Chapter 20

DNA Damage: Sperm Chromatin Structure Assay

Sperm Chromatin Structure Assay Test on its Fortieth Anniversary

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20.1 Introduction

The year 2020 is the fortieth anniversary of the introduction of the concept of "sperm DNA fragmentation" as related to human and animal male factor fertility. This concept was introduced by Donald Evenson in a *Science* article (1980) that also introduced the first test for its detection, the Sperm Chromatin Structure Assay (SCSA®). Sperm DNA fragmentation is defined as sperm single and double DNA strand breaks. Experiments on bulls, boars and stallions clearly show that the SCSA test identifies the highest fertile animals. Thousands of measurements on clinical human semen samples also clearly show that when more than 25 percent of sperm (DFI pecentage) in an ejaculate have measurable DNA fragmentation the probability of live birth pregnancy is significantly diminished. SCSA data on percentage DFI and Mean DFI are the same, meaning that the SCSA test measures the total of sperm DNA strand breaks detected with acridine orange staining.

20.2 Sperm Chromatin Structure Assay History 1980–2020

In 2011 John Aitken wrote the Forward to the book *Sperm Chromatin: Biological and Clinical Applications in Male Infertility and Assisted Reproduction*, edited by A. Zini and A. Agarwal. Aitken wrote: "The impetus to study the composition and integrity of sperm chromatin from a clinical perspective can be traced back to the pioneering studies of Don Evenson, who not only initiated research in this area long before it became fashionable but also pioneered one of the major analytical techniques used in assessment of sperm chromatin, the Sperm Chromatin Structure Assay (SCSA). This assay has now become the industry standard against all other techniques."

Going forward to 2017 for an assessment on the value of our anniversary finding there is a quote from C. O'Neill and G. Palermo writing: "Many assays have been introduced into male infertility testing over the past two decades regarding varying aspects of spermatozoal competence. However, none have provided more clinically relevant data and insight into male fertility potential than the study of DNA fragmentation in the male gamete" [1].

Going to PubMed and entering our coined term "Sperm DNA fragmentation" yields 2400 manuscripts. Entering "Sperm Chromatin Structure Assay" yields 804 manuscripts. Our pioneering SCSA clinical paper [2] is among the top three papers ever cited from Human Reproduction. The evidence is clear, the SCSA test has a highly significant impact on the outcome for patients struggling with infertility.

The SCSA test has been extensively evaluated and validated for biochemical and fertility outcome soundness. Sperm from many hundreds of animals exposed to a variety of agents and environmental conditions have been analyzed to understand the SCSA test. On this fortieth anniversary of the SCSA test it can be solidly stated that the SCSA test is the most statistically robust assay for understanding the concepts of sperm DNA fragmentation.

20.3 The Sperm Chromatin Structure Assay Test

The original SCSA test [3] used heating of sperm (100^0C/five minutes) to open the sperm nuclear DNA strands at sites of single (ss) and double (ds) DNA breaks. These DNA strand breaks are captured by staining with fluorescent acridine orange (AO). AO intercalated into ds DNA fluoresces green while AO stained ss DNA collapses into a crystal that upon

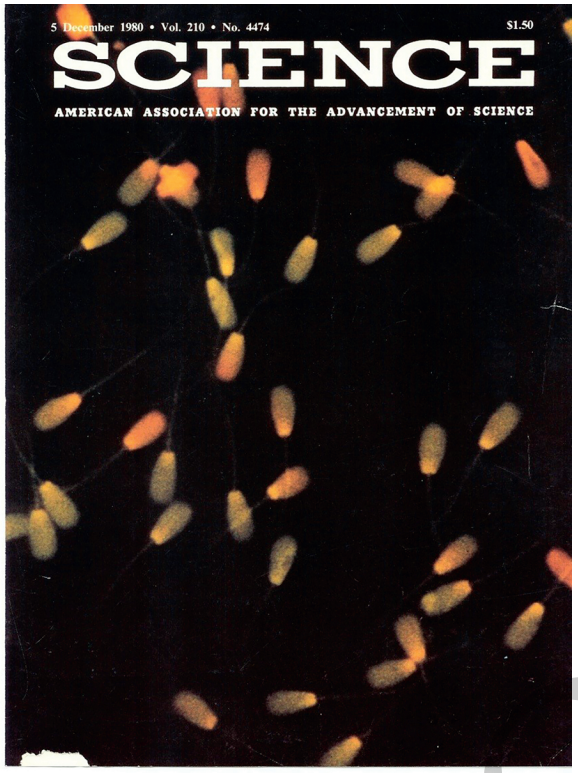


Figure 20.1 Cover of *Science* (1980) showing AO stained heated bull sperm.

exposure to 488 nm light has a metachromatic shift from green to red fluorescence as seen in the *Science* cover (Figure 20.1).

While many have tried to quantitate sperm DNA fragmentation with light microscopy of AO stained sperm [4], it is now clear that due to many technical problems this can not reliably by done by light microscopy [5] but is, however, very accurately accomplished by flow cytometry [2, 6].

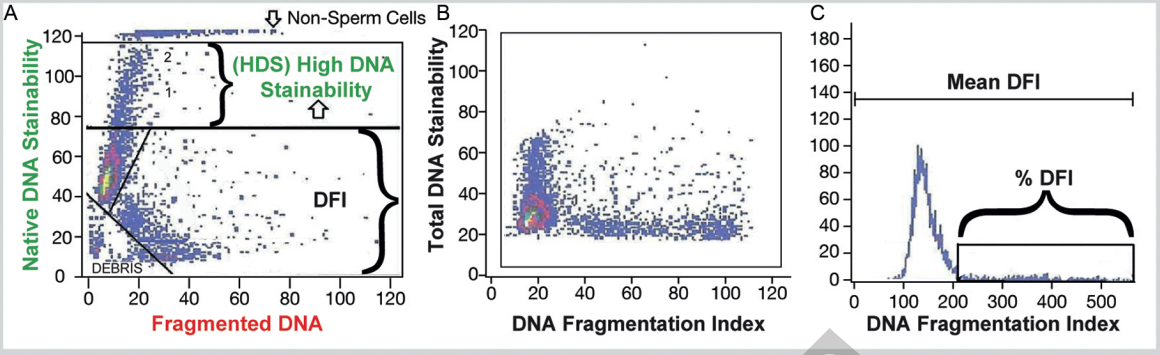
This *Science* article [3] showed the increased shift from green to red fluorescence in sperm from sub-infertile bulls and humans. Sub-fertile bulls and infertility clinic patients had 2.6x and 1.6x increased mean alpha t (Mean DFI) respectively, relative to highly fertile bulls and men. These data were very encouraging for the prospect of using the SCSA test in the human infertility clinic; however, before using this new test in the highly sensitive area of human infertility, this test was evaluated in numerous ways including toxicology, reproductive biology and animal fertility experiments.

20.4 The Sperm Chromatin Structure Assay Test Protocol

Switching from heating of sperm to the new acid denaturation protocol is done as per the following and has been described [5–7]. Individual frozen semen samples are thawed in a 37^0C water bath just until all ice has melted and then immediately diluted with TNE buffer (0.01M TRIS, 0.15 M NaCl, 1 mM EDTA, 4°C) to a final concentration of 1–2 x 10^6 sperm/mL. A 200 µL sperm suspension is admixed with 400 µL acid solution (0.1 percent Triton X-100, 0.15 M NaCl, and 0.08 N HCI, pH 1.20, 4°C) for 30 seconds followed by addition of 1.20 mL of acridine orange (AO) staining solution (containing 6 µg chromatographically purified AO (Polysciences Inc., Warrington, Pennsylvania) per mL of AO buffer (370 mL of stock 0.1 M citric acid, 630 mL of stock 0.2 M Na2HPO4 disodium hydrogen phosphate , 1 mM disodium EDTA. 0.15 M NaCl, pH 6.0, 4°C) as previously described in detail [6, 7]. Individual samples are placed into a flow cytometer (for our lab an Ortho Diagnostics L30 flow cytometer (FCM)) and after ~2 minutes of hydrodynamic equilibration of sample and sheath flow, 5000 sperm are measured at rates of ≤250 cells/second. All samples are measured independently twice. The mean values of the two independent measurements are then calculated. These mean data are processed through SCSAsoft® software (or equivalent) to produce a clinical report. These reports are sent back to the clinics via a secured WEB address.

Figure 20.2 shows SCSA raw data obtained by the acid denaturation protocol and processed though our SCSAsoft software.

Very importantly, all semen samples are measured by **exactly** the same strict protocol. Prior to measurements, laser focus is accomplished by maximizing red and green fluorescence values of fluorescent polystyrene beads [7]. Also, a positive (high percentage DFI) and a negative (low percentage DFI) semen sample are measured to verify the results as previously established. Then a reference semen sample from a set made up with hundreds of samples of small aliquots stored in LN_2 are used to set the red and green photomultipliers tubes to the same (± 5/1024 channels) X, Y coordinates; approximately 540/1024 green versus 130/1024 red. This setting allows the capture of high DNA stainable (HDS) sperm. When one set of reference samples is nearly depleted, another set is



File	% DFI	% HDS	Mean DFI	Std Dev DFI
1	17.66	8.40	222.4	182.2
2	18.00	8.80	224.7	186.9
mean	17.8	8.6	223.6	184.6
std dev	0.2	0.3	1.6	3.4

Figure 20.2 SCSA test data. **A)** Raw data from a flow cytometer showing each of 5000 sperm as a single dot on a scattergram. Y axis = green fluorescence with 1024 gradations (channels) of DNA stainability. X axis = red fluorescence with 1024 gradations of red fluorescence (ss DNA). Axes shown are 1024/10. Dotted line at Y = 75 marks the upper boundary of DNA staining of normal sperm chromatin; above that line are sperm (dots) with partially uncondensed chromatin allowing more DNA stainability (HDS sperm). Bottom left corner shows gating out of seminal debris**. B)** Raw data from left panel are converted by SCSAsoft® software (or equivalent) to red/red + green fluorescence. This transforms the angled sperm display in left panel to a vertical pattern that is often critical for accurately delineating the percentage of sperm with fragmented DNA. Y axis = total DNA stainability versus X axis = red/red + green fluorescence (DFI). **C)** Frequency histogram of data from middle panel showing computer gating into percentage DFI and Mean DFI. Bottom box. SCSAsoft calculations of mean of two independent measures of: percentage DFI, percentage HDS, mean DFI and SD DFI. **Note:** Mean DFI are presented here with range from 0–1024 flow cytometer channels. Some studies have shown this in a range from 0–1; e.g. 0.22.

made, even if from a different individual with different mean red (X) and green (Y) values. This is accomplished by first measuring the previous reference sample, then the new reference semen sample is measured at the same red/green photomultiplier gains and noting the new mean red and green fluorescence values. In this fashion, samples measured years ago can be measured again with the new reference sample and obtain the near exact same results [7].

20.5 Examples of Sperm Chromatin Structure Assay Validation Experiments

20.5.1 Toxicology

Triethylenemelamine, a trifunctional alkylating agent, has a highly negative effect on mammalian spermatogenesis as seen in Figure 20.3. For the purpose of this chapter, two important factors were learned about the SCSA test from our study on TEM treated mice [8].

1. Fresh sperm and frozen/thawed sperm produce the same near exact results.
2. Measurements made of freshly isolated sperm at repeated times following toxicant exposure provided the same results as when aliquots of the frozen and stored samples were measured months later; thus showing that the flow cytometer variables can be repeated with exacting results.

20.5.2 Human Air Pollution

Since the 1950s the residents of Teplice, Czech Republic, had been exposed to high levels of air pollution generated from the combustion of high-sulfur coal used for local industry and home heating. The air pollution was severe during the winter when climate inversion smog conditions existed. Infertility and miscarriage were a significant problem. The Czech Republic government and the US EPA came in to evaluate the problem. The study protocol had semen

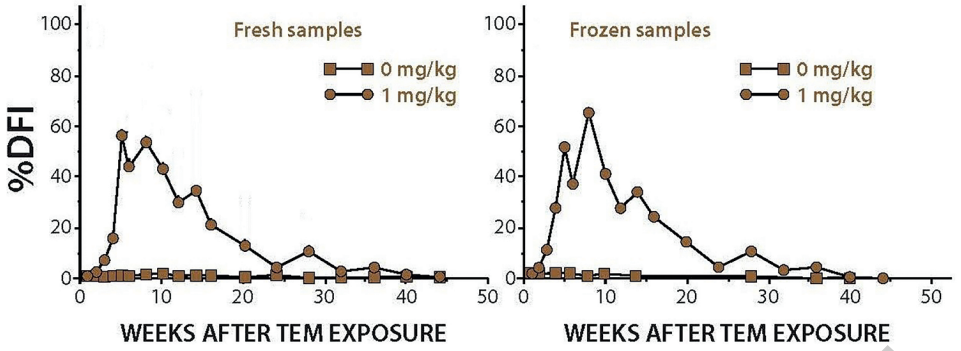


Figure 20.3 Epididymal sperm from mice treated with triethylenemelamine (TEM) and over 45 weeks harvested with one fresh sample measured at the time of isolation and frozen/thawed aliquots measured months later.

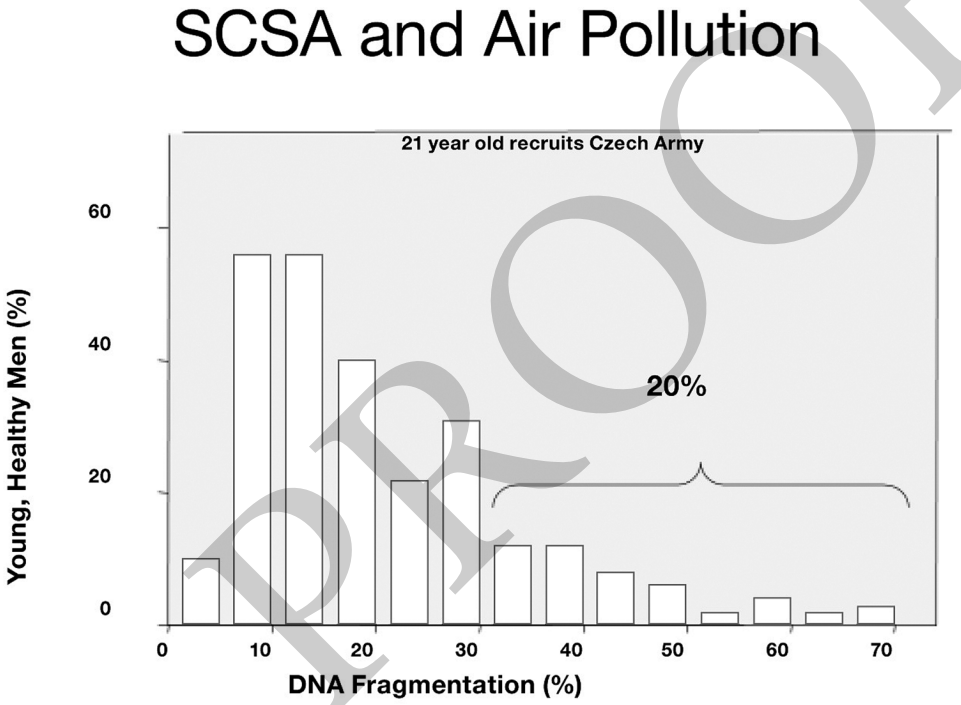


Figure 20.4 Percent of men with extent of sperm percentage DFI fragmentation. Twenty percent of men had pathological (>30 percent DFI) levels of DNA fragmentation.

sample testing of young men over two years that included both winter-time smog and summer-time clean air exposure. Figure 20.4 shows that the Teplice donors had very poor SCSA percentage DFI data during wintertime smog exposure [9]. While the great majority of semen samples should normally have been in the 5–10 percent DFI range, the majority of these samples were above that level, with 20 percent being in the pathological range above 30 percent DFI. Interestingly, semen samples from Teplice men exposed to the winter-time air pollution had an increase of sperm motility.

20.5.3 Reproductive Biology

Analysis of monthly semen samples obtained over eight consecutive months from donor men [10] showed that although SCSA data are heterogeneous

among men, individual men provide highly repeatable SCSA data from month to month as seen in Figure 20.5.

Experimental studies on animal reproduction have some great advantages over human studies that require more ethical standards. One of the best means to evaluate the fertility potential of sperm from different animals is done with heterospermic insemination. For example, equal numbers of motile sperm can be mixed from e.g. a black and white bull, and that mixture used to inseminate 100 cows. If 80 black and 20 white calves are born that would clearly show the superiority of the sperm from the black bull. Figure 20.6 shows that SCSA data of percentage DFI and SD DFI are highly correlated with the known competitive index of bulls [11].

Another study [12] with boars provided data showing that SCSA data are significantly correlated with the number of successful pregnancy outcomes. Also, boars with poorer percentage DFI had fewer pigs/litter likely due to death of embryos as seen in Table 20.1. Semen from 18 sexually mature boars with known fertility information was bred to 1867 females. 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 (Table 20.1).

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 fertilized the oocytes and the resulting embryos implanted in the female only to be lost at a later time when likely needed proteins are lacking due to a broken DNA/gene(s) required for supplying vital proteins.

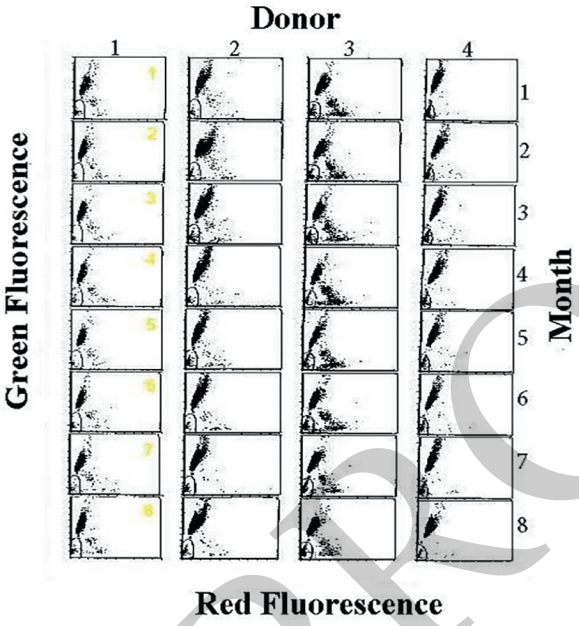


Figure 20.5 Sperm Chromatin Structure Assay cytograms of semen samples from four donors over eight consecutive months of collections.

Table 20.1 Boar Fertility and Average Pigs Born versus Sperm Chromatin Structure Assay Percentage DFI and Mean DFI

	FR	APB
% DFI	-0.55[a]	-0.54[a]
SD DFI	-0.67[b]	-0.54[c]

[a] P <0.01 [b] P <0.002 [c] P <0.02

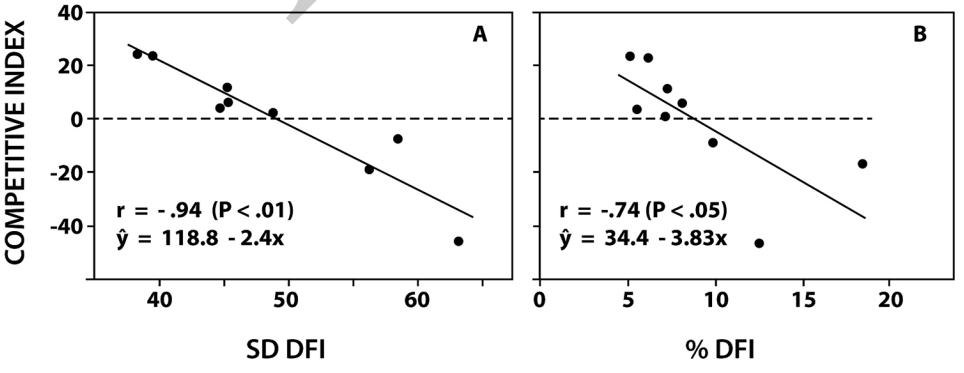


Figure 20.6 Bull heterospermic competitive index versus SCSA SD DFI and SCSA percentage DFI of bulls with different phenotypes.

20.6 Sperm Chromatin Structure Assay for Humans

20.6.1 Sperm Chromatin Structure Assay and Pregnancy by Intercourse

The first definitive study for SCSA-defined clinical utility was done in the "Georgetown study" [2]. This study uniquely evaluated 165 couples, without known female infertility factors, who had time sensitive intercourse over 12 menstrual cycles. During the first three months biochemical testing for pregnancy was performed. SCSA data from male partners of 73 couples (group 1) who achieved pregnancy during months 1–3 were compatible with "high fertility". The SCSA values from 118 semen samples from 40 couples achieving pregnancy in months 4–12 were significantly ($p<0.01$) higher than those from couples achieving pregnancy within three menstrual cycles. Also, the 89 semen samples obtained from 32 couples not achieving pregnancy by month 12 had highly significant ($p<0.001$) increase in SCSA values. Finally, the 115 semen samples from men attending an infertility clinic had highly significant ($p<0.001$) increases in SCSA values; the percentage DFI was 115 percent greater from these men in comparison to couples achieving pregnancy within three months (Table 20.2).

20.6.2 High DNA Stainable Sperm

Since AO stained histone complexed DNA stains 2.3x more than protamine complexed DNA [13], this HDS sperm fraction with increased histones is easy to detect with the SCSA test (Figure 20.7). Flow cytometer sorted HDS population of human sperm showed that these sperm nuclei are more rounded, consistent with lack of full sperm chromatin maturity [5, 14]. These sorted HDS sperm were negative for pH 10 Comets and thus they contained no DNA strand breaks (percentage DFI) [5]. These sorting experiments also showed that SCSA defined moderate level DFI sperm had DNA strand breaks but also a totally normal morphology [5,14], meaning that picking up a sperm for ICSI with a normal morphology is not a guarantee of normal DNA integrity.

Abnormal sperm nuclear condensation involves a complex sequence of events including topological rearrangements, transition of DNA binding proteins, transcriptional alterations, nucleosomal structure loss and abnormal condensation of chromatin resulting in disturbances in the organization of genomic material in the sperm nuclei and decreasing sperm functional ability. Ultimately this reduces normal fertilization, affects early embryonic development and interferes with the primary mission of the sperm DNA which is reliable transmission of paternal genetic information.

Interestingly, while it is known that abnormally high levels of HDS lead to early embryo death and miscarriage [2, 15, 16], the rationale for this is controversial with suggestions that it is related to increased aneuploidy [15, 16]. Alternatively, it is more likely related to abnormality of tertiary chromatin structure, thereby causing an abnormal read out of early embryo proteins needed for embryo growth and differentiation [17, 18]. Interestingly, while percentage DFI increases with age of men the percentage HDS goes down with age [16, 19].

20.6.3 Sperm Chromatin Structure Assay and Clinical Assisted Reproductive Technology Lab Pregnancies

1. **IUI**. The subsequent studies of Bungum et al. [20] showed that DFI >25 percent reduced IUI pregnancy success to almost nil.
2. **IVF/ICSI**. The study of Oleszczuk et al. [21] summarized SCSA outcomes on 1633 IVF and ICSI cycles. The percentage DFI values were

Table 20.2 Sperm Chromatin Structure Assay Outcomes from the Georgetown Study and Infertility Clinic

Pregnancy Outcomes	n	X DFI	SD DFI	% DFI	%HDS
PG in 3 months	73	234.6	137.9	11.2	8.95
PG 4-12 months	40	255.1**	157.9**	15.5**	8.78
No Pregnancy	31	270.3***	173.7***	17.2***	15.03***
Infertility Clinic	115	308.6***	194.2***	24.0***	

placed into four intervals: DFI<10 percent (reference group), 10 percent≤DFI≤20 percent, 20percent<DFI≤30 percent, DFI>30 percent. For the three latter intervals, the following outcomes of IVF/ICSI procedures were analyzed in relation to the reference group: fertilization, good quality embryo, pregnancy, miscarriage, and live births. In the standard IVF group, a significant negative association between DFI and fertilization rate was found. When calculated per ovum pick up (OPU), odds ratios (ORs) for at least one good quality embryo (GQE) were lower in the standard IVF group if DFI>20 percent. OR for live birth calculated per OPU was significantly lower in the standard IVF group if DFI>20 percent (OR, 0.61; 95 percent CI: 0.38–0.97; p=0.04). No such associations were seen in the ICSI group. OR for live birth by ICSI compared to IVF were statistically significantly higher for DFI>20 percent (OR, 1.7; 95 percent CI: 1.0–2.9; p=0.05). OR for miscarriage was significantly increased for DFI>40 percent (OR, 3.8; 95 percent CI: 1.2–12; p=0.02). These results suggest that ICSI may be a preferred method of in vitro treatment in cases with high DFI. Extensive SCSA data on infertility patients have shown that when a patient has <20 percent DFI, such semen sample with regards to sperm DNA integrity is consistent with normal pregnancy by intercourse or IUI unless other classical semen analysis shows one or two abnormal scores which decrease the odds for pregnancy [22]. Decreasing odds are present with >20 percent DFI and at 25 percent DFI the odds become poor for pregnancy by intercourse or IUI. At 30 percent DFI, reasonable success requires

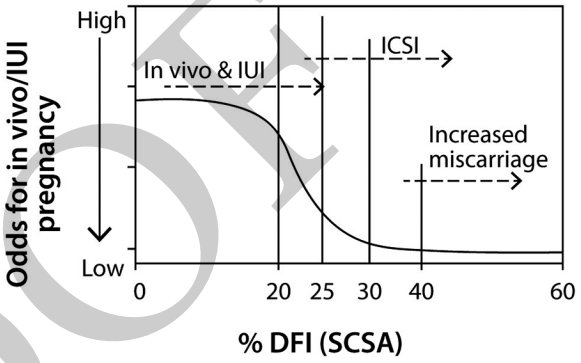


Figure 20.7 Odds for live birth versus percentage DFI.

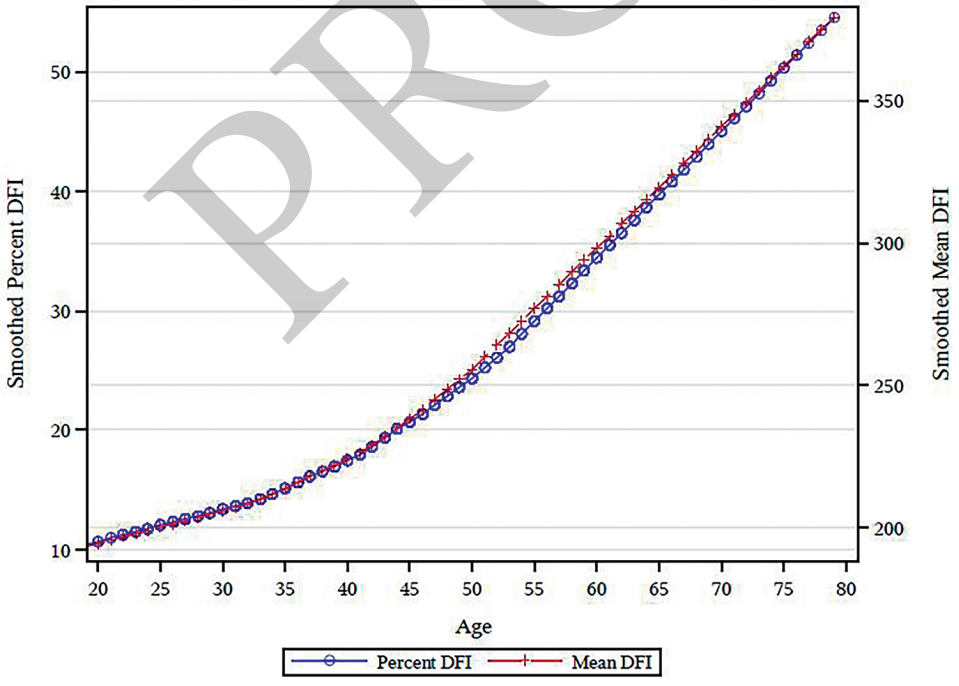


Figure 20.8 Percentage DFI versus Mean DFI (n=3K human semen samples).

ICSI. At 40 percent DFI the odds become very poor for pregnancy and increased odds for miscarriage. Values above 50 percent may rarely achieve pregnancy, but the odds are indeed poor. Figure 20.7 graphically summarizes the live birth thresholds for SCSA: the 20–25 percent DFI has been considered the "grey zone" [21–22], suggesting that fertility problems may start to occur when SCSA percentage DFI reaches this level. It is noted, however, that our SCSA diagnostic center has seen natural full-term pregnancy with up to 68 percent DFI. This observation illustrates that these percentage DFI clinical thresholds are statistical values and not absolute values.

Figure 20.7 summarizes the decreasing statistical chances of a full-term successful pregnancy with increasing SCSA percentage DFI of the male partner. Success begins to drop off at 15–20 percent DFI. Twenty-five percent DFI is considered the statistical threshold for in vivo and IUI fertilization and when IVF/ICSI should be used in the ART lab. Greater than 40 percent DFI significantly increases the risk for miscarriages.

20.7 Comparison of Percentage DFI versus Mean DFI

Percentage DFI is determined by visual computer gating of the sperm (dots) that correspond to increased red fluorescence due to single (ss) and double (ds) DNA strand breaks. Mean DFI is the measure in FCM channels (0–1024) of increased red fluorescence of the entire sperm population. As seen in Figure 20.8 the SCSA data show both the percentage DFI and Mean DFI.

For the first years of SCSA reporting the Mean DFI was used [e.g. 20, 22] to express the level of sperm DNA damage in the measured samples. Since it was easier for reproductive medicine personnel to understand percentage DFI, i.e. percent good versus percent bad sperm, we changed our quantitation of DNA damage to use of percentage DFI in both our research manuscripts and clinical reports. The question has been asked whether the percentage DFI accounts for the entirety of total DNA strand breaks. Figure 20.8 shows that the curves of percentage DFI and Mean DFI are the same thereby proving that the percentage DFI method accounts for the total of the sperm DNA breaks.

It is highly recommended that all users of the SCSA test use percentage DFI since the use of Mean DFI requires highly strict attention to being certain that the green and red fluorescence values of reference semen samples are the same for measurements of all clinical samples. It is known from published cytograms that some laboratories are not consistent in placing the reference sample with exactly the same (±5 channels) photomultiplier (PMT) gains for red and green fluorescence. Thus, it is strongly suggested that only percentage DFI be used to characterize SCSA-derived sperm DNA integrity.

20.8 Sperm Chromatin Structure Assay Testing with Different Flow Cytometers in Different Locations

Since there are a variety of flow cytometers worldwide it was important to determine whether different types of instruments could produce the same results. Figure 20.9

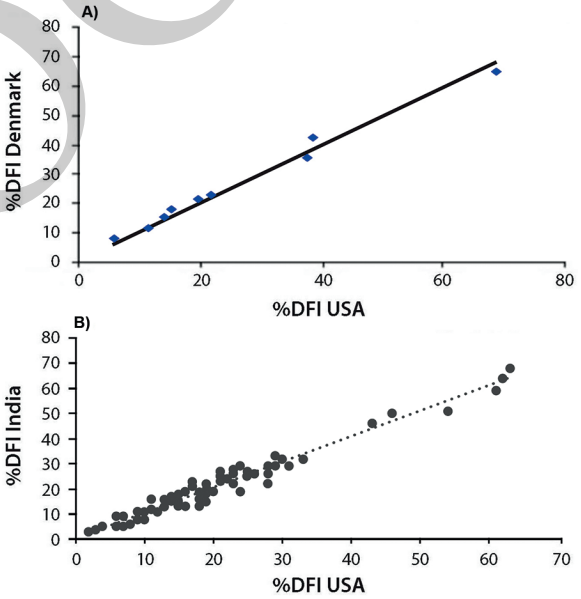


Figure 20.9 Correlations between SCSA data obtained on three continents. **A)** Correlation between SCSA percentage DFI on ten frozen/thawed human samples on two different brands of flow cytometers (Cytofluorograf 30; Ortho Diagnostics) at SCSA Diagnostics, Inc. in South Dakota, USA and (FACScan, Beckton Dickenson) at the University of Copenhagen, Denmark. (Correlation: R^2=0.961). **B)** Correlation between SCSA percentage DFI on 57 frozen/thawed human samples on two different brands of flow cytometers (Cytofluorograf 30; Ortho Diagnostics) at SCSA Diagnostics, Inc. in South Dakota and a Beckman Coulter flow cytometer in the Andrology Lab, Coimbatore, India. (Correlation: R^2=0.9812)

shows that data on frozen/thawed semen samples from humans measured on different types of flow cytometers in three countries are nearly exactly the same.

20.9 Conclusions

The SCSA test has proven to be a very rapid, highly reliable and statistically robust test to measure sperm DNA integrity as related to pregnancy outcomes. The SCSA test is considered to measure the total of sperm DNA fragmentation. It is the most direct DNA fragmentation assay in that starting with a fresh or frozen/thawed raw semen sample, SCSA data on 5000 sperm can be obtained in less than 10 minutes. Using raw semen samples assures that all cell types present are measured, and none lost or biochemically changed by experimental manipulations. Somatic cells are also measured as non-sperm cells as seen in Figure 20.2. Being assured that frozen/thawed samples produce the exact data as fresh samples means that semen samples obtained anywhere in the world can be frozen and shipped by air carriers such as Federal Express to a lab with expertise in SCSA testing. Likewise, samples frozen for various reasons, such as cryopreservation in sperm banks, can be retrieved and measured.

Importantly for the human infertility clinic, SCSA thresholds of sperm DNA fragmentation have been determined as seen in Figure 20.7 allowing couples striving for a pregnancy to help understand whether the male partner's sperm DNA quality is adequate to have a good probability of pregnancy success.

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