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Chapter title: Sperm Chromatin Structure Assay (SCSA)

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Douglas T. Carrell, PhD, HCLD and Kenneth T. Aston, PhD

SUMMARY: The SCSA® is the pioneering assay for the detection of damaged sperm DNA and altered proteins in sperm nuclei via flow cytometry of acridine orange (AO) stained sperm. The SCSA® is considered to be the most precise and repeatable test providing very unique, dual parameter data (red vs. green fluorescence) on a 1024 x 1024 channel scale, not only on DNA fragmentation, but abnormal sperm characterized by lack of normal exchange of histones to protamines. Raw semen/sperm aliquots or purified sperm can be flash frozen, placed in a box with dry ice and shipped by overnight courier to an experienced SCSA® lab. The samples are individually thawed, prepared and analyzed in ~10 min. Of significance, data on five thousand individual sperm are recorded on a 1024 x 1024 dot plot of green (native DNA) and red (broken DNA) fluorescence. Repeat measurements have virtually identical dot plot patterns showing that: A) the low pH treatment that opens up the DNA strands at the sites of breaks and B) staining by acridine orange (AO) is very highly precise and repeatable (CV's of 1-3%) and the same between fresh and frozen samples. SCSAsoft® software transforms the X-Y data to total DNA stainability vs. red/red + green flouresence (DFI) that provides a more accurate determination of % DFI as well as the more sensitive value of standard deviation of DFI (SD DFI) as shown for animal fertility and dose-response toxicology. 25% DFI is currently the established clinical threshold for placing a man into a statistical probability of: a) longer time to natural pregnancy, b) low odds of IUI pregnancy, c) more miscarriages, or d) no pregnancy. Changes of lifestyle as well as medical intervention can lower the %DFI to increase THE probability of natural pregnancy. Couples of men with >25% DFI are counseled to try ICSI and when in the >50% range may consider TESE/ICSI. The SCSA® simultaneously determines the % of sperm with high DNA stainability %HDS) related to retained nuclear histones consistent with immature sperm; high HDS values are predictive of pregnancy failure.

The SCSA® is consider to be the most: a) technician friendly, b) time and cost efficient, c) precise and repeatable, with most data and the only one with an accepted clinical threshold for placing a man at risk of infertility. SCSA® data are more predictive of male factor infertility than classical semen analyses.

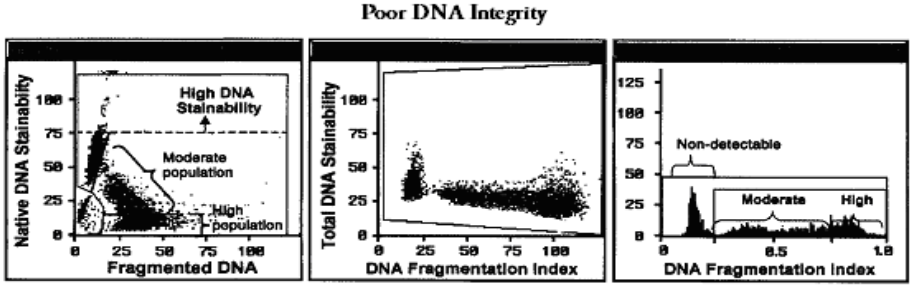
I.UNIQUENESS OF THE SCSA®. The SCSA® is considered to be the most precise and repeatable test providing very unique, dual parameter data (red vs. green fluorescence) on a 1024 x 1024 channel scale, not only on DNA fragmentation, but abnormal sperm characterized by lack of normal exchange of histones to protamines. These abnormal protein/DNA complexes allow a greater access of acridine orange dye (AO) to sperm DNA thus producing a High DNA Stainable (green fluorescence) fraction (HDS). HDS data are highly correlated ($r=0.610$, $p<0.001$) with data derived from other tests for excess histones such as the CMA_3 test (3). In one study (1), the HDS fraction contained unprocessed P2 protamines, likely resulting in structural chromatin abnormalities that allowed an increased amount of DNA being available for AO staining.

The SCSA® is ideal for researchers and students since the reagents cost only about 10 cents per test rather than dollars per test allowing many repeat experimental tests to be done at very little cost provided that a relatively inexpensive , two parameter flow cytometer is available.

II. DIRECT VS INDIRECT SPERM DNA FRAGMENTATION TESTS. Some authors have classified the various sperm DNA fragmentation tests as being either a direct or indirect test. For example, the Tunel test is listed as a direct test on the assumption that the DNA strand break marker has “direct” access to the broken strand. The SCSA® is listed as an indirect test since it requires that an agent, heat or low pH, to open the DNA double helix at the sites of DNA strand breaks. However, when one considers that the Tunel test requires extensive pelleting and

washing of the sperm, permeabilization of the cell membranes, agents (e.g. DTT) in some cases to open up the S-S bonds of chromatin structure that is a maze of chromatin fibers (2), incubation with enzymes of potential varying activity and washing out of non-specific label, it hardly seems appropriate to consider this as a direct test that takes at least 4 hours to conduct. In sharp contrast, the SCSA® only requires that fresh or frozen/thawed semen is treated for 30 sec with pH 1.2 buffer to open, with extremely high and repeatable precision, the DNA at the sites of strand breaks followed by staining with AO that stays in equilibrium with the cells as they are measured. Thus, this author considers the SCSA® to be as much or more a "direct test" of sperm DNA fragmentation.as others.

III.SCSA® DATA. Fig. 1 illustrates SCSA® raw data and SCSAsoft® converted data on human sperm.



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 1. Left panel: Green vs. red scattergram (cytogram) showing 5000 dots, each representing a single event with specific green (native DNA) and red (fragmented DNA) coordinates on a scale from 0 to 1024. The horizontal dashed line lays at the top of the highest green fluorescence values for normal sperm. Sperm above this line have "High DNA Stainabilty" (HDS) and are characterized by immature sperm lacking full protamination.

Center panel: SCSAsoft® software (SCSA Diagnostics., Brookings, SD) converts the data in the left panel to total DNA stainability vs. the DNA Fragmentation Index (DFI). This re-orients the data into a vertical/horizontal pattern of dots.

Right panel: The data in the middle panel is converted to a frequency histogram of DFI which is divided into a) non-detectable DNA fragmentation, b) moderate level of DNA fragmentation, and c) high level of DNA fragmentation. Total %DFI is Moderate + High level of DNA fragmentation, a parameter that is most frequently used in expressing the extent of sperm DNA fragmentation in a sample. This method, derived from SCSAsoft®, provides a much more accurate calculation of total %DFI due to the difficulties to accurately gate between the populations with none or moderate fragmentation in the left hand panel. Fig. 4 is an example of a sample that requires SCSAsoft for correct interpretation.

IV.CHARACTERIZATION OF SPERM POPULATIONS IDENTIFIED IN SCSA® ANALYSIS

Flow cytometer-- sorted SCSA processed human sperm (3) showed that Feulgen stained --moderate %DFI sperm had normal nuclear shape while high DFI sperm nuclei had a smaller area. The HDS fraction, known to be immature sperm, had significantly more nuclear area and roundness as would be expected from immature sperm. Comet analysis showed that approximately 75% of the sperm with moderate and high DNA fragmentation had positive Comets while the population without sperm DNA fragmentation (Norm) and the population of sperm with high DNA stainability (HDS) only showed a minor degree of background noise level. Several conclusions can be drawn from this: 1) Sperm with fragmented DNA in a SCSA® analysis demonstrate true DNA strand breaks, 2) HDS sperm having an increased ratio of histones to protamines do not have any significant amount of DNA strand breaks.

V. POWER OF THE SCSA® TEST: Uniquely, biochemical interactions between AO and DNA/chromatin are precisely repeatable with any single sample. This is proven by comparing cryptograms (X vs. Y scatter plots) of repeat measures of a single semen sample. The dot pattern from replicate measures is virtually identical on a 1024 x 1024 scatter plot. Thus, both the 30 sec low pH induced opening of the DNA strands at site of DNA breaks and the AO labeling are highly specific and repeatable in exacting patterns.

VI. PRECISION AND REPEATABILITY OF SCSA® DATA OVER TIME

The visual patterns of scatter plots of 5,000 sperm/sample are strikingly exact repeat patterns (4) and the CV of the repeat data typically 1-3% as seen for consecutive monthly semen samples from four men (Fig. 2).

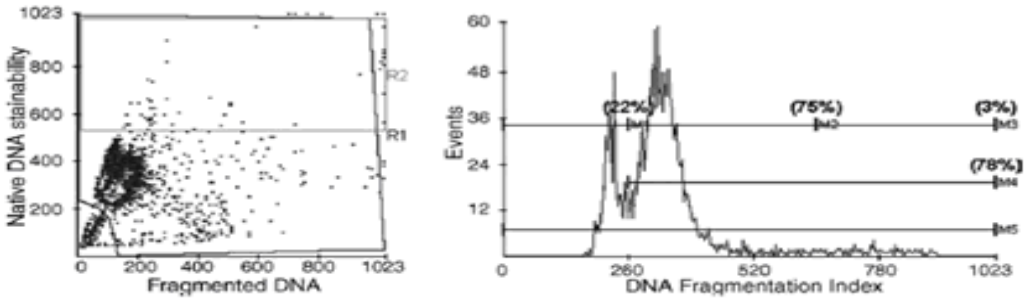


Fig 2. SCSA® data from a sample with a high frequency of sperm with moderate DNA fragmentation. In this case, it is impossible to gate between sperm with no or moderate DNA fragmentation in the FCM dot-plot (left panel). With the SCSAsoft® gating between the two populations is unproblematic (right panel).

The SCSA® values have a very high level of repeatability provided that no significant event has happened between collection times, e.g., hot tubs, medications, fever, illness, etc... One report (5) showed a considerable CV of 30% for human patient semen samples obtained over several years from the same patients. This is, in part, expected as patients presenting with a high %DFI could have factors that cause DFI fragmentation such as varicocele, obesity, poor diet, extreme sports, routine hot tub/sauna use, infections, disease, pesticide/chemical exposure, spinal cord damage, use of medications such as SSRI's. When such factors are removed or treated, the level of %DFI may well have significant decreases. In fact, it can be stated, that unless changes can be made by personal lifestyle and/or medical intervention, the use of SCSA® in clinical settings would have lesser utility.

VII. REPEATABILITY OF SCSA® DATA BETWEEN FRESH AND FROZEN/THAWED EPIDIDYMAL SPERM ON LONG TERM RECOVERY FROM TOXICANT EXPOSURE

11 month recovery. A fresh, raw semen or sperm sample (e.g. mouse epididymal sperm) can be measured immediately or flash frozen, thawed and analyzed; the data are highly repeatable between the fresh and frozen/stored/thawed samples (6) as seen in Fig. 3.

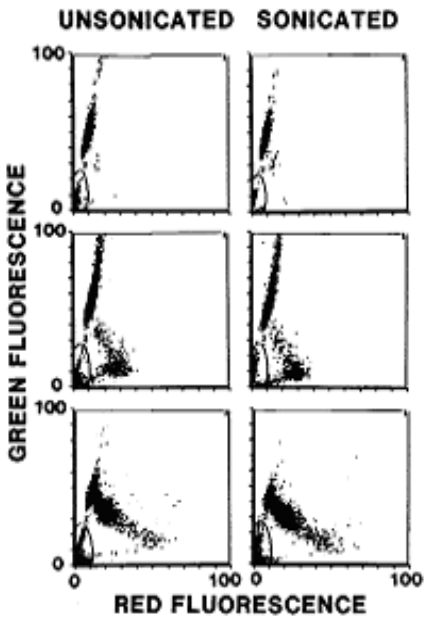


Fig 3. Each human semen sample was diluted to a final volume of 0.5 ml with TNE buffer to obtain a count of approximately 2 x 10^6 sperm/ml. The samples in the right column were sonicated for 30 sec with a Branson 450 Sonifier operating at a power setting of 3 and utilizing 70% of 1 sec pulses.

VIII. SCSA DATA ON EPIDIDYMAL SPERM FOLLOWING EXPOSURE TO THE MUTAGEN, METHYL METHANESULFONATE (MMS) AS RELATED TO DOMINANT LETHAL MUTATIONS/ EMBRYO DEATH.

Exposed males (MMS) were mated and dominant lethal mutation deaths were scored at 1-4, 5-8, 9-12, 13-16 and 17-20 days post conception. The first deaths were seen at the 5-8 day period while 85% of sperm had extensive DNA damage at 1-3 days post exposure (7). Thus, the SCSA® data likely showed the molecular precursor events (DNA breaks) that led to embryo deaths.

IX. SCSA DATA ON ANIMAL FERTILITY PROVIDING HIGHLY CONVINCING ARGUMENT THAT SCSA DATA PREDICT MALE SUB/INFERTILITY

The SCSA® has established clinically significant thresholds for male factor sub/infertility for both large domestic animals [bulls (8, 9) and stallions [10] and humans. (11-14). When ≥20-25% of sperm in a semen sample have increased red fluorescence due to (ss) or (ds) DNA breaks, the patient/animal is placed into a statistical category of: a) taking a longer time to pregnancy, b) more IVF cycles, c) more miscarriages or d) no pregnancy. At the 30% DFI level, the odds ratio (OR) is reduced 8-10 fold for a successful natural or IUI pregnancy. Several studies (15, 16) have shown the exception for boars where pregnancy rates and pigs/litter are reduced when the %DFI is >6%. Sperm with severely damaged DNA fertilize eggs that may lead to embryo death.(15-17).

X. POTENTIAL RNA STAINING ARTIFACTS FOR SCSA®

Since AO stains both single stranded DNA and RNA the fluorescent color red, it was very important to know if cytoplasmic or nuclear RNA contributed to the red fluorescence that might be erroneously attributed to denatured DNA. We addressed this question (4) by sonicating whole bull, mouse, and human sperm, purifying each sample of nuclei through a sucrose gradient and measuring both the sonicated and non-sonicated sperm by SCSA®. As illustrated in Fig. 4, the unsonicated and sonicated sperm produced cytograms that were virtually identical.

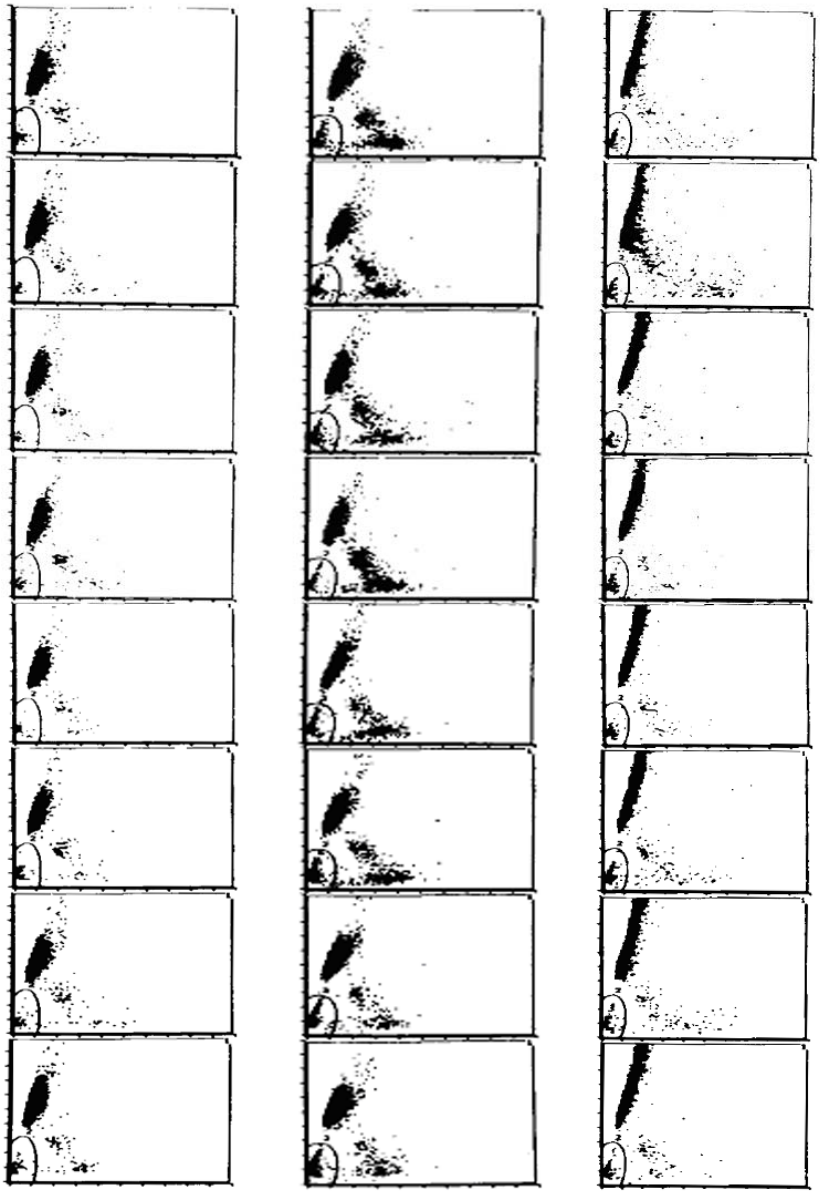


Fig 4 Green vs. red fluorescence cytograms from monthly semen samples provided by 3 donors. Examples are selected from the 45 men illustration of different types of cytogram patterns (10).

XI. CLINICAL UTILITY OF SPERM DNA FRAGMENTATION TESTS

A. Confusion from multiple DNA fragmentation tests. All sperm DNA fragmentation tests report results as %DFI, as was initially coined for the SCSA® test. However, these % DFI numbers may mean different things for different tests. For example, light microscope Tunel data give different %DFI than flow cytometry Tunel. Also, Tunel %DFI may not be the same as for flow cytometry SCSA®. This is most likely due to the requirements of Tunel to have large protein enzymes enter into the highly compact and tangled nuclear chromatin (2) to tag DNA strand breaks. In sharp contrast, the SCSA® molecular tag is a very small acridine orange molecule (MW 265) that can penetrate the maze of nuclear chromatin and, furthermore, remains in equilibrium with the sperm during SCSA® measurement. In attempts to increase access to fragmented DNA sites for the Tunel test, some investigators have used disulfide bond reducing agents in the protocol to open the highly complex nuclear chromatin with disulfide bond reducing agents. However, since some sperm nuclei of "normal WHO samples" have diffuse chromatin and others highly compact chromatin (2), there would likely be differential effects from a DTT treatment. This was dramatically shown by Evenson et al (18) when measuring the kinetics of human and mouse sperm chromatin uptake of a DNA stain following DTT and/or protease treatments. The mouse nuclei responded uniformly while the sperm from a fertile man were highly heterogeneous in response to DTT treatment.

B. Power of SD DFI. The use of SCSAsoft® software to re-orient the raw green vs. red dot plots to green vs. green/ (red + green) dot plots followed by the generation of a frequency histogram of the converted dot plots provides a more accurate determination of %DFI, especially those samples that have poor resolution between normal and moderate DFI (Fig 5).

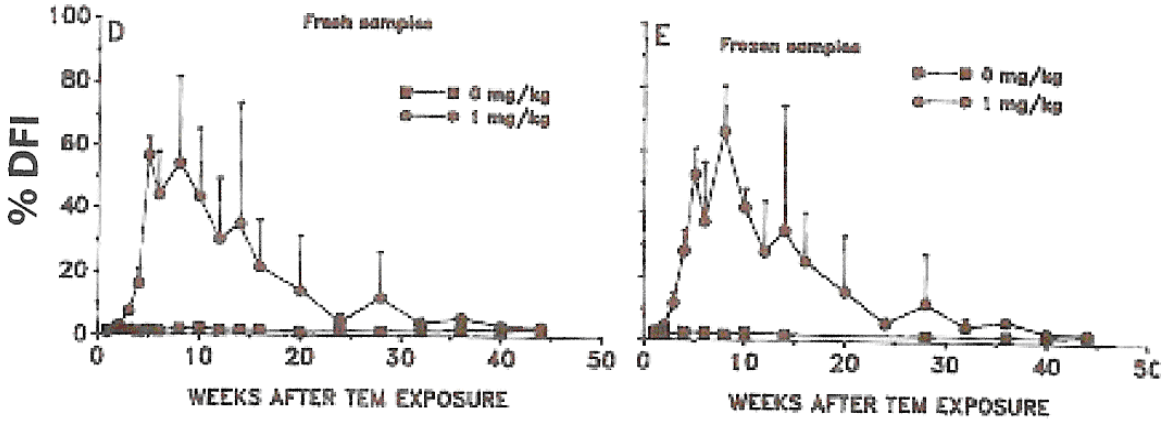


Fig 5. Effects of 1.0 mg/kg (daily x 5) TEM on %DFI in epididymal sperm during a 44 wk period. Left: %DFI on fresh samples. Right: Aliquots of the same samples frozen and measured later at a single time period.

XII. ANIMAL FERTILITY STUDIES related to %DFI, DFI and SD DFI

Importantly, the value of SD of DFI (SD DFI) is often a more sensitive measure of sperm DNA damage than % DFI alone. One reason for this difference is that DFI is determined on the whole population of sperm measured and is not subject to dividing the population into normal and increased red fluorescence populations by computer gating. This has been solidly shown in animal fertility and animal toxicology studies.

A. Bulls Semen from individual bulls is often used for hundreds to thousands of cow inseminations. Thus, fertility rankings can be made between bulls in a stud service. Negative correlations were seen between fertility ratings and both SD DFI (-0.58, $P < 0.01$) and %DFI (-.40, $P < 0.01$). Inherent in studies as above, and much more so with human studies, are the variables in the females and a host of other factors such as experience of the artificial

insemination team. To get around this problem, animal studies can use what is known as heterospermic insemination protocols in which equal numbers of motile sperm from two or more phenotypically different bulls are mixed prior to insemination. The parentage of calves resulting from these matings is determined and, based on the number of calves sired with each phenotype, a competitive fertility index is derived for each bull (9). Correlations of SD DFI and %DFI with competitive index were -0.94 ($P < 0.01$) and -.74 ($P<0.05$), respectively.

B. Boars. Multiparous pigs allow for a determination of both fertility rate and number of piglets per litter. The SCSA® was used (15) retrospectively to characterize sperm from 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. In contrast to humans and other mammals studied where the threshold for reduced fertility is an approximate 25-30% DFI, the threshold for boars is about 6% DFI. The %DFI and SD DFI showed the following significant negative correlations with FR and ANB; %DFI vs. FR, $r = -0.55$, $P < .01$; SD DFI vs. FR, $r = -0.67$; %DFI vs. ANB, $r = -0.54$, $P <.01$ and SD DFI vs. ANB, $r= -0.54$, $P <.02$. The present data suggest that boar sperm possessing fragmented DNA can affect embryonic development corroborating earlier studies in mice showing that fertilization occurs whether the sperm has damaged DNA or not (17) but may cause embryonic death.

XIII. SCSA® DEFINED ETIOLOGIES OF INCREASED DNA FRAGMENTATION

The most likely common factor in causing sperm DNA fragmentation is oxidative stress (ROS) (19). Factors related to increased %DFI include: age (20), genetics (21), air pollution (22), varicoceles (23-27), cancer and cancer and cancer treatment (27, 28), pesticides (29), environmental heat (30-31), fever (32), medications (33), and diabetes (34-36).

IVX. HUMAN CLINICAL UTILITY OF THE SCSA® The American Society for Reproductive Medicine (ASRM) consensus committee on *"the clinical use of sperm DNA fragmentation assays for clinical patients"* has stated that Sperm DNA fragmentation tests are recommended for certain patients but it has not been recommended as a part of the ***routine*** semen analysis although some clinics have implemented this approach already. At the time of the 2006/08 ASRM reports (37) there were only two SCSA® reports on the odds ratios (OR) of natural pregnancy. These studies reported an odds ratio of 8-10 fold reduction for pregnancy when the %DFI was >25-30%. Unfortunately, the ASRM consensus report erroneously showed this as OR of 2.08 (37) which implied lesser utility for classifying men's sperm DNA fragmentation levels and reduced fertility. Since that time, the studies of Givercman et al (14) on natural fertility OR, using the same SCSA® protocol, confirmed an even higher odds ratio when the SCSA® %DFI was > 20%.

XV. CONCLUSION. The SCSA® is the easiest and most rapid DNA fragmentation test in the laboratory. The SCSA is also the most precise and repeatable with CV of ~1-3%. Due to the low cost of supplies, numerous measurements can be made for both research and clinical applications.

XII. REFERENCES

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