t (α_t) which is the ratio of red to total (red + green) fluores-

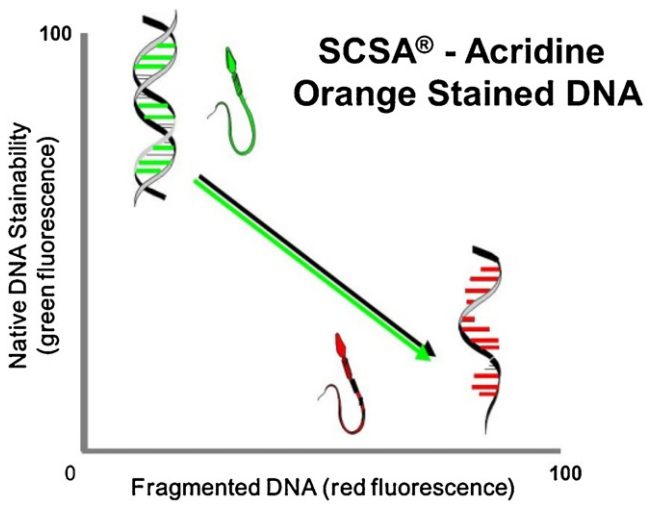


Fig. 2. Schematic of fluorescence color shifting from normal DNA (green) to fragmented DNA (red).

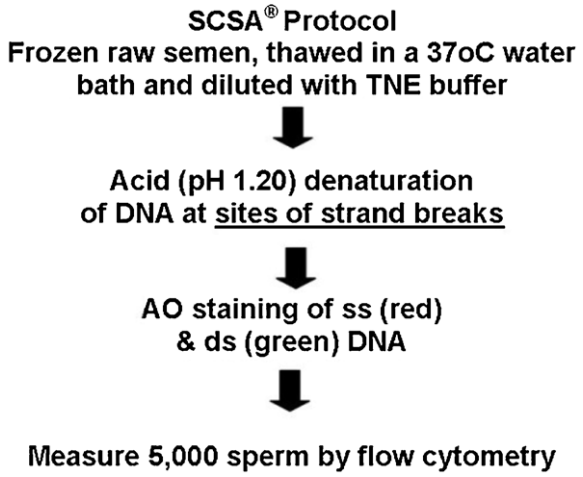


Fig. 3. SCSA test protocol (Evenson et al., 2002a).

cence intensity (Darzynkiewicz et al., 1975). For bulls, the increase of mean α_t values for known sub- fertile bulls was 1.6 times that of fertile bulls. The mean α_t value for men attending the infertility clinic relative to known fertile men (successful pregnancy within two years) was 2.25 times higher (Fig. 2).

The heated sperm protocol used in early experiments was not only time consuming, but it also caused a significant random loss of sperm nuclei due to sticking to the various plastic and glass tubes that were tested for the heating protocol. Therefore, it was great news to see that treating sperm with low pH buffer (1.20) for 30 s to open up the DNA strands at the sites of DNA strand breaks produced the same results as seen with the heated sperm (Evenson et al., 1985) (Fig. 3).

Since the heat and acid treatments do not break the DNA strands and both treatments produced the same results (Evenson et al., 1985, Evenson, unpublished), it was concluded that the red fluorescence was due to the DNA strands parting (denaturing) at the sites of single or double strand breaks and the AO stacks on the single strands that collapse into a complex that produces a metachromatic shift to red fluorescence [luminescence (Darzynkiewicz et al., 1975)]. Importantly, after obtaining a fresh semen sample, or thawing a frozen sample, the acid SCSA technique can be accomplished in about five minutes which is much faster than any other current sperm DNA fragmentation protocol.

It is to be strongly noted that over the past quarter century with an estimated one to two hundred thousand sperm samples measured on two dozen or more animal species, our SCSA test has been done *EXACTLY* the same as described in detail (Evenson, 2011; Evenson, 2012; Evenson et al., 2002a). This *IS* the *SCSA*® test; any deviation from this exact protocol is NOT the SCSA® test. While the other sDF tests (TUNEL, COMET, HALO, AO) all have variations of their protocols, the SCSA test has only one protocol that is now accepted internationally.

The Sperm Chromatin Structure Assay (SCSA®) was named as such since it measures both sDF and abnormal chromatin structure.

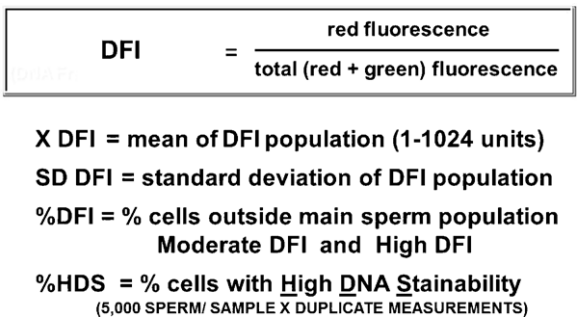


Fig. 4. Newer terminology for SCSA data. DFI = DNA Fragmentation Index (Evenson et al., 1985).

The major use of the SCSA test has been to determine the percent of sperm with fragmented DNA. The original term for describing the % of sperm in a semen sample with fragmented DNA was Cells Outside the Main Population (COMP α_t). The High DNA Stainable (HDS) sperm population in a semen sample have an abnormally high level of DNA staining due to lack of full protamination and thus an increased amount of retained histones. Determination of the % HDS fraction has been useful to characterize sperm chromatin (protein) defects (Evenson et al., 2002b; Evenson et al., 1999a).

Due to suggestions from human medical andrology interests that the acronym COMP α_t did not explain well what this meant, we changed the COMP α_t terminology to % DFI (DNA Fragmentation Index). Thus the three equivalent values [original]: (new)] that describe the extent of DNA fragmentation are: [% COMP α_t,]: (%DFI); [X α_t]:(X DFI) and [SD α_t]:(SD DFI). All of the other current sperm DNA fragmentation tests have now adopted our % DFI expression of % sperm with fragmented DNA. However, in the animal andrology field, the original terms have been kept by most authors. Thus, both terminologies have been kept in this chapter as per the use of the author's terminology. (Sorry for the confusion!)

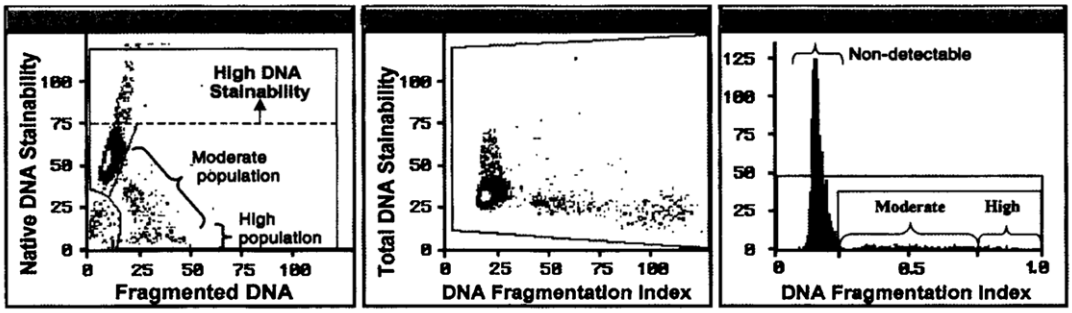
Fig. 4 shows the newer terminologies that our lab and many other medical labs use currently for both human and animal studies and clinical reports.

2. Clinical reports on SCSA data

Thousands of frozen human clinical semen samples have been shipped internationally to SCSA Diagnostics, Inc. (Brookings, SD) as well as other SCSA certified centers (e.g., Andrology Center, Coimbatore, India).

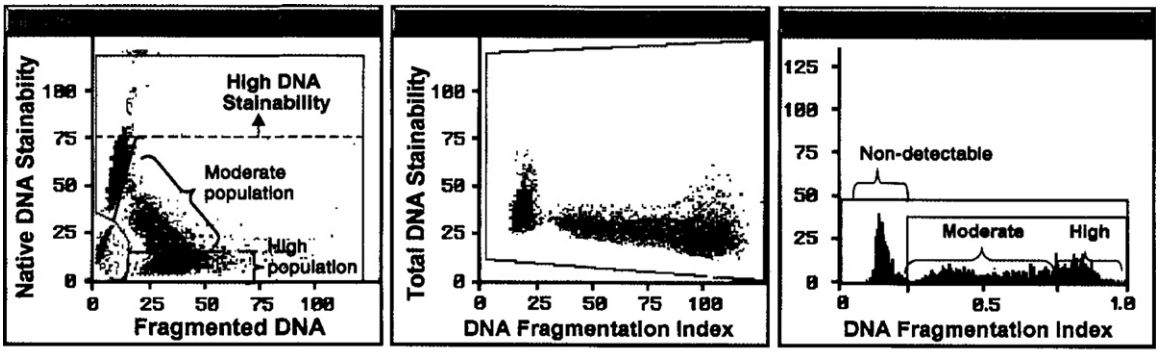
Aliquots of flash frozen semen samples, typically in 2 mL cryovials, are thawed one at a time, processed and measured by flow cytometry. Raw data are scattergrams (cytograms) as seen in the left panels of Figs. 5 and 6. SCSAsoft® software (SCSA Diagnostics), or equivalent, manipulates data seen in the left panel to the altered cytogram in the middle panel. The frequency histograms in the far right panels are produced from data in the middle panel.

SCSA Diagnostics, Inc. *ALWAYS* makes two independent measurements of *every* single sample to ensure that the first measurement is not an artifact of laser drift or



Patient	Date	Measurement	DFI	SD DFI	% DFI	% HDS
7272-87	####	1	213.5	111.6	6.8	5
		2	221.2	118.1	8.3	5.4
		mean	217.4	114.8	7.5	5.2
		sd	5.4	4.6	1.1	0.2

Fig. 5. SCSA clinical report on a human semen sample with relatively good sperm DNA integrity (low % DFI) and chromatin condensation (low % HDS) (Evenson et al., 2002a).
Left panel—raw data from flow cytometer showing each of 5000 sperm as a dot on the scattergram. *Y* axis—green fluorescence with 1024 gradations (channels) of DNA stainability. *X* axis—red fluorescence with 1024 gradations of single stranded DNA stainability. Axes shown here are 1024/10. Dotted line at *Y* axis at 75—upper boundary of DNA staining of normal sperm chromatin; above that line are sperm (dots) with abnormal sperm chromatin allowing more DNA stainability [(High DNA stainable fraction (HDS)]. Three levels of sperm DNA integrity: (a) Normal, (b) moderate and c) high level of DNA fragmentation. Bottom left corner is the gating out of seminal debris.
Middle panel—raw data from left panel are converted by SCSAsoft® software (or equivalent) to red/red + green fluorescence (DFI or α_t). This transforms the angled normal sperm display in left panel to a vertical pattern that is important for a more accurate delineating % of sperm with fragmented DNA. *Y* axis—total DNA stainability vs. *X* axis—DNA fragmentation index (DFI) or α_t).
Right panel—frequency histogram of data from middle panel showing computer gating into three categories: normal, moderate and high DFI. Moderate + High DFI = total % DFI or (% COMP α_t).



Patient	Date	Measurement	DFI	SD DFI	%DFI	%HDS
7272-113	####	1	563.7	307	64.9	6.4
		2	561.4	304.8	64.9	7.2
		Mean	562.6	305.9	64.9	6.8
		sd	1.2	1.1	0	0.4

Fig. 6. Clinical report on a human semen sample with very poor DNA integrity (High % DFI) and moderate chromatin condensation (% HDS) (Evenson et al., 2002a).

clogged flow channel (very important for human infertility patients). In Table on the bottom of Fig. 6, note the extremely low standard deviation (SD = 0.0) of the % DFI thus showing the incredible precision and repeatability of the SCSA test. No other andrology test has shown such precision and repeatability on an independent repeat measurement of 5000 sperm.

3. Characteristics of SCSA defined sperm populations

A Mean and standard deviation (SD) of DFI or SDα_t.

Fig. 7 illustrates populations of sperm obtained from mice exposed to a range of doses of the mutagen ethyl

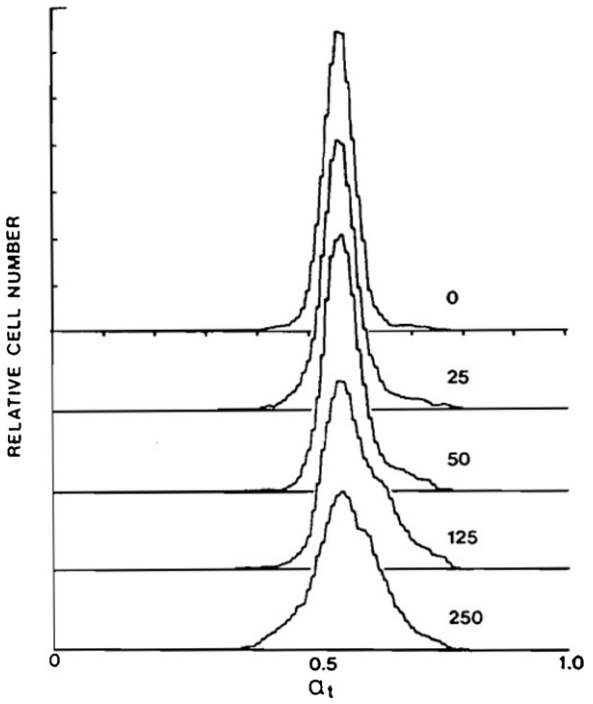


Fig. 7. Frequency histograms of sperm from mice exposed to various dosages of ethylnitrosourea showing increased DNA damage by the SD α_t parameter while the mean value of DFI remains constant (Evenson et al., 1985).

nitrosourea (ENU). Note that the samples have the same X α_t but have dose responsive changes to SD α_t, i.e., showing increased variation with increased dosage (Evenson et al., 1985).

The SCSA derived SD α_t has been shown in a number of experiments to have a higher correlation with animal fertility (Ballachey et al., 1987; Ballachey et al., 1988) and

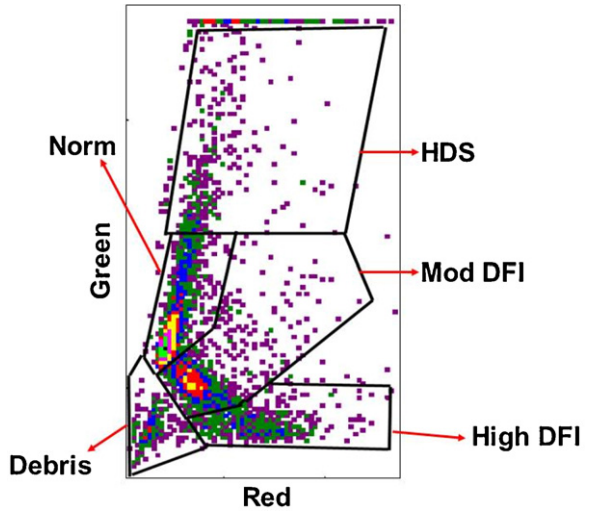


Fig. 9. Gating of SCSA data of human sperm into Normal DFI (no measureable DFI) Moderate DFI and High DFI and HDS. This flow cytometer physically sorted these different populations onto glass slides that were later analyzed as shown in Figs. 10 and 11 (Evenson, 2011).

toxicology dose (Evenson et al., 1989a; Evenson et al., 1986).

Fig. 8 shows the cytograms of a sample with high %DFI and another with high % HDS.

B FCM sorting of different SCSA populations to correlate SCSA data with sperm morphology and Comet derived DNA strand breaks.

A flow cytometer (FACSort, Becton Dickenson, San Jose, CA) was used to make two different sorted populations (Evenson, 2011). Using the gates seen in Fig. 9, sorted sperm were collected in tubes, spun down, resuspended and then forced onto a glass microscope slide using a Cytocentrifuge

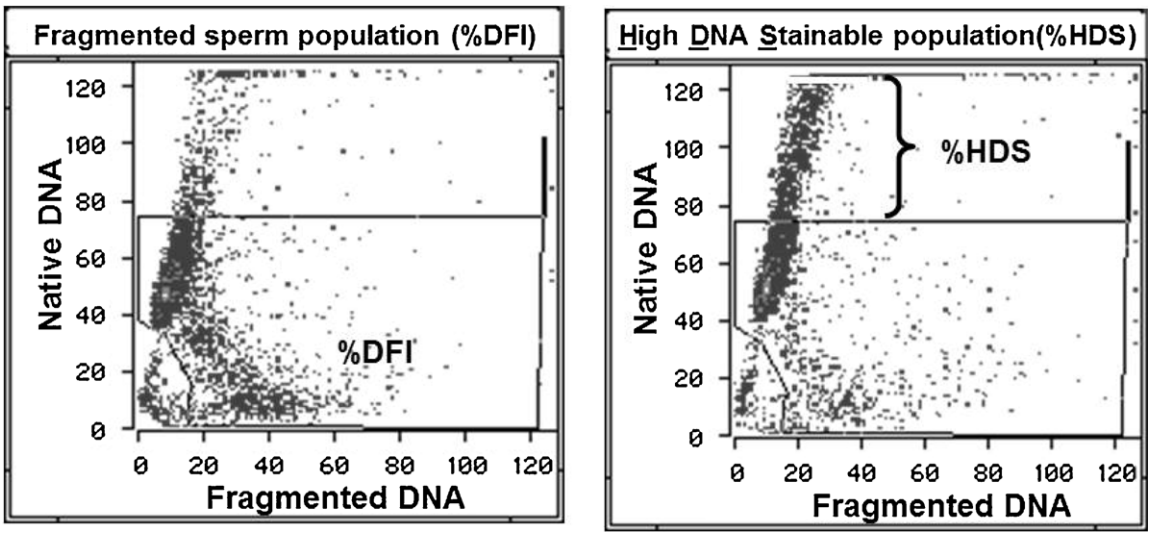


Fig. 8. Illustration of one human semen sample with high % DFI and another human sample with a high % HDS (Evenson et al., 2002a).

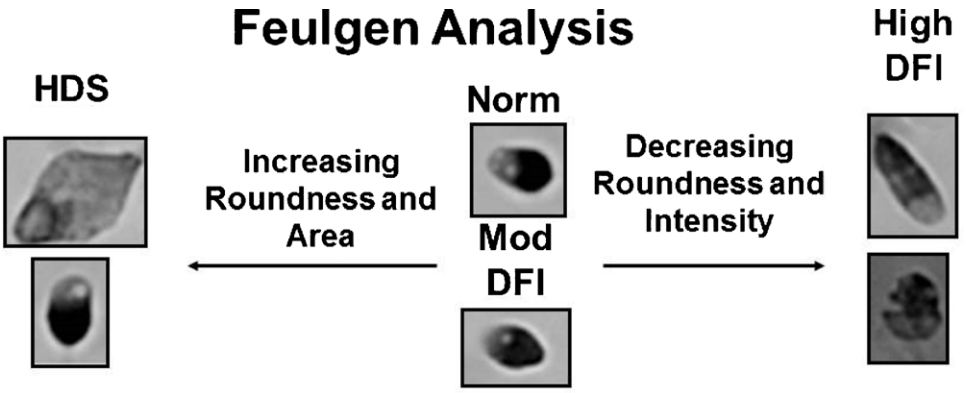


Fig. 10. Feulgen stained nuclei of sorted sperm (Evenson, 2011).

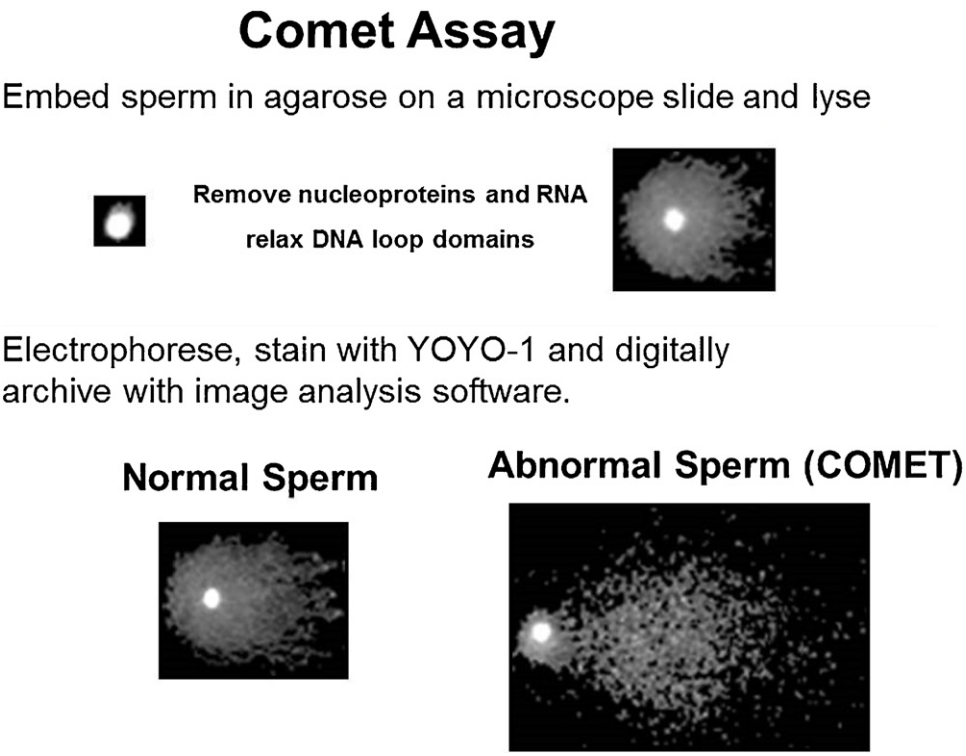


Fig. 11. COMET assay showing sperm without fragmented DNA (normal) and with fragmented DNA (Evenson, 2011).

Shandon Cytospin II, Minneapolis, MN that concentrates the sperm into a small region.

Fig. 10 shows representative Feulgen stained sperm nuclei photographed with a light microscope interfaced to image analysis software.

Of interest, both the SCSA normal population *and* the moderate DFI fractions had normal nuclear morphology. On the right are representative sperm from the High DFI sorted group showing elongated nuclei and those showing signs of apoptosis. HDS sperm were characteristically rounder with immature sperm morphology.

The second set of FCM sorts was on glass slides that were then subjected to a pH 10 (neutral) Comet assay that identifies: normal sperm and abnormal (Comet positive sperm) (Fig. 11).

The main population and HDS population had very few Comets, while half of the moderate DFI population had Comets and all of the High DFI population had Comets. This study confirmed the presence of ds DNA strand breaks in the SCSA DFI populations. An alkaline Comet assay was not run; it is hypothesized that both moderate and High DFI fractions would show 100% alkaline Comets.

C Characteristics of the HDS population.

HDS sperm have abnormal nuclear proteins that prevent normal condensation thereby exposing more DNA to AO staining of dsDNA. FCM measures comparing SCSA vs. Chromomycin A3 (CMA_3) staining of human sperm (Jeffay et al., 2006) support the view that increased CMA_3 stain-

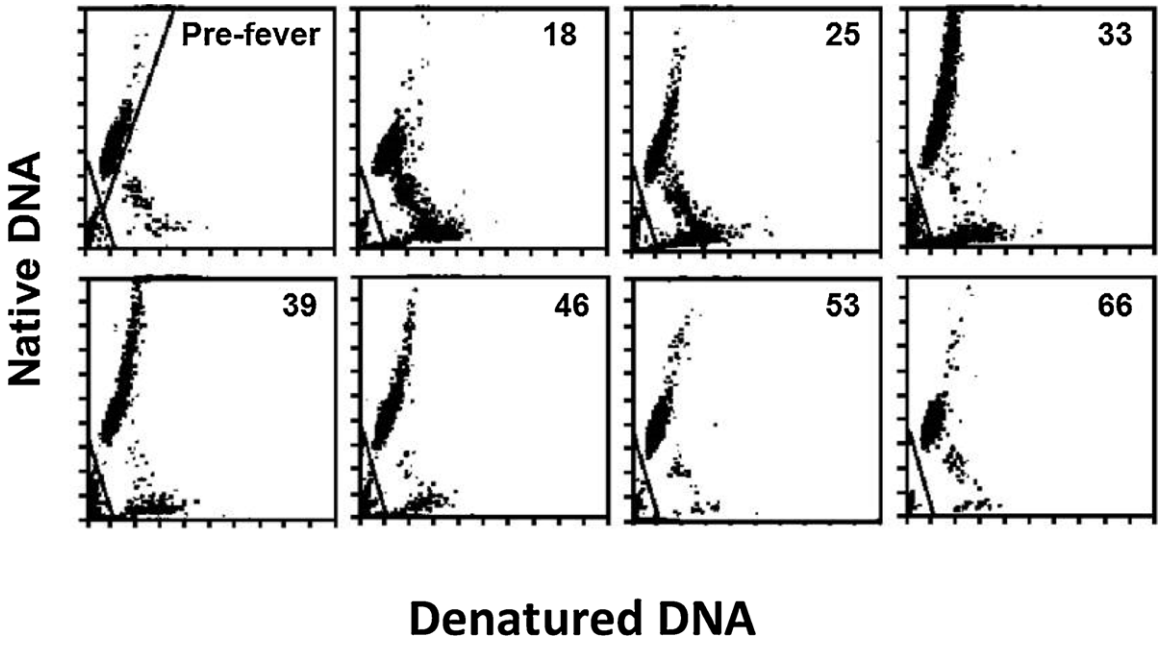


Fig. 12. SCSA measurements of sperm from a human donor who had a fever (104 °F) for one day and then recovered over days 18–66 (Evenson et al., 2002b).

ing (histones) is highly related to AO staining of dsDNA complexed with histones.

High fever for all animals likely has an effect on the integrity of sperm DNA. SCSA measurements (Evenson et al., 2002b) were made on sperm from a human donor who had a fever (104 °F) for one day and then recovered over 66 days. The first effect seen was sDF at day 18 followed by high % HDS at day 33. Sperm nuclear proteins were isolated from the sample at day 33. Gel electrophoresis (Rod Balhorn, Lawerence Livermore National Laboratory) showed an increased ratio of histones to protamines (Evenson et al., 2002b) (Fig. 12).

Also, base sequence analysis of day 33 sperm showed that a precursor to protamine 1 retained the end amino acids which are normally clipped off (Evenson et al., 2002b) This abnormally large protein likely prevented proper condensation of chromatin that then allowed more AO staining of dsDNA.

D Repeatability of SCSA data within individuals over time.

It is well known that semen parameters such as count, motility and morphology can vary widely over short periods of time.

Fig. 13 shows very high repeatability of three SCSA cytogram "patterns" from month to month over eight months; the same was seen for the 45 men in this study (Evenson et al., 1991). This trait has been seen many times with other animals as long as no significant genotoxic or other agent interferes with spermatogenesis. This repeatability of the cytogram patterns and the % DFI has been seen for humans, stallions and bulls over years of time. One can identify many males simply by their cytogram pattern. Practically, this means that a SCSA test at one time point is likely to be the same months later as long as the individual has no negating interference with sperm production.

E E Relationship between sperm DNA fragmentation data and classical semen parameters.

For the many publications on numerous species using the five sperm DNA fragmentation tests, the correlations between sDF and classical semen parameters including sperm count, motility and morphology is generally low showing that sDF is a relatively independent parameter (Evenson, 2011). It is concluded that sperm DNA fragmentation is primarily an independent parameter that adds different information on the quality of the semen sample as related to potential for pregnancy outcome. The highest correlation with SCSA data is usually with sperm motility (0.4–0.6) based on the hypothesis that oxidative stress [reactive oxygen species (ROS activity)] damages cell membranes, including the mitochondrial membrane, and also breaks DNA.

4. Other sperm DNA fragmentation tests

Due to the limited space allowed for this publication, it is not possible to discuss in detail the other four sperm DNA fragmentation tests. Reviews of these tests can be found in a recent book (Zini and Agarwal, 2013).

A TUNEL

The TUNEL test (terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling)(Gorezyea et al., 1993; Sailer et al., 1995a; Sharma et al., 2013) is likely the next best method for analysis of sDF, primarily

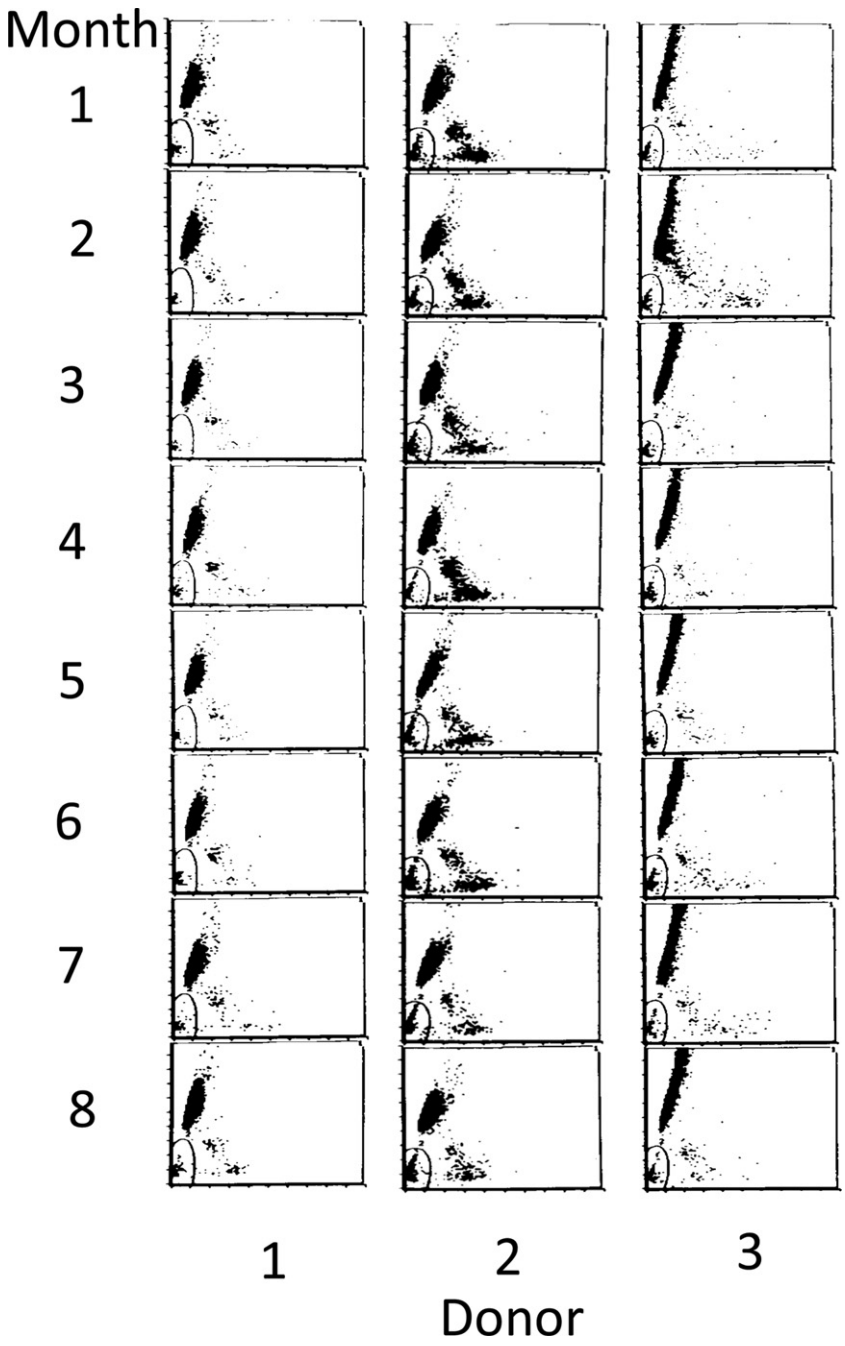


Fig. 13. Data on three men providing one semen sample per month for 8 mo. The three selected individuals show different kinds of dot patterns, from the left to right: normal, high HDS, high % DFI and high % HDS (Evenson et al., 1991).

since it can also measure thousands of sperm per sample by flow cytometry; however, in contrast to the SCSA test, the TUNEL data are only single parameter. The TUNEL test requires many laboratory steps over several hours of time including washing, treating with dithiothreitol (DTT), fixing with paraformaldehyde, permeabilization with Triton X, staining with a commercial terminal deoxynucleotidyl transferase (TdT) enzyme kit e.g., In Situ Cell Death Detection Kit (Roche, Indianapolis, Indiana, U.S.A.) washed and measured. The TdT enzyme identifies nicks, or points of fragmentation, in the DNA. TUNEL data are shown as a ratio of total sperm to FITC-positive sperm (staining of free 3 hydroxyl ends of ss DNA).

B Comparisons between SCSA and TUNEL tests

Due to the highly condensed nuclear chromatin, two large protein molecules, the TdTA enzyme used for the Tunel test and a large fluorescent tagged antibody, not only need to find access to broken DNA strands, but the non-

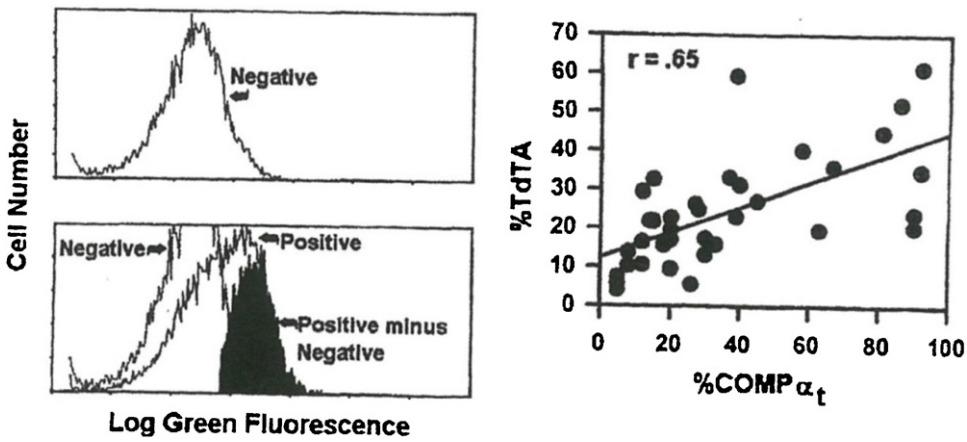


Fig. 14. Regression analysis depicting the relationship between % COMPα_t vs. % TdTA positive sperm. Right panel: Stallion sperm. $n = 36$, $r = 0.65$, $P < 0.001$. % COMPα_t resulted from the analysis of α_t histograms as shown in Fig. 5. Determination of % TdTA positive sperm (shaded population) resulted from the subtraction of TdT control green fluorescence frequency histograms (upper panel and left histogram on bottom panel) from TdT-positive green fluorescence frequency histogram (middle histogram) as shown in bottom panel (Sailer et al., 1995a).

bound marker must be washed out prior to flow cytometric measurement.

Fig. 14 shows raw TUNEL data (left panels). The panel on the right (% COMP α_t) vs. (% TdTA) shows the increased sensitivity of the SCSA test.

Fig. 15 shows % DFI (TUNEL) vs. % DFI (SCSA) data from human, bull, ram and stallion. The relatively high level correlations suggest that SCSA and TUNEL are measuring the same DNA defect; however, note the one-third higher level

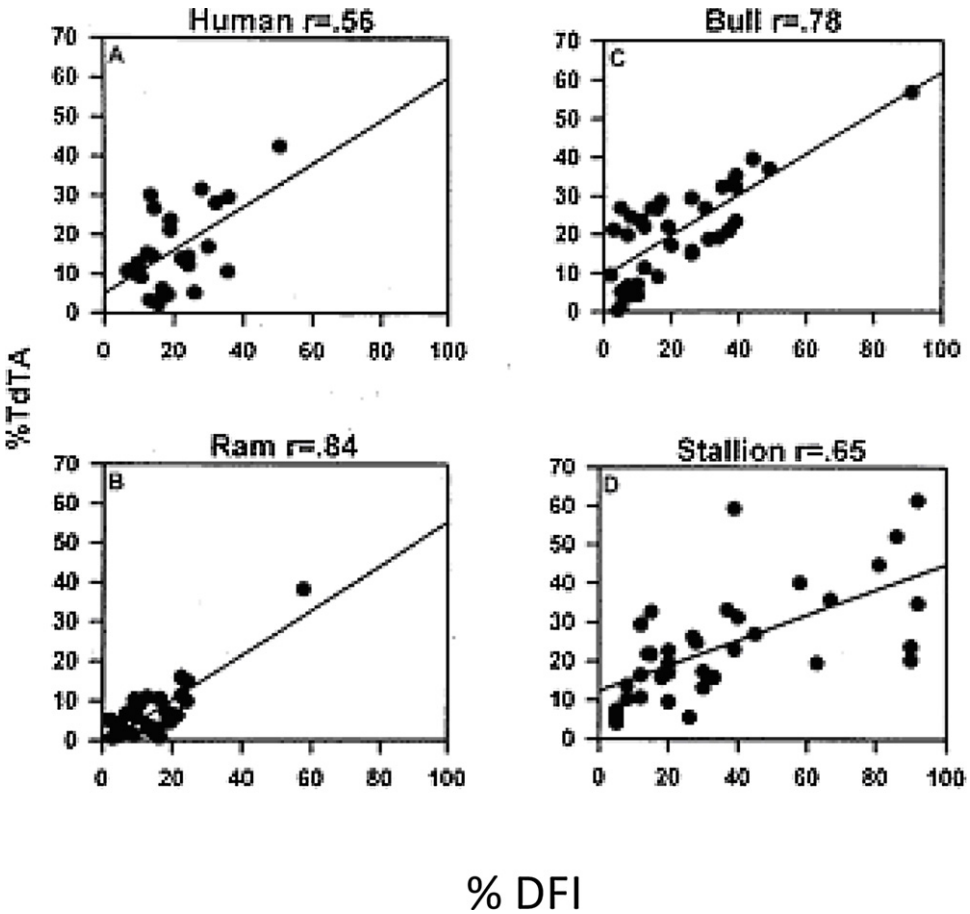


Fig. 15. Regression analysis depicting the relationship between % COMP at and % TdTA. (A) Human, $n = 25$, $P = 0.004'$ (B) ram, $n = 29$, $r = 0.84$, $P < 0.002$; (C) bull, $n = 36$, $r = 0.78$, $P < 0.001$; (D) stallion, $n = 36$, $r = 0.65$, $P < 0.001$. % COMP and % TdTA calculated as described in Fig. 14 (Sailer et al., 1995a).

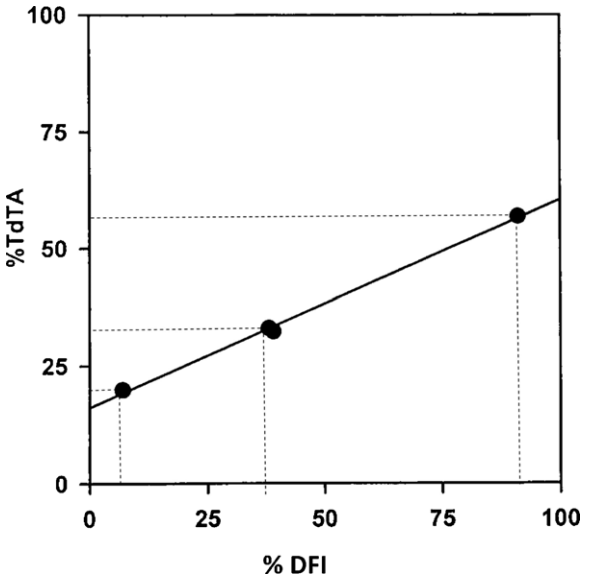


Fig. 16. % DFI vs % TdTA data from aging bull at three time points. Note that the % TdTA values are about 1/3 less than the values for % DFI (Sailer et al., 1995a).

of sensitivity of %DFI from SCSA measurements (Sailer et al., 1995a).

Fig. 16 shows regression analysis depicting the highly significant relationship ($r = 0.999$; $P = 0.001$) between % COMPα_t and % TdTA positive sperm for a single bull with four collections dates over 4.5 years. with the observed negative correlation between decreased fertility with age and increased sDF (Sailer et al., 1995a).

Fig. 17 illustrates how the SCSA test has greater sensitivity for measuring DNA strand breaks throughout the entire chromatin complex in contrast to the TUNEL test. The schematic in Fig. 17 (Gawecka et al., 2015) shows the intact sperm chromatin consisting of the protamine toroid connected to toroid linkers. The linkers include matrix attachment regions (MARs) by which the DNA is attached to the sperm nuclear matrix. The TUNEL test requires the TdT enzyme to add dUTP to broken DNA ends. However, due the high degree of compaction of sperm chromatin, its requirement for TdT almost certainly restricts its access to a limited fraction of the in situ DNA, most likely only to the torroid linker regions (Gawecka et al., 2015). In sharp contrast, the SCSA test requires only the very small AO molecule that likely detects lesions in a broader fraction of the compact sperm chromatin (Gawecka et al., 2015).

Data shown in Fig. 16 and the schematic in Fig. 17 (Gawecka et al., 2015) suggest why the SCSA test is more efficient in staining sperm DNA in the complexed chromatin due to its very small MW marker molecule.

Advantageously, the TUNEL assay can be done with sperm on a glass slide and is especially useful when the sperm count is very low. It is noted however, that the % DFI are not the same between the two techniques so that values obtained by TUNEL FCM cannot be directly compared to the light microscope TUNEL. Both assays use a fluorescent marker that identifies both ssDNA and ds DNA breaks.

Many manuscripts have continued to cite early reviews (Alverez, 2005) saying that the TUNEL test detects *existing* DNA strand breaks, but that the SCSA detects *potential* DNA strand breaks suggesting that the acid step causes DNA strand breaks. However, heating of sperm and subjecting sperm to low pH buffers give the same results (Evenson et al., 1985; Evenson, unpublished). Since neither heat nor acid will break the DNA phosphate backbone we have concluded that the SCSA detects *EXISTING DNA* strand breaks just as the TUNEL assay.

Some authors (Alverez, 2005; Stahl et al., 2015) continue to suggest that the SCSA and TUNEL tests measure different aspects of sDF. In contrast, Z. Darzynkiewicz, co-author of the SCSA test (Evenson et al., 1980a,b) and co-author

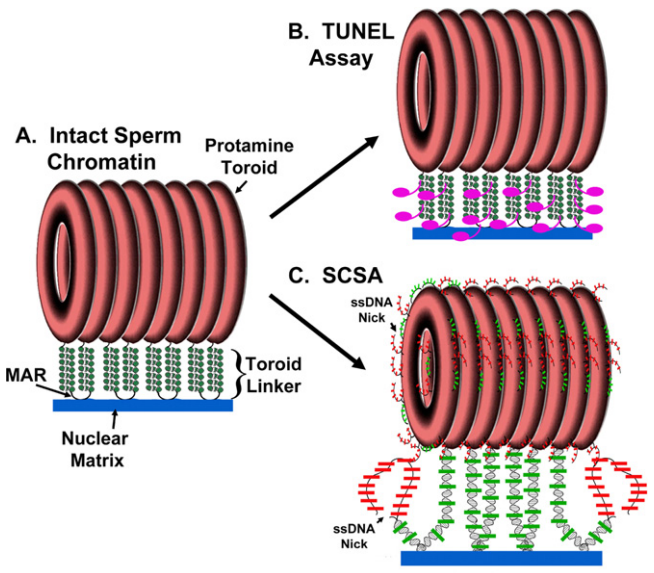


Fig. 17. Schematic model of mammalian sperm chromatin stained either by TUNEL or SCSA tests showing greater access of AO to chromatin and limited access of the TUNEL staining due to the large proteins required by the TUNEL test (Gawecka et al., 2015).

of the pioneering sperm TUNEL test (Gorezyea et al., 1993) showed a correlation of (r = 0.87; P < = .05) between the two tests. Those data plus the data shown above is convincing that the two tests measure the same EXISTING DNA strand breaks although the SCSA test is about one-third more efficient.

For those laboratories that have access to a flow cytometer, it is noted that the cost of materials for the SCSA test is about $0.20/sample while the TUNEL test bears the cost of the light microscope, commercial TdT enzyme, antibodies and fluorescent markers.

C Acridine Orange test (AOT)

The photomicrograph of red and green sperm on the Science cover (Evenson et al., 1980b) showing the AO red and green stained sperm (Fig. 1) was the impetus to develop the "AO technique" to assess sperm DNA breaks in human sperm samples (Tejada et al., 1984). The data in the Tejada manuscript (Tejada et al., 1984) have never, to my knowledge, been repeated with the supposedly outstanding correlation between sperm DNA fragmentation and human pregnancy outcome. The Tejada AOT method stated that the slides of AO stained sperm were prepared at one time and then viewed at "some time over an eight hour span". We know from our own testing (unpublished) that a slide read at 1 h and 8 h do not give the same results.

Three significant problems exist for the AOT.

1. AO is adsorbed by glass. Thus, for the photomicrograph seen on the cover of Science (Fig. 1), the AO concentration was made 10X greater than what is used for the FCM measurements. The glass surfaces of routine commercial laboratory slides and the glass cover slips are not perfectly smooth at the molecular level. Consequently, those areas of a slide/coverslip that have a "pocket", the AO concentration is so high that the intercalating AO molecules can force DNA denaturation thus transforming what would be green fluorescing nuclei toward the red. Then, where the two glass surfaces are both smooth at the molecular level, it forces out AO to such a low concentration that what might be red fluorescing sperm to be pushed toward a green fluorescing sperm. This problem can be seen by the lack of random distribution of red and green sperm on the prepared slide. Often one observes areas of mostly green or mostly red colored sperm (unpublished, DPE)
2. A second problem is the rapid fading of AO fluorescence under a light microscope (Evenson, 2011).
3. The AOT suffers from being a light microscope test which then is magnified by knowing that fluorescent fading is occurring while measuring the slide and the operator rushes the test to overcome this.

Even so, the AOT has been used by many labs over the quarter century since publication of the Tejada article (Tejada et al., 1984). However, this author (DPE) has never seen any data showing good repeatability between slides, times of incubation and different observers. While the AOT may give an indication of samples being excellent or very poor, anywhere in between is largely unreliable and should not be used especially for the highly sensitive human infertility clinic. In summary, the AOT is not credible.

D COMET test.

Single-cell gel electrophoresis (COMET test) allows the distinction between ssDNA and dsDNA breaks, depending on whether alkaline denaturing or neutral conditions are performed, respectively. There are many variations of the COMET assay, both for the neutral and alkaline pH versions.

Simon and Carrell (Simon and Carrell, 2013) have described in detail the alkaline version of one COMET test as a "relatively simple and sensitive method" for measuring DNA strand breaks in individual sperm. For this COMET test, sperm are embedded in a thin layer of agarose on a microscope slide and lysed with detergent under high salt conditions. This process removes protamines and histones allowing the nucleus to form a nucleoid-like structure containing supercoiled loops of DNA. Alkaline pH conditions result in unwinding of double-stranded DNA, and subsequent electrophoresis results in the migration of broken strands toward the anode, forming a "comet tail," when observed under fluorescence microscope. (see Fig. 11). The relative fluorescence in the tail compared with its head serves as a measure of the level of DNA damage.

A review article by Cortés-Gutiérrez et al. (2014) on the COMET test included methods to detect single-stranded (SSBs) and double-stranded DNA (DSBs) breaks on the same sperm. The review highlights the two-dimensional Two-Tailed COMET assay (TT-comet) that quantitates the amount of DSBs and SSBs of each sperm. TT-comet assay has been used to investigate the structure and function of sperm DNA across a diverse range of mammalian species (eutheria, metatheria, and prototheria) (Vilfan et al., 2004).

E SCD (Sperm Chromatin Dispersion) or HALO test

The SCD test is a "simple" method in kit form. Unlike all the other tests, it measures the absence of damage rather than the damaged DNA in sperm. It does not rely on either color or fluorescence intensity making the test simple to use with light microscopy. As shown in Fig. 17, mammalian sperm chromatin is organized in loops. If there are no breaks in the DNA, the molecular torsion of chromatin will open the chromatin into coils; DNA breaks will prevent dispersion.

López-Fernández et al. (2007) reported on the validation of the SCD for stallion sperm and its application to semen that was chilled (4° C; $n = 10$) or frozen-thawed ($n = 13$). The results of this investigation revealed that there was no significant difference in the sperm DNA fragmentation index (sDFI) of sperm evaluated initially after collection compared to those tested immediately after chilling or cryopreservation. However, within 1 h of incubation at 37 °C, both chilled and frozen–thawed spermatozoa showed a significant increase in the proportion of sDFI; after 6 h the sDFI had increased to over 50% and by 48 h, almost 100% of the sperm showed DNA damage. While the sDFI of individual stallions at equivalent times of incubation was variable, an analysis of the rate of change of sDFI revealed no difference between stallions or the way in which the semen was

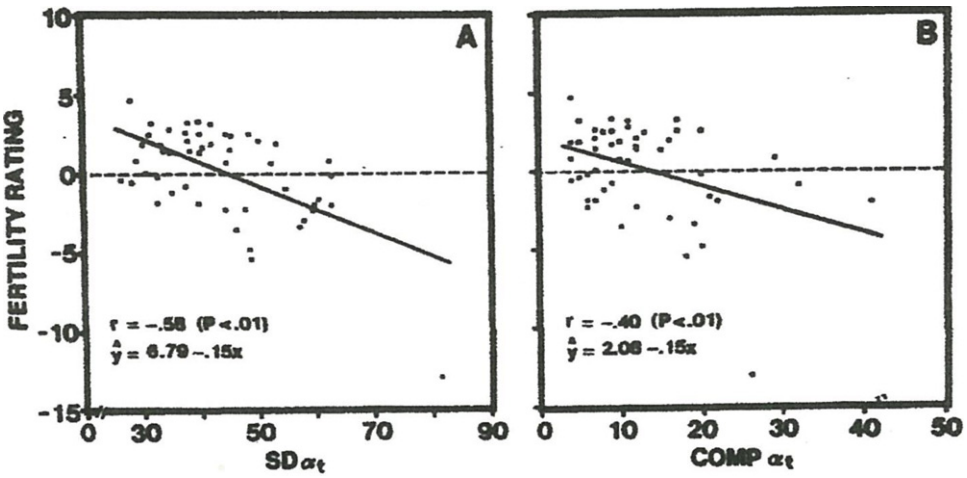


Fig. 18. Bull stud fertility rating vs. SCSA SD α_t and SCSA % COMP α_t. Fertility ratings were based on nonreturn rates adjusted for various environmental effects and expressed as a deviation from zero (Ballachey et al., 1987).

preserved. In terms of sperm DNA fragmentation dynamics, the highest intensity of sperm DNA damage occurred in the first 6 h of incubation.

Martínez-Pastor et al. (2009) compared SCSA and SCD data to assess the chromatin status of three bull samples that were cryopreserved semen and then analyzed after thawing and after 6 h at 37 °C with and without oxidative stress (1 mM FE(2+)). SCD could not discriminate between samples with and without oxidizing treatment [Area Under the Curve (AUC): 0.52]. SCSA (%DFI) showed a high discriminating ability between treatments (AUC: 0.96). The repeatability coefficient was also higher for SCD (5.9) than for % DFI (1.8), indicating lower repeatability for SCD. "Overall, the SCSA %DFI might be the most useful parameter for assessing sperm chromatin on bull".

The SCD test is done on commercial test kits obtained from Halomax Inc. (Halotech DNA SL, Madrid, Spain). The cost per test becomes relatively expensive when large numbers of repeat samples are made, in contrast to the consumables used for SCSA test which is about $0.20/sample. Thus, students/researchers can do hundreds of SCSA tests and experiments for very little expense.

While the SCSA is the *exact same test* at all times, the TUNEL, COMET and HALO tests have many variations. For SCD, custom made test kits are manufactured for different species or groups of species.

5. Sperm DNA fragmentation (sDF) data related to fertility potential

A Bulls at AI centers.

Ballachey et al. (1987) correlated the field data of bulls at the Eastern Artificial Insemination Coop (Ithaca, NY) with SCSA data as shown in Fig. 18.

Fertility ratings of the bulls ranged from −13.05 to +4.72; higher values were associated with increased fertility. Fertility was correlated significantly with COMPα_t (−0.40, $P < 0.01$) and SD α_t (−0.58, $P < 0.01$).

B Heterospermic inseminations to eliminate female factors

Perhaps the best way to eliminate many of the variables in AI field trials is to conduct heterospermic inseminations. Thus, e.g., if equal numbers of motile sperm from a black bull and a white bull are inseminated into 100 females, the ratio of black and white calves shows which bull has the greater fertility potential.

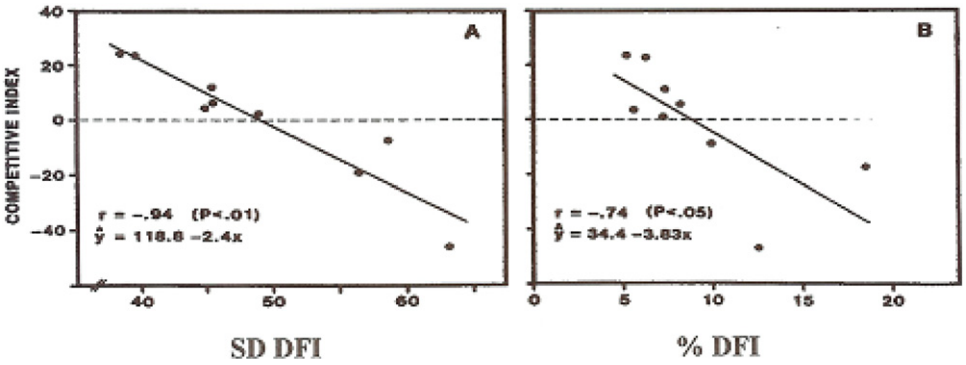


Fig. 19. Bull Heterospermic Competitive Index vs. SCSA SD α_t and SCSA % COMP α_t (Ballachey et al., 1988).
A heterospermic trial is done by mixing equal numbers of motile sperm from two or more males differing in traceable phenotypes.

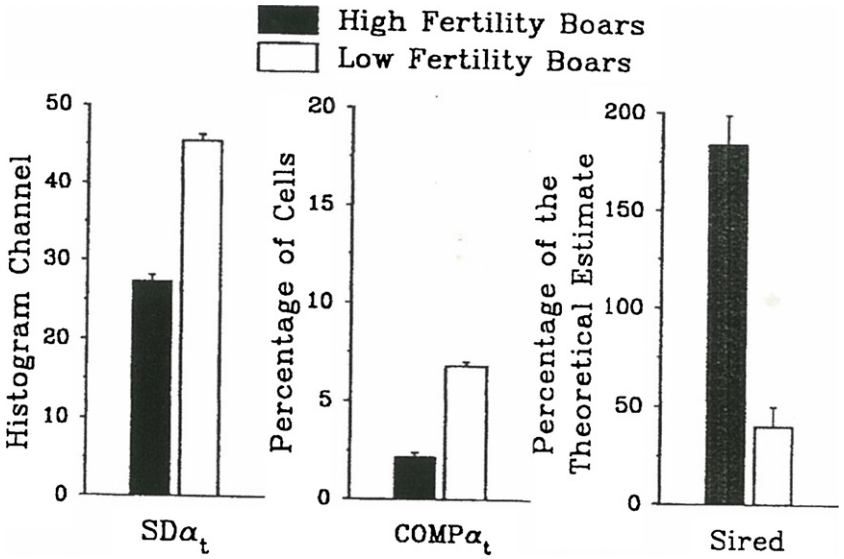


Fig. 20. Heterospermic Competitive Index for 3 phenotypic different boars as scored by SD α_t and SCSA % COMP α_t and percent of the theoretical estimate of piglets (Evenson et al., 1994a). Equally competitive sperm would produce litters containing one-third of each phenotype.

Fig. 19 shows the relationship of SCSA data and heterospermic insemination (Ballachey et al., 1988). SCSA test data on sperm from nine bulls were correlated with fertility, measured by heterospermic performance (−0.94, $P < 0.01$) and by alternate tests of sperm quality, including motility, acrosome integrity, Sephadex filtration and morphology of sperm (all significant at $P < 0.05$ to $P < 0.01$).

The SCSA data on sperm chromatin structure stability was as highly correlated with the heterospermic performance of semen as the best of the classical tests for semen quality.

	FR	APB
DFI	-0.60[a]	-0.59[a]
SD DFI	-0.68[b]	-0.55[c]

a p<0.01
b p<0.003
c p<0.02

Fig. 21. Boar fertility (FR) and average pigs born (APB) vs. SCSA %DFI and SD DFI (Didion et al., 2009).

C Boars

In a similar heterospermic competitive study (Evenson et al., 1994a), semen from 6 boars was mixed in equal sperm numbers in six 3-way combinations and inseminated into at least 3 Duroc gilts per combination. Four of the six combinations yielded 2 litters, while the remaining 2 combinations yielded 3 litters.

As seen in Fig. 20, the SCSA correctly predicted both the high and low fertility boars based on a ratio of offspring as deviated from the theoretical percentage.

A great advantage for investigating not only fertility data on single-birth animals is to use multiparous animals that can help detect embryo loss in vivo as related to male factor.

Didion et al. (2009) evaluated 18 sexually mature boars having fertility information. Boar fertility was defined by farrow rate (FR) and average total number of pigs born (ANB) per litter of gilts and sows mated to individual boars. Fertility data were compiled for 1867 matings across the 18 boars (Fig. 21).

It is of great interest to note the significant correlations between DFI values and average number of piglets per liter. Since oocytes do not discriminate against sperm with damaged DNA, these DNA damaged sperm likely fertilize the eggs and the resulting embryos implant in the female. Those embryos fertilized with high sDF sperm may be lost at a later time when likely needed proteins are lacking due to a break in the DNA/gene required for supplying that vital protein.

Boe-Hansen et al. (2008) studied the relationship between SCSA data from extended and stored boar semen and field fertility. Three ejaculates from each of 145 boars for a total of 3276 inseminations born (litter size) for Hampshire, Landrace and Danish Large White boars was, respectively, 0.5, 0.7 and 0.9 piglets smaller per litter when % DFI values were above 2.1% as opposed to below this value likely due to loss in vivo. It is surprising to see that such a low threshold level of % DFI for fertility and litter size (2–6% DFI). This number is so low that it suggests that perhaps some other factor(s) is being measured besides DNA strand breaks. In any case the data strongly support the use of the SCSA to screen boar semen for fertility and litter size.

D Stallions

The SCSA success with bulls and boars led to a collaboration with Dr. Robert Kenney, Kennett Square, U. of Penn., for an evaluation of stallion sperm DNA and fertility potential (Kenney et al., 1995).

STALLION FERTILITY OUTCOMES

Fertility Classes	% Seasona l PG Rate	% DFI
Fertile	86	16
Subfertile	38	28
Genetically Abnormal	37	39
Functionally Sterile	0.5	41

Fig. 22. Stallion % DFI vs. fertility (Kenney et al., 1995).

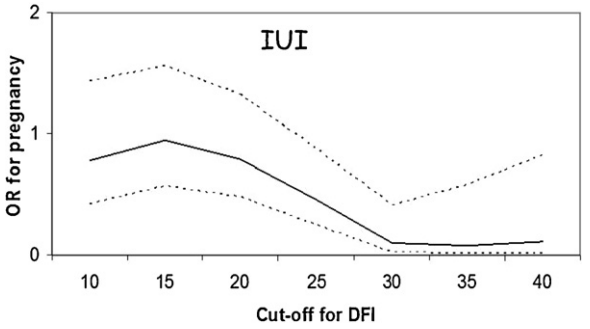


Fig. 23. % DFI threshold for intra-uterine insemination (IUI) fertilization in humans (Bungum et al., 2007).

Fig. 22 shows the relationship between stallion breeding success and SCSA parameters. Even considering the complexities of equine breeding variables, these data are in agreement with the boar and bull data that the SCSA is a valuable test for breeding soundness determinations.

Fortunately, these stallion studies included a Ph.D. student, Charles Love, who has gone on to use the SCSA test for stallion breeding soundness at Texas A&M. Dr. Love and colleagues have done many valuable studies on stallion sperm and these data have clearly shown that the SCSA test is valuable for equine breeding success (Love, 2005).

E Humans

The threshold for human male infertility risk is 25–27% DFI for both natural or IUI fertilization (Bungum et al., 2007; Evenson et al., 1999b; Virro et al., 2004).

As shown in Fig. 23 using IUI insemination, semen samples >27% DFI produced only 4 pregnancies from 87 inseminations. This being the same threshold for natural conception shows that IUI is of poor value for patients having >27% DFI in an ejaculate. Such patients are often referred to Intra-Cytoplasmic Sperm Injection (ICSI) treatments.

6. Etiology of sperm DNA fragmentation

Characterization of mechanisms and causes of sDF is not easy, because there are many intrinsic and extrinsic factors involved. Different factors causing sDF have been proposed. Principally, oxidative stress (Aitken and De Iuliis, 2007), endogenous endonuclease and caspase activation (Maione et al., 1997), alterations to chromatin remodeling during spermiogenesis (Carrell et al., 2007) and apoptosis of germ cells at the beginning of meiosis (Sakkas et al., 2004) have been identified as intrinsic factors. External factors causing DNA damage have also been described, such as radiotherapy, chemotherapy and environmental toxicants (Sailer et al., 1995b; Evenson and Wixon, 2005; Rubes et al., 2005). All of these mechanisms can affect DNA integrity in various manners, producing, in the end, ss DNA or dsDNA breaks (Ribas-Maynou1 et al., 2012). Below are some examples of extrinsic factors affecting sperm DNA integrity.

A Effects of semen preparations, freezing raw semen, cryopreservation and storage on sperm DNA fragmentation.
B Freeze and freeze/thaw
a Mouse

Mice were injected with triethylmelamine (Evenson et al., 1989b) a genotoxic chemical, and over 45 days post injection epididymal sperm were harvested and fresh sperm were measured by the SCSA test. Aliquots of the same samples were flash frozen and stored in LN_2. Two months later the frozen samples were thawed and measured. Fig. 24 compares the %DFI of fresh vs. frozen samples.

Note the very high correlation between the histogram profiles from the fresh measured sperm and that of the same samples frozen and thawed. These data clearly show two very important factors regarding the SCSA test. (1) The sperm chromatin is not altered by the freeze/thaw pro-

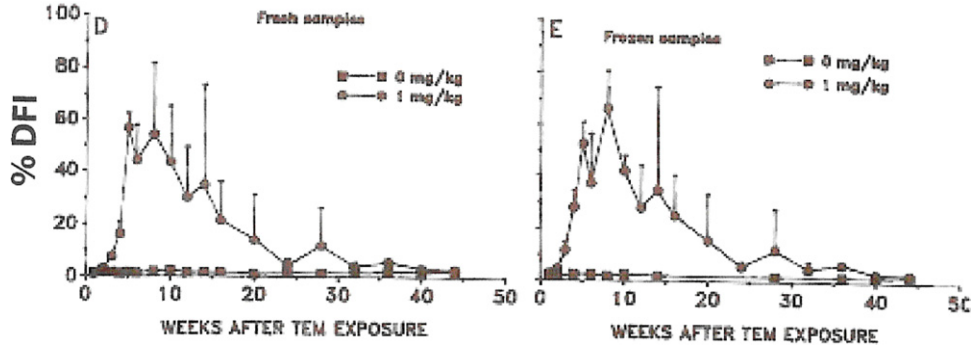


Fig. 24. Sperm from mice treated with triethylmelamine (TEM) and over time harvested with one fresh set measured by SCSA and an aliquot frozen and measured later by SCSA (Evenson et al., 1989b).

cedures, and (2) the flow cytometer is capable of exact repeated measurements even when done months later.

b Large animals

Semen cryopreservation and artificial insemination (AI) offer many advantages to the livestock industry, particularly in conjunction with genetic evaluation and selection programs (Maxwell, 1984). However, the biggest obstacle to exploiting cryopreserved semen of many species is that cooling, freezing, and thawing generally damage sperm membrane structures, leading to fewer viable and motile cells post thaw (Hammerstedt et al., 1990). Consequently, fertility following AI is poorer than with fresh semen in most species (Maxwell, 1984).

i Bull

In a study by Karabinus et al. (Karabinus et al., 1991), semen from four Holstein bulls was evaluated to compare effects of four extender treatments on post thaw semen quality. SCSA data demonstrated differential extender effects on post thaw semen quality and indicate that altering extender composition or sequence of adding extender components may improve post thaw quality of chromatin in cryopreserved sperm.

ii Ram

Ram sperm samples ($n = 12$) underwent the SCSA and semen quality tests, including motility parameters, and viability (Peris et al., 2004) including effects of 20 h of incubation in synthetic oviductal fluid (SOF; 39 °C, 5% CO_2). The SCSA $X\alpha_t$ and $SD\alpha_t$ were higher because of cryopreservation ($P < .05$, $P < .001$, respectively) and 20 h in SOF. For both fresh and frozen spermatozoa, SCSA values ($X\alpha_t$, $SD\alpha_t$, %COMP α_t) increased during incubation in SOF. Motility was negatively correlated with both $SD\alpha_t$ and $\%COMP\alpha_t$, ranging from −0.39 ($P < .01$) to −0.59 ($P < .001$) for both fresh and cryopreserved semen; variation among ejaculates within ram was observed ($P < .01$).

iii Stallion

Semen collected and extended from nine fertile stallions (4 Arabian thoroughbreds and 5 cold bloods) were kept at +4 °C. Increased sperm DNA fragmentation was not seen at 24 h but was observed at 48 h (Krakowski et al., 2013).

Lopez-Fernandez et al. (2007) measured the DFI of chilled (4 °C, $n = 10$) and frozen–thawed ($n = 13$) stallion sperm incubated at 37C by the SCD test. After 1 h both samples showed a significant increase of %DFI, at 6 h over 50% DFI and near 100% at 48 h. The rate of %DFI change showed no difference between stallions or between samples chilled or frozen–thawed.

iv Boar

Hernández et al. (2006) tested the hypothesis that differences in sperm freezability, shown by stud boar semen as 'good' or 'bad' freezers by conventional analyses, could be attributed to differences in chromatin structure. All SCSA parameters were low, but significantly ($P < 0.05$–0.001) higher for 'bad' freezers, showing they had lesser quality sperm chromatin than the 'good' freezers. "The results indicate that SCSA data outcome complements conventional assessment of frozen–thawed (FT) boar sperm, disclosing differences in their ability to sustain freezing and thawing".

Fig. 25 shows that SCSA measures of sperm in semen samples that had been frozen in different ways as well as having different cryoprotectants had no effect on sperm DNA integrity (Evenson et al., 1994b).

7. Other factors affecting sperm DNA fragmentation

A S═S bonds between sperm nuclear protamines.

The redox status of −SH groups on the cysteine residues of sperm nuclear protamines are thought to stabilize

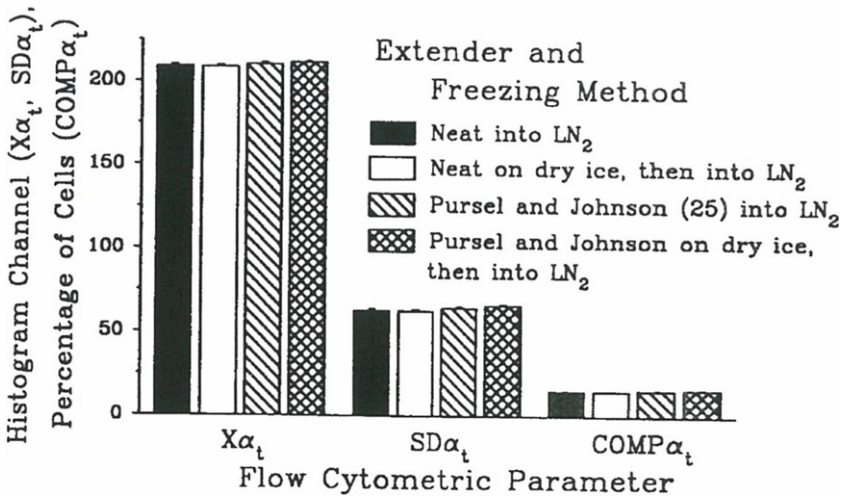


Fig. 25. SCSA variables vs. extender and means of freezing boar sperm (Evenson et al., 1994b).

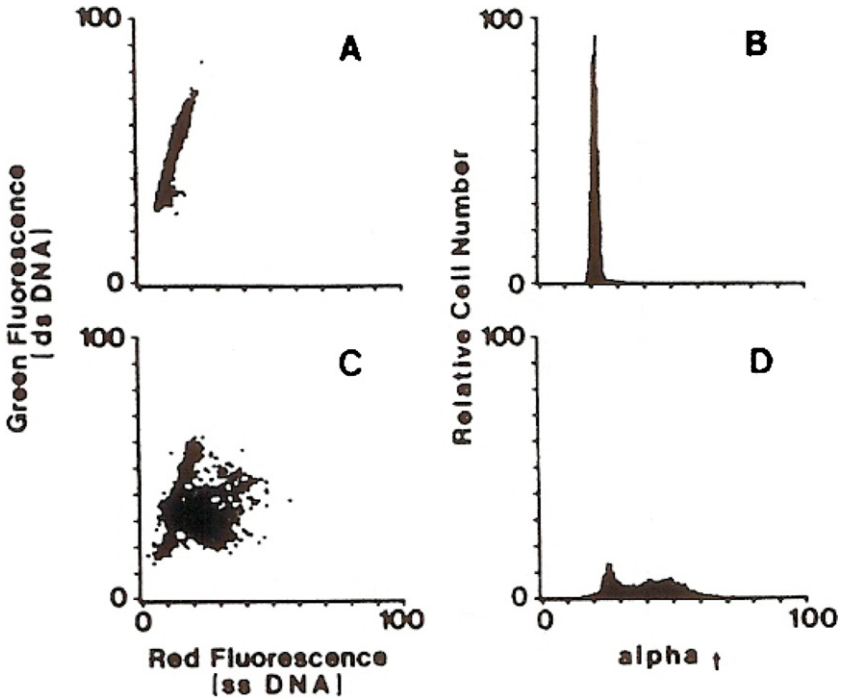


Fig. 26. Effects of Ralgro® supplement to a single bull on sperm DNA fragmentation (sDF) (Ballachey et al., 1986).

chromatin. However, these bonds between sperm protamines likely interfere with access of large protein marker molecules used for the TUNEL test. Some investigators are treating sperm with a S═S reducing agent, dithiothreitol (DTT) prior to the Tunel test to minimize this problem.

Evenson et al. (2000) studied the relationship between stallion ($n = 30$) %DFI and % sperm with free nuclear −SH groups. Sonication freed sperm nuclei were purified and measured by the SCSA test and a −SH specific fluorochrome CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin). This study showed no correlation ($r = -0.199$, $P = 0.31$) of −SH stainability with the extent of DNA denaturation. It is concluded that −SH redox status does not interfere with the SCSA test.

B Growth hormones.

Zeranol (Ralgro®), a resorcylic acid lactone used to increase animal growth causes sDF (Greathouse et al., 1983; Ballachey et al., 1986) as shown in Fig. 26.

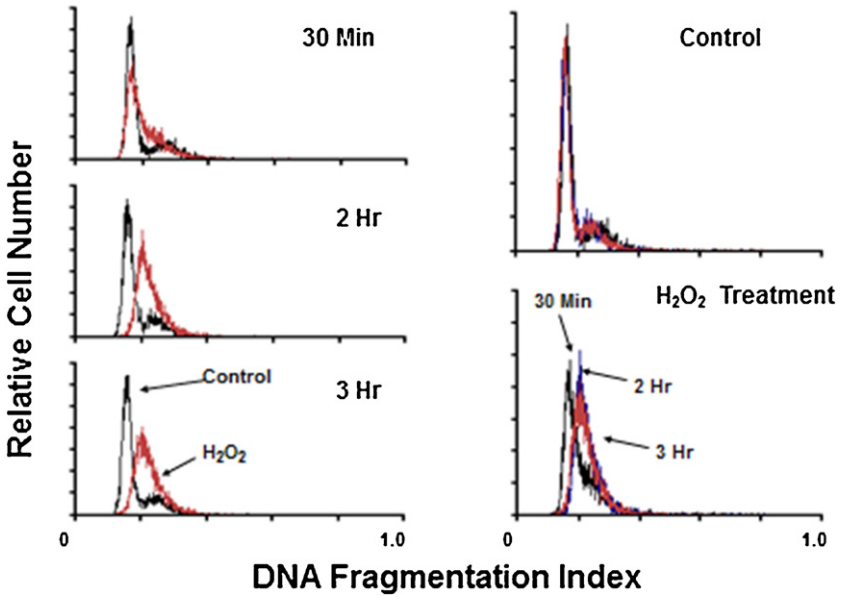


Fig. 27. Effects of in vitro incubation of bull sperm with H_2O_2 on SCSA DFI [unpublished, DPE].

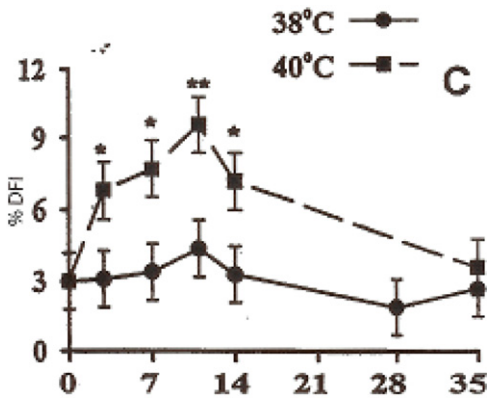


Fig. 28. Effect of heat on mouse epididymal sperm collected 28 days after heat (38° C and 40 °C) applied to mouse testis in water bath (Sailer et al., 1997).

C Oxidative stress

DNA damage in sperm is primarily from oxidative stress (Aitken and De Iuliis, 2007). Oxidative stress [reactive oxygen species (ROS)] activity is of major concern for sperm in vivo, during incubation in vitro, and during passage through the female reproductive tract. Two trials on bull and stallion sperm point out some of the issues.

The effects of ROS in vitro as related to SCSA data are sjown in Fig. 27. Note that the frequency histograms for undamaged and damaged DNA have the same frequency histogram patterns as seen from damaged DNA in sperm from infertile animals or animals exposed to genotoxic chemicals. Thus, both in vivo and in vitro ROS has similar effects.

Wnuk et al. (2010) found a negative correlation between total antioxidant capacity of stallion ($n = 23$) and sperm DNA damage (8-oxoG immunostaining, TUNEL and COMET tests). These authors postulated that the redox status of seminal plasma may be an additional important parameter for evaluation of equine semen quality. In a different view, Morte et al. (2008) correlated oxidative stress caused by ROS and DNA damage with classic semen parameters in sperm and seminal plasma of fertile and subfertile stallions. The authors concluded that the levels of ROS production never seemed to result in compromised sperm DNA integrity (TUNEL), indicating that measurements were within physiological levels and/or that there is an efficient antioxidant activity in stallion sperm cells. From a variety of studies, it appears to this author (DPE) that between the tests of sperm DNA fragmentation and the measurement of antioxidant capacity that the more important of the two is the actual measurement of DNA fragmentation to assess successful pregnancy potential.

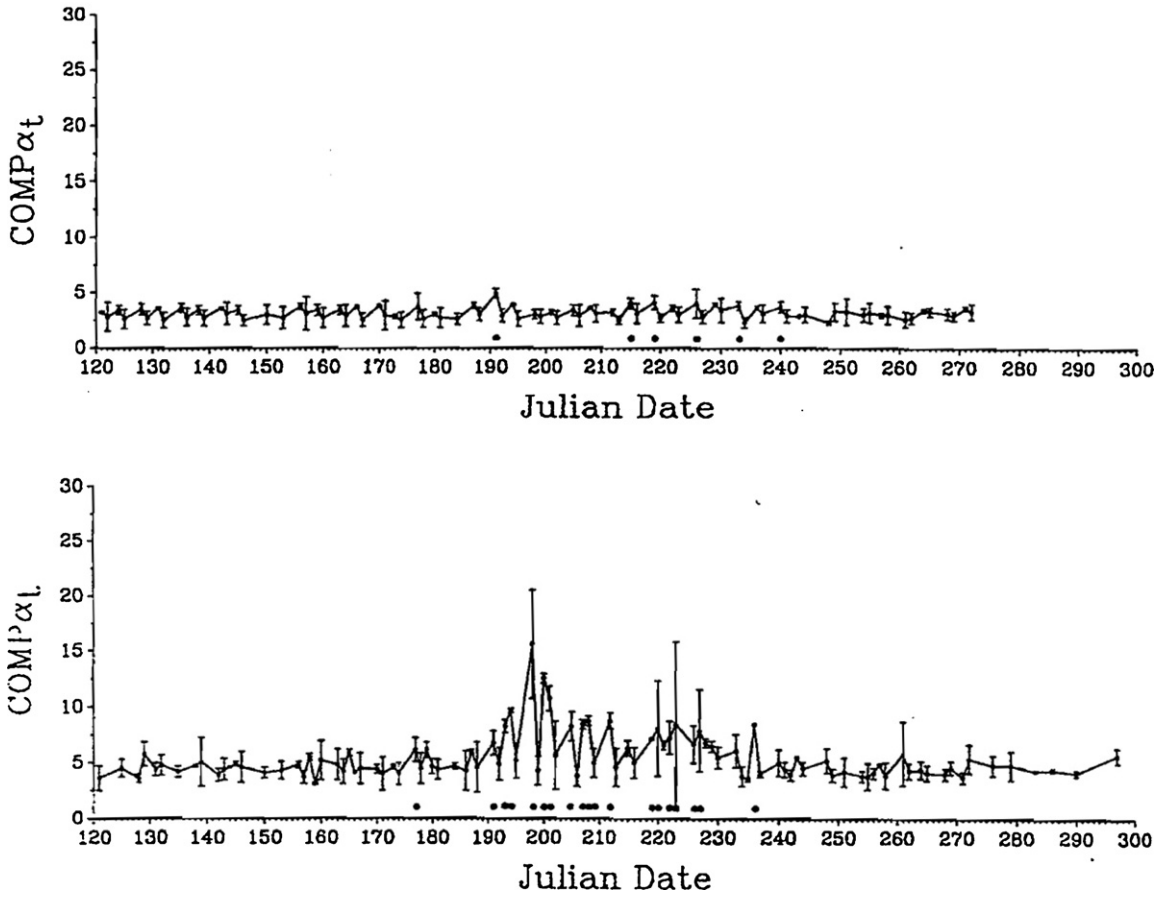


Fig. 29. Effects on sDF on sperm collected over 295 days from bulls with or without sensitivity to environmental heat. [unpublished, Karabinus, Evenson].

D Heat to testis
E Mouse

Fig. 28 shows the effects of heat to mouse scrotum on sDF (Sailer et al., 1997).

2 Bull
a Wool sock on testis.

Semen was collected from six bulls after 48 h of scrotal insulation (SI) with an insulated sock. Increased SCSA values were elevated for the first collections (day +3) and for all other collections on days 3–21 after SI, except for day +9 ($P < 0.001$) (Karabinus et al., 1997).

b Environmental heat

It is known that high daily temperatures can have a negative effect on semen quality of some animal types and also have differences within that specie. Fig. 29 shows effects on bulls that were responsive or not responsive to environmental heat.

E Age of male and %DFI
F Bull

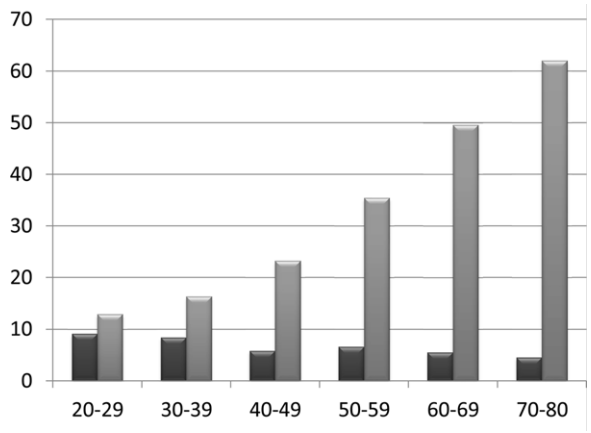


Fig. 30. Effects of aging from 21–80 years in healthy men on sperm SCSA % DFI (grey bars) and % HDS (solid bars) values (Wyrobek et al., 2006).

Aging has a negative effect on bodies including the testis, likely due in large part to oxidative stress. Fig. 16 above showed negative effects of bull aging on sDF.

2 Human
3 Healthy donors

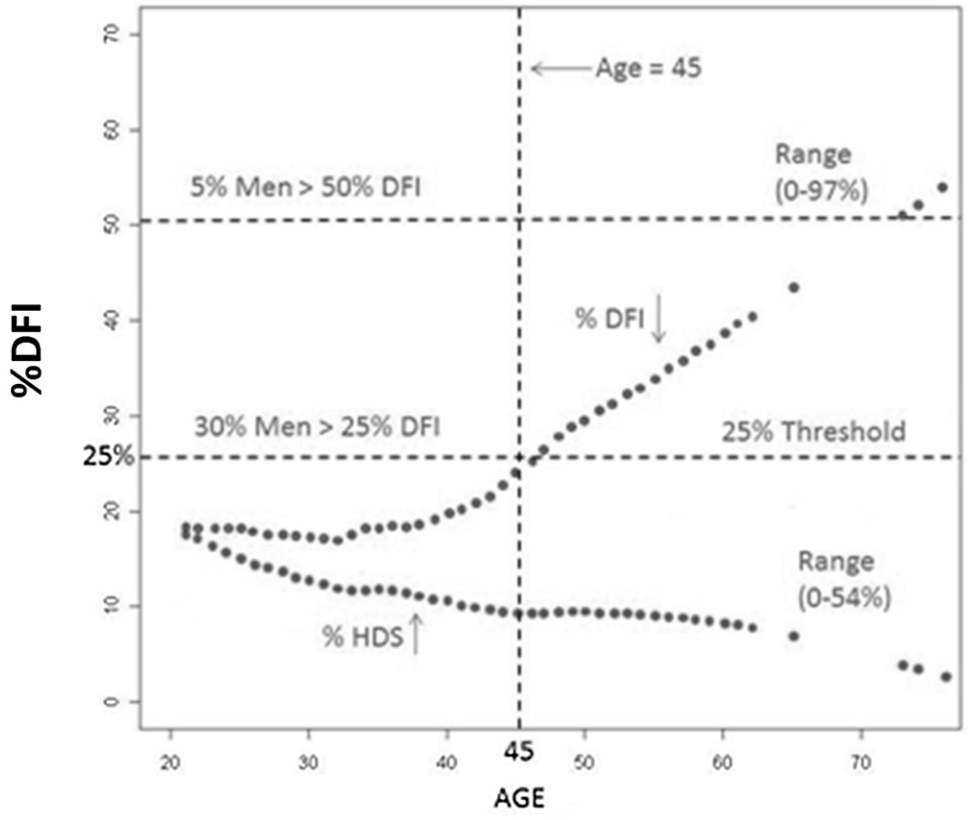


Fig. 31. Means of SCSA % DFI and % HDS of sperm from 3,044 patients attending fertility clinics ages 22–75. The horizontal line at 25% DFI is the human threshold above which places the man at risk for longer time for pregnancy, more miscarriages or no pregnancy. The horizontal line intersects the vertical line showing that on average a man is placed at statistical risk of infertility at age 45 (Evenson et al., 2014). This is the same threshold for healthy donors of the sameage (Wyrobek et al., 2006).

SCSA data on 100 healthy donors' ages 21–80 (Fig. 30) shows that %DFI steadily increased with age. However, % HDS values decreased showing the independence of these two SCSA parameters (Wyrobek et al., 2006).

ii Patients from infertility clinics (Evenson et al., 2014)

In Fig. 31, the horizontal line at 25% DFI is the human % DFI threshold above which places the man at risk for longer time for pregnancy, miscarriages or no pregnancy. This line intersects the vertical line showing that, on average, a man has a statistical risk of reaching >25% DFI by age 45.

8. What is the primary interest of individuals and commercial breeding firms regarding sperm DNA integrity and pregnancy outcomes?

The primary interest of sDF data has been the % DFI as related to the threshold considered for that species. However, a problem in the field of sDF is that the five different DNA fragmentation tests done on different species, produce different estimates of that threshold. The estimated threshold above which the SCSA % DFI has a detrimental impact on fertility varies across species e.g., pigs: 6%, bulls 10–20%, horses: ~28%, humans: 25–30%.

This chapter on the relationship between sperm DNA integrity as measured by five different tests and successful reproduction must leave the reader feeling somewhat confused clinics. Given the hundreds of manuscripts on effects of sperm DNA fragmentation and pregnancy success in both animal and humans, the overall conclusion is that sperm DNA damage has a negative effect on embryo quality and pregnancy outcome. The decision by an animal breeder whether to undergo the cost of sperm DNA fragmentation testing obviously depends on the value of that male breeding soundness testing.

In the commercial world for bovine, porcine and ram, semen from a single male may be used for thousands of cycles; thus the cost of sDF testing seems very small. For equine, where the breeding is focused on performance of the male, the cost of stud fees is relative to that performance. For this author, even given all the complexities shown in this article, testing for the integrity of sperm DNA is considered to have many personal and commercial benefits.

Conflict of interest

I, Donald Evenson, confirm that the manuscript has been written, read and approved by myself and that there are no other persons who fit the criteria for authorship but are not listed. I am the owner and president of SCSA Diagnostics.

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