Chapter 4
Sperm Chromatin Structure Assay (SCSA®): Evolution from Origin to Clinical Utility

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4.1 Origin, Standardization, and Verification of the SCSA Test as Marker of Male Sub-/Infertility

4.1.1 Origin of the SCSA Test

Thin section electron microscopy of ejaculated human sperm shows significant heterogeneity of nuclear chromatin structure between different men and within individuals [1]. Since sperm nuclear morphology is related to chromatin condensation and other nuclear phenomena occurring during spermatogenesis, it was hypothesized, as have others [2], that misshaped sperm nuclei have an altered chromatin structure. Furthermore, since the resistance of in situ DNA to thermal denaturation is related to counter ion and protein interactions with DNA [2, 3], it was further hypothesized that an altered chromatin structure would reflect in an abnormal DNA denaturation profile.

The hypothesis was introduced that if isolated and purified sperm nuclei were heated at 100 °C for 5 min, the denaturation of nuclear DNA would be heterogeneous between samples from high and low fertility humans and animals. Semen samples were obtained from three sources: (a) men of known fertility and men

This chapter is dedicated to the memory of Marcello Spano who died of a fatal heart attack in his ENEA lab in Rome, December, 2016. In 1979, Marcello invited me to his lab to set up the SCSA test. In 2000, he published a seminal paper, Sperm Chromatin Damage Impairs Human Fertility. The Danish First Pregnancy Planner Study Team. Fertil. Steril. 73:43-50. His frequent collaboration with Aleksander Giwercman and Mona Bungum in Sweden brought a wealth of valuable SCSA clinical data on sperm DNA fragmentation as related to male factor infertility. We are grateful for his excellent collaboration and warm friendship; he will be greatly missed.

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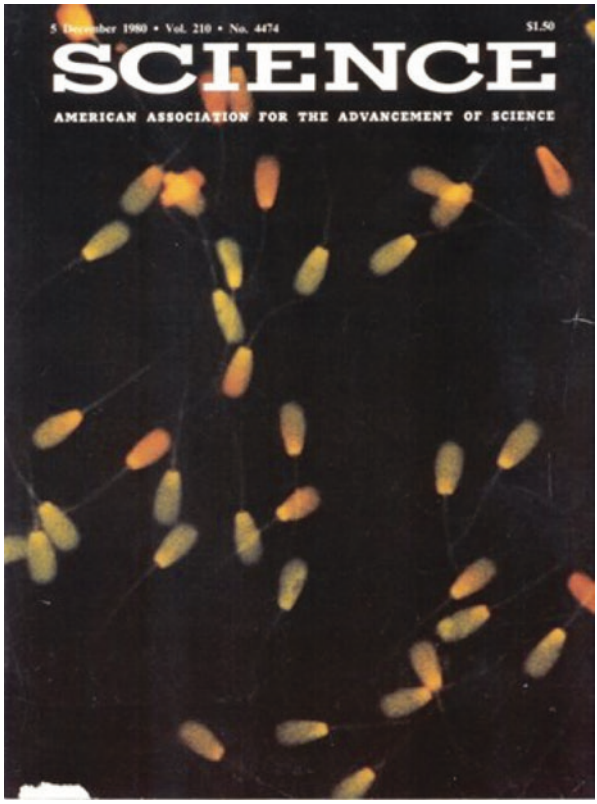


Fig. 4.1 Fluorescence photomicrograph of bull sperm nuclei heated and stained with acridine orange [4] (From Evenson et al. [4]; used with permission)

attending an infertility clinic, (b) bull semen from known fertile bulls and subfertile bulls, and (c) sperm from mice on a normal diet and diet of Zn^{+2} deficiency, a known factor required for intact sperm nuclear chromatin structure. Human and bull sperm from known subfertile donors as well as mice on a Zn^{+2}-deficient diet had two to four times greater red fluorescence (broken and denatured DNA) as seen by light microscopy (Fig. 4.1) and precisely quantitated by flow cytometry [4].

This new concept and solid data were the origin of the first publication [4] of flow cytometry-measured in situ sperm DNA denaturation as related to fertility both by men at an infertility clinic and bulls of known levels of high and low fertility. The ranking of the five bulls by their degree of sperm DNA denaturation was inversely the same as their ranking of field fertility by the Eastern Artificial Insemination Cooperative (Ithaca, NY).

Importantly, not only did the in situ DNA of misshaped sperm nuclei have significantly decreased resistance to thermal denaturation, but many morphologically normal nuclei derived from subfertile donors had abnormal susceptibility to in situ thermal denaturation of their DNA. This important point has been confirmed in various human clinical studies. For example, Avendaño et al. [5] found that in infertile

men with moderate and severe teratozoospermia, the sperm with apparently normal morphology present in the motile fractions after swim-up may have broken DNA.

Studies by Wyrobek et al. [6] showed that sperm from genotoxin-exposed mice had high dose-response correlations with sperm head morphology. Studies, shown below, also demonstrated a very high dose-response correlation between abnormal sperm head morphology and SCSA data on sperm from genotoxin-exposed mice [7].

4.1.2 Standardization of the SCSA Test: Changes to the Finalized and Federal Registered Protocol

4.1.2.1 Problems with the Heated Sperm Nuclei Protocol

A high percentage of the nuclei stuck to the heated containers including surfaces of glass, plastic, polypropylene, siliconized surfaces, and others. Also, measuring whole sperm was equivalent to data on isolated nuclei [7]; thus, the time to prepare the samples was long and very technician unfriendly.

4.1.2.2 Low pH to Denature DNA at DNA Break Sites

Fortunately the two-step acid procedure used for somatic cells [3] gave the same results as the heat protocol [4, 7]. Technician time and effort were dramatically reduced. This procedure, as well as specific steps for preparation, measurement, and data processing, has been the FIXED SCSA® protocol for over three decades. Table 4.1 briefly outlines the protocol. Extensive details are published elsewhere [8–10].

Table 4.1 SCSA® Protocol

1. Prepare and measure one semen sample at a time
2. Transfer vial of frozen semen in LN2 tank near FCM to a 37° C water bath and immediately dilute with TNE buffer to ~1–2 × 10^6 sperm/ml
3. Acid (pH 1.20 for 30 s) denaturation (open up) DNA double helix at sites of ss or ds DNA breaks
4. AO staining of ss (red) and ds (green) DNA
5. Immediately place in flow cytometer and run sample/sheath for 1–2 min to establish fluidic equilibrium
6. Measure 5000 sperm by flow cytometry at rates <250/s
7. Computer calculations of data for clinical report
8. Send report to clinic by secure WEB site

Detailed protocol: ask for PDF (don@scsatest.com)

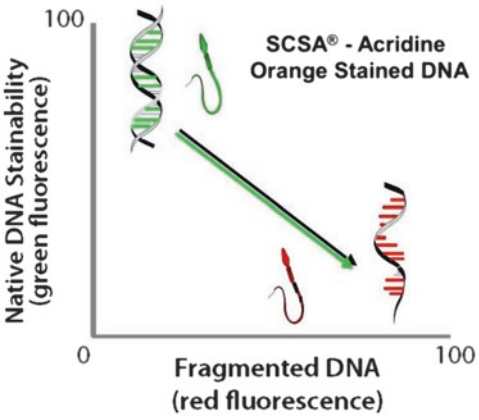


Fig. 4.2 Schematic of sperm nuclear DNA shift of staining from intact DNA (*green*) to high levels of DNA denaturation at sites of single-strand (ss) and double-strand (ds) DNA breaks (*red*)

4.1.3 Biochemistry of Acridine Orange (AO) and Sperm DNA Interactions of the SCSA Test

Figure 4.2 illustrates AO intercalated into dsDNA and stacked on ssDNA. At sites with ss or ds DNA strand breaks, the heat or acid locally denatures or "opens" the ds to ss DNA. AO stacks on the ssDNA that then collapses into a crystal and when exposed to blue laser light has a metachromatic shift to red fluorescence [3]. With an increasing number of DNA breaks, there is a concomitant decrease of green fluorescence and an increase of red fluorescence.

A very significant advantage of the SCSA test is that its marker for DNA strand breaks is the very small (MW 265), flat planer acridine orange (AO) molecule. Thus, AO likely penetrates the entire highly compact nuclear chromatin structure [11, 12]. In contrast, the TUNEL assay requires the large terminal deoxynucleotidyl transferase enzyme to label at sites of DNA strand breaks, except those breaks without a 3′OH end, and it is likely that the protamine toroid is not penetrable by this enzyme, thus reducing the efficiency of flow cytometric TUNEL testing by about 1/3 [11, 12]. Research from the lab of J Aitken [13] shows that the TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality. Efforts are described in using a S-S reducing agent (DTT: dithiothreitol) to open up the S-S compacted chromatin.

The light microscope TUNEL further reduces the %DFI from that measured by flow cytometry (FCM). Figure 4.3 illustrates the different potential staining sites by the SCSA and TUNEL tests.

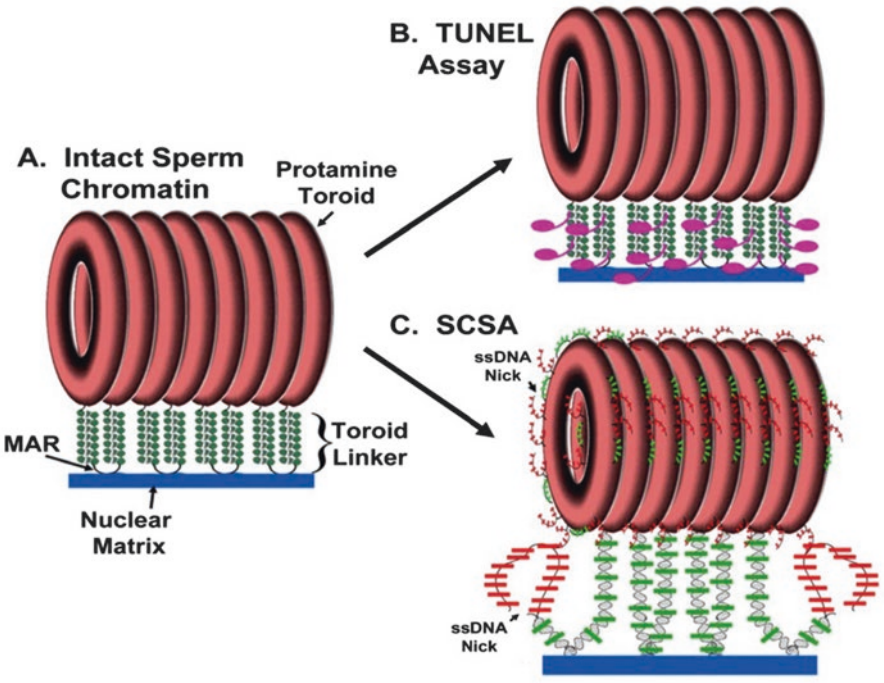


Fig. 4.3 SCSA vs. TUNEL accessibility to sperm chromatin for detection of DNA strand breaks. (**A**) Model of sperm chromatin, (**B**) TUNEL assay accessible sites, (**C**) SCSA accessible sites. SCSA = AO; TUNEL = TdTA + fluorochrome; TUNEL % DFI values 1/3 less than SCSA values [10, 11] (From Gawecka [11]; used with permission).

4.1.4 Does the SCSA Test Measure Potential or Existing Sperm DNA Strand Breaks?

Early publications of SCSA data stated that AO stained sites of decreased resistance to in situ denaturation leaving open any interpretation of mechanism [4]. The term "resistance to in situ denaturation" was later spoken of as sites of "sperm DNA fragmentation" leading to the expression "DNA fragmentation index" or % DFI, as adopted by users of other sperm DNA fragmentation (SDF) assays. A current expression is "sites of ss or ds DNA strand breaks" [12]. Previous literature often stated the concept that the TUNEL assay was a "direct" measure of DNA strand breaks, while the SCSA test was an "indirect test" measuring "potential DNA breaks." A recent review [14] stated that the "SCSA starts with an acid denaturation step and depends on the principle that abnormal DNA is more prone to further fragmentation by acid denaturation than intact DNA." Does that imply that the acid causes fragmentation, i.e., DNA strand breaks? No, all data to date strongly suggest

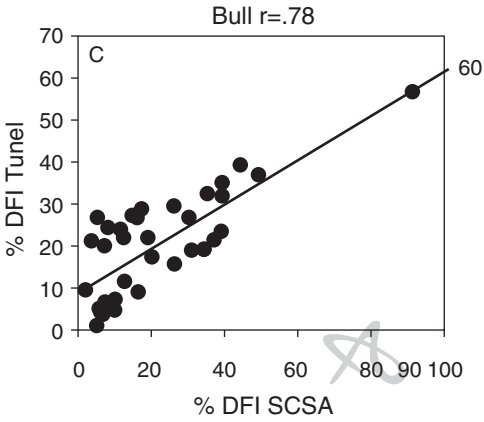


Fig. 4.4 Regression analysis depicting the relationship between %TdTA staining and % DFI staining of bull ($n = 38$) sperm. $r = 0.78$, $P < 0.001$ [16]. Note the top right dot at 60% DFI (TUNEL) vs. 90% DFI (SCSA) (From Sailer et al. [16]; used with permission)

that the function of the heat or pH 1.20 treatment for 30 s is to denature (open) the two DNA strands at the sites of *existing single or double DNA strand breaks*, i.e., "normal DNA" with single- or double-strand breaks.

Since neither heat (100 °C, 5 min) nor acid (pH 1.20/30 s) breaks the DNA phosphodiester backbone, both the TUNEL (listed as a "direct test") and the SCSA (listed as an "indirect test") are measuring existing DNA breaks *available* to each specific molecular probe. This view is supported by the following:

1. The first and likely foremost evidence needs to come from the co-founder, Z. Darzynkiewicz, of both the SCSA test [4] and TUNEL test [15]. These two tests, most importantly done by an expert in the same laboratory using the same flow cytometer, showed a correlation of $r = 0.87$; $P < 0.05$. This is a strong evidence suggesting that these two tests measure the same sites available to each specific probe.
2. Studies using bull semen samples showed a remarkably high correlation (0.99) between the TUNEL and SCSA tests for consecutive collections from a single bull [16]. However, the data suggest a one-third (60/90) less efficiency in labeling sites of DNA strand breaks using the TUNEL assay. Figure 4.4 shows data [16] on 38 bull semen samples measured by the SCSA and TUNEL tests ($r = 0.78$, $P < 0.001$). These data confirm the observations from Aitken's lab [13] that the TUNEL test underestimates DNA strand breaks.

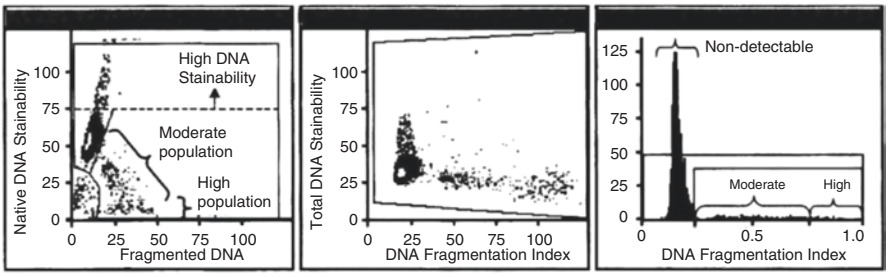
4.1.5 Change in SCSA Terminology

The Sperm Chromatin Structure Assay (SCSA®) was named as such since it measures both *sperm DNA fragmentation* and *abnormal chromatin structure*. The major use of the SCSA test has been to determine the percentage of sperm with fragmented DNA. The original term for describing the percentage of sperm in a semen sample with fragmented DNA was cells outside the main population (COMP α_t).

Due to suggestions from human medical andrology interests that the acronym COMP α_t did not explain well what this meant, the COMP α_t terminology was changed to %DFI (DNA fragmentation index) [9]. 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 the concept of %DFI expression of the percentage of sperm with fragmented DNA. However, in the animal andrology field, the original SCSA terms have been kept by most authors.

4.1.6 Clinical Report

Figure 4.5 shows typical SCSA clinical data on ejaculated sperm from men attending an infertility clinic. These raw and computer converted data are inserted into a clinical report that includes suggestions for clinical intervention.



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. 4.5 SCSA test data. Top Box. Left panel. 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 uncondensed chromatin allowing more DNA stainability. Three levels of sperm DNA integrity: normal, moderate, and high levels of DNA fragmentation. Bottom left corner shows gating out of seminal debris. Middle panel. Raw data from left panels are converted by SCSAsoft® software (or equivalent) to red/red + green fluorescence. This transforms the angled normal sperm display in left panel to a vertical pattern that is often critical for accurately delineating the % of sperm with fragmented DNA. *Y* axis = total DNA stainability vs. *X* axis = red/red + green fluorescence (DFI). Right panel: Frequency histogram of data from middle panel showing computer gating into three categories: normal, moderate, and high DFI (moderate DFI + high DFI = total %DFI). Bottom box. SCSAsoft calculations of mean of two independent measures of mean and SD of DFI, SD DFI, and % DFI and %HDS

4.1.7 Relationship Between Sperm DNA Fragmentation Data and Classical Semen Parameters

Investigation of the male partner of infertile couples is traditionally based on the conventional WHO semen analysis, which includes an assessment of sperm count, motility, and normal sperm morphology. This analysis has, however, a limited value both as a diagnostic tool and as a guide to selection of the therapeutic procedure [17]. In numerous studies using the SCSA test, many investigators have recorded correlations between %DFI and the standard semen parameters. These stated correlations vary widely; however, the consensus is that the correlations are weak enough to conclude that the SCSA %DFI is a relatively independent parameter. The most highly correlated parameter is usually with motility. The rationale is that reactive oxygen species (ROS) activity breaks DNA and damages cellular membranes, including the mitochondrial membranes, likely inhibiting motility.

4.2 Biochemical Characteristics of SCSA-Defined Sperm Populations

Some investigators using other DNA fragmentation techniques that employ light microscopy have stated that their method advantageously analyzes single cells, while the SCSA measures groups of cells but not single cells. No, the very essence of flow cytometry is that every single cell is measured one at a time at a fast rate. Any single cell or cluster of cells can be characterized on a 1024 × 1024 grid on the computer monitor as seen in Fig. 4.5. As an example, a single cell, or cluster, may have a characteristic *Y* value of 540 nm green fluorescence (native DNA) and *X* value of 650 red fluorescence (broken DNA). Sperm with such values can be flow cytometry (FCM) sorted out for further morphological and biochemical characterization.

4.2.1 FCM Sorted SCSA Populations to Analyze Sperm Nuclear Morphology

A FACsort flow cytometer (Becton Dickenson, San Jose, CA) was used to separate four (normal, moderate DFI, high DFI, and HDS) SCSA populations [18]. Using the computer gates seen in Fig. 4.5, the sorted sperm were collected in tubes, spun down, resuspended, and then forced onto a glass microscope slide using a cytocentrifuge (Shandon Cytospin II, Minneapolis, MN) that concentrates the sperm into a small region of the glass slide.

For the first experiment, Feulgen-stained nuclei were photographed with a Nikon 800 light microscope interfaced to computer image analysis software. Three

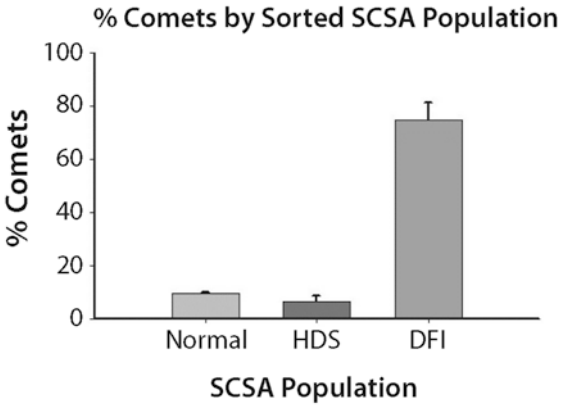


Fig. 4.6 Flow cytometry-sorted SCSA sperm populations of normal, HDS, and DFI populations. These sperm on glass slides were processed by the pH 10 (neutral) comet assay. Five hundred sperm per group were scored for the % with comets indicative of double-strand DNA breaks

measured slides/population for a total of 600 nuclei were analyzed for each sorted population. Of interest, both the SCSA normal population AND the moderate DFI population had nearly identical nuclear morphology images. Of clinical interest, these sperm may be picked up for ICSI due to their normal morphology, but they likely contain fragmented (broken) DNA.

In the second experiment [18], each of the four populations that was sorted onto glass slides was subjected to pH 10 (neutral) comet assay that identifies (a) sperm *without* dsDNA breaks and (b) sperm *with* dsDNA breaks having a pattern of an astrological comet. The main population and HDS population had few (background noise) comets. As seen in Fig. 4.6, about 75% of the sperm with moderate and high DNA fragmentation also had positive pH 10 comets indicative of dsDNA breaks, thus confirming the presence of dsDNA breaks measured by the SCSA test.

An alkaline comet assay was not run; it is hypothesized that both moderate and high %DFI fractions would show 100% alkaline comets, thus confirming SCSA measurements of *both ds and ssDNA breaks,* i.e.*, breaks in the phosphodiester backbone of one or both of the DNA strands. This has also been described as DNA fragmentation* (Latin: *fragmentum*—a broken piece—thus, DNA with pieces of broken ss or ds DNA).

4.2.2 Characteristics of HDS Population: New Emphasis for the ART Lab

HDS sperm have abnormal nuclear proteins and/or other factors that prevent normal chromatin condensation thereby exposing more DNA to AO staining of ds DNA; this includes excess histones and other proteins such as unprocessed protamines [19]. Histone-complexed DNA has a 2.3 X greater AO staining than protamine-complexed DNA [20].

In a study by Zini et al. [21], samples from men ($n = 87$) attending an infertility clinic showed a significant relationship between sperm morphology defects according to strict criteria and SCSA parameters (%DFI and %HDS), i.e., normal sperm forms and both %HDS ($r = -0.40$) and sperm motility ($r = 0.32$). The observed relationship between sperm head defects and %HDS suggests that sperm head abnormalities may, in part, be due to incomplete sperm chromatin condensation.

Of importance, it is becoming clearer that a high %HDS is correlated with increased probability of early embryo-grown cessation and miscarriage [22–25]. The laboratory of Menezo [22] has been at the forefront in providing evidence on the importance of the decondensed chromatin population. Menezo's lab has called the %HDS fraction "DNA decondensation state index" (SDI) measured by aniline blue (AB) or by SCSA, which fortunately can simultaneously measure both DFI and HDS. Some gene families that are highly important for early embryo development are associated with histones in human spermatozoa [24]. "While it is well known that the oocyte can repair limited sperm DNA breaks, its capacity to improve tertiary structure is rather limited." Menezo's lab/clinical data [26] suggest that defective methylation linked to methylenetetrahydrofolate reductase (MTHFR) may contribute to sperm pathogenesis via increased %HDS (%SDI) [26].

The negative impact of high sperm chromatin decondensation (high HDS) may occur at the time of early developmental arrests up to miscarriages [23–25].

In a study of 1417 ART patients [26] where the man had an SCSA test, 77% had less than 20% HDS, 10% had 20 to <25% HDS, and 12% had >25% HDS. High %HDS values result in a large embryo loss at an approximate eight-cell stage. A very preliminary study at our SCSA diagnostic lab has seen ~80% embryo failure when HDS >35%, while the %DFI values were at acceptable levels.

It is of great interest that the negative influence of HDS on pregnancy outcomes follows closely to the curve shown in Fig. 4.7 for %DFI. Specifically, all is well with

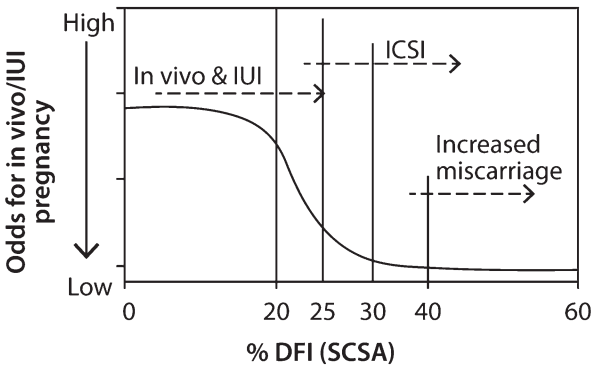


Fig. 4.7 Odds for in vivo/IUI/IFV pregnancy vs. % DFI. The curve was estimated from data from intercourse [8, 27], IUI [54] and IVF/ICSI [28] data. Below 15–20% DFI is without a known problem. Threshold for in vivo and IUI fertilization is 25%, and at that level ICSI should be considered. At ~40% DFI presents a high risk for no pregnancy and increased probability for miscarriage

<20% HDS. However, the outcomes become poorer from 20 % to 30% HDS, and at >30% HDS there is a high level of cessation of early embryo growth [25, 26].

Menezo's group have described how defective methylation linked to MTHFR may contribute to sperm pathogenesis via increased SDI (HDS). While the egg has repair capacity for broken sperm DNA, it has no capacity to fix the lack of organization found in the uncondensed chromatin (HDS) that may be critical to synthesizing the specific needed proteins for growth of the embryo.

4.3 Validation of SCSA Clinical Thresholds

4.3.1 Humans

The early SCSA human experiments suggested that the threshold for male factor subfertility via intercourse was ~25–27% DFI [8, 27]. Spano et al. [27] showed that pregnancy rate via intercourse *begins to drop* with >20% DFI [27]. Note that this represents TWO different statistical thresholds, namely, *20%DFI for the beginning level for fall off reproductive outcomes* and *25% as a statistical threshold for* in vivo *success*. Furthermore, a *third threshold is at >40%DFI* for very low success by any fertilization method and an increased level of miscarriages [27, 28]. The most common question asked by patients is "If 25% of my sperm have fragmented DNA, why can't the other 75% be sufficient for attaining a pregnancy?" More dramatically, the threshold for boars has repeatedly been shown to be 6%DFI [29]. An answer to this question is described as the "iceberg phenomena" [9]. The human threshold at 25% is equivalent to an iceberg with 25% of its mass above the water line. However, the 75% of the iceberg under the water line likely have sperm with negative factors such as pre-apoptotic sperm.

4.3.2 Animals

As stated by Barratt and De Jong [30], validation of sperm DNA fragmentation tests needs to include animal models where the breeding can be controlled to a much greater degree than for humans. To achieve this recommendation, known fertility data from bulls and boars were correlated with SCSA data.

4.3.2.1 Bulls

Perhaps the best way to eliminate many of the variables in potential female factor assessment of male fertility is to conduct heterospermic inseminations that are possible only in animal studies. 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

Table 4.2 Pearson correlation coefficients (r) of SCSA variables and fertility for 18 boars bred to 1867 females

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

[a] $p < 0.01$
[b] $p < 0.003$
[c] $p < 0.02$

white calves shows which bull has the greater fertility potential. SCSA test data [31] on sperm from nine bulls showed a very high correlation with a known fertility competitive index, measured by heterospermic performance (%DFI, −0.74, $P < 0.05$; SD DFI, −0.94, $P < 0.01$).

4.3.2.2 Boars

Heterospermic trial. Encouraged by the field [32] and heterospermic bull data [31], similar heterospermic experiments were done with boars [33]. Semen from six phenotypically different boars was mixed in equal motile sperm numbers in six three-way combinations and inseminated into at least three Duroc gilts per combination. The SCSA correctly predicted both the high and low fertility boars based on a ratio of offspring as deviated from the theoretical percentage. The "low fertility boars" had 3.0 times higher %DFI values than for the high fertility boars. The offspring of the high fertility boars were 4.8 times more than from the low fertility boars.

Multiparous animals. 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. [29] 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 (Table 4.2).

The boar fertility rate had a high correlation with the %DFI ($r = -0.60$, $P < 0.01$) and SD DFI ($r = -0.68$, $P < 0.003$) [29]. It is of great interest to note the significant correlations between %DFI and SD DFI values and average number of piglets born (APB)/liter. Since oocytes do not discriminate against sperm with damaged DNA [34], these sperm with damaged DNA likely fertilize and the resulting embryo *implants* in the female only to be *lost later* when likely needed proteins are lacking due to a break in the DNA/gene required to supply that vital protein. Human data have clearly shown that DFI >30% are related to increased miscarriage rate [8, 27]. As stated by Borini et al. [35], high %DFI can compromise "embryo viability," resulting in pregnancy loss.

4.4 Validation of the SCSA Test for Precision and Accuracy

4.4.1 Invaluable Use of Flow Cytometry

Of critical importance for validation of any test is its precision and accuracy. Without a doubt flow cytometry is invaluable to achieve this requirement for the SCSA test. Flow cytometry (FCM) measuring of cells is highly rapid with exceptional *mechanical precision* that avoids human eye biases. Both TUNEL and SCSA tests are amenable for use with flow cytometry; SCSA has a significant advantage of being a dual parameter measurement. Thus, *each* sperm is characterized by 1024 × 1024 units (channels) of green vs. red fluorescence seen as a dot plot on the FCM monitor (Fig. 4.5). And for accuracy, i.e., the extent to which a given measurement agrees with the standard value for that measurement, it is near perfect for the SCSA test. Thus, EVERY SINGLE SPERM in a SCSA measurement can be characterized by the *exact extent* of DNA damage.

Figure 4.8 provides evidence for two important features of the SCSA test [36]: (1) lack of difference of %DFI between fresh and frozen samples and (2) the ability of setting up the flow cytometer for exact repeat measurements by the use of reference samples consisting of numerous frozen aliquots of a semen sample with about 10–15% DFI [9, 10]. These reference samples are used to set the mean green and red fluorescence values to the same exact (+ 5 channels) *X* and *Y* coordinates each time the FCM is set up for measuring samples.

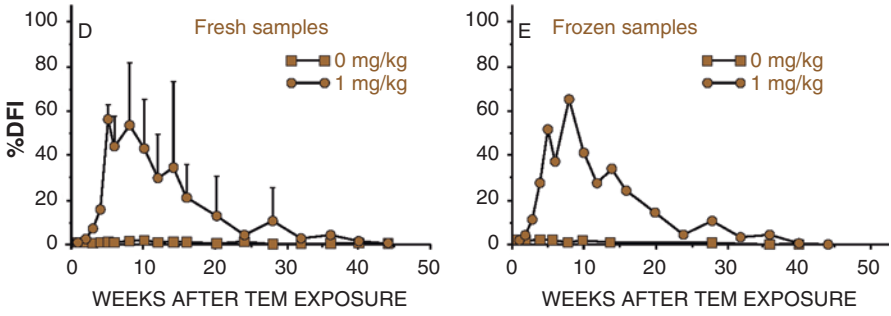


Fig. 4.8 Epididymal sperm from mice treated with triethylenemelamine (TEM) and over 45 weeks harvested with one fresh set measured by the SCSA test and a frozen aliquot measured months later by the SCSA test [36]

4.4.2 Repeatability of SCSA Data

4.4.2.1 Within Human Donors Over Time

It is well known that semen parameters such as count, motility, and morphology can vary widely over short periods of time [17]. For this reason, it is often recommended that a man has at least two classical semen tests over some weeks of time. In contrast, the evidence is strong that the SCSA test data are highly stable over months of time for healthy men [37] as seen in Fig. 4.9.

Note the consistent, unique cytogram patterns from month to month within individuals. Left column, excellent DNA integrity; middle column, poor DNA integrity with high % DFI; and right column, high %HDS and near absence of DNA breakage. Note that if the clinical report on the latter only listed %DFI, this would score as a very normal sample; however, the very high %HDS changes the clinical report to an increased probability of early embryo cessation of growth [22–26]. The CV of intra-individual eight monthly samples of 45 men was 10% [37].

Some studies have stated that the intra-individual CV for SCSA measures is as high as 30% [38]. This was a retrospective study of 282 consecutive patients referred for ART with repeated (2–5) SCSA measurements. The mean CV of DFI for repeated SCSA measurements was 29%. Thirty-seven percent of patients with DFI >30.0% in the first test had DFI <30.0% in the second test. Also, 27% of patients with 21–30% DFI values in the first test had DFI >30% in the second test. The authors concluded that with this high intra-individual variability in %DFI of repeated SCSA measurements, repeat SCSA measurements are recommended. *However*, a problem with this conclusion is that patients with an *initial value* of, e.g., 29.9% and a *follow-up value* of 30.1% would be scored as changing categories, while it is obvious that these two numbers are statistically the same.

To help resolve this problem, a new study [39] was done in which SCSA analyses were performed on 616 samples from men between 18 and 66 years of age. A calculation was performed using an interval of 29–31% instead of the 30.0% cutoff value (switch from <29 to >31% or vice versa*).* "When the DFI interval 29–31% was used instead of the 30% cut-off level, 12% of the subjects switched categories. Thus, in the clear majority of the subjects, repeated SCSA testing does not result in a switch in DFI category, in relation to the clinical cut-off level of 30%. This repeatability adds to the utility of the SCSA %DFI as a valuable tool in the investigation of men from infertile couples." There is a highly likely reason why the CV of % DFI is greater in patients than what is seen in non-patient donors. When a man at an infertility clinic has a high % DFI with the realization that pregnancy would be more easily obtained with a lower % DFI, the patient is often encouraged to ingest antioxidants [40], keep the testes cool [41], lower BMI values, avoid some medications (e.g., selective serotonin reuptake inhibitors (SSRIs) [42], reduce stress, fix large varicoceles [43, 44], and overall move to a healthier lifestyle. Many of the changes are known to reduce %DFI by a significant amount. And consequently,

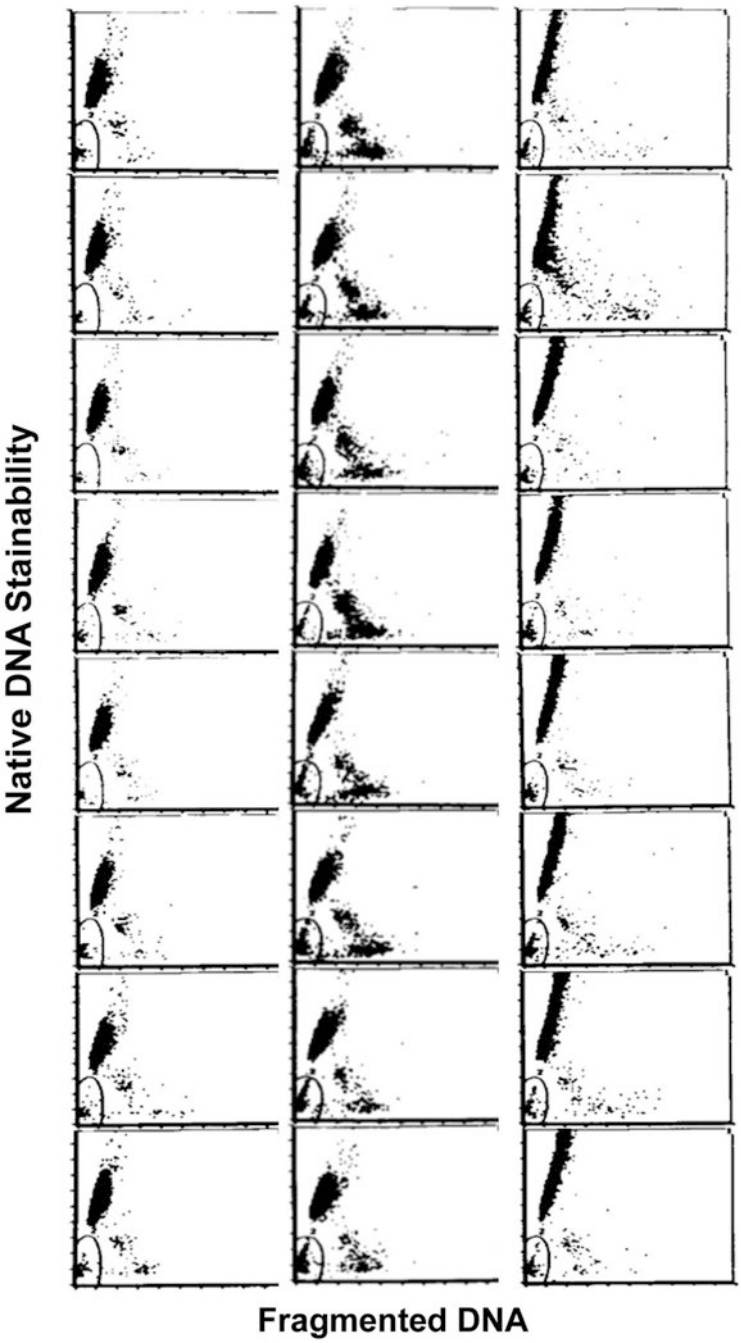


Fig. 4.9 Repeatability of SCSA measures of donor sperm over time. Shown here are semen samples from three donors obtained for eight consecutive months. Note the highly consistent patterns for each man despite a significant difference between the men shown [37] (From Evenson et al. [37]; used with permission)

there is often a greater CV for repeat measurements due to the patient and not the SCSA test.

Data on repeatability of %DFI in a single non-smoking fertile donor over 10 years (age 40–50) showed that semen parameters and sperm DNA integrity remained normal, and no trend was observed over the study period. Of interest, the %DFI was less than 20% [45].

4.4.2.2 SCSA Data Using Different Flow Cytometers Internationally on Sperm from Eight Different Mammalian Species

Now that flow cytometers are available in numerous laboratories and medical institutions around the world, it is very important to know whether multiple types of flow cytometers are compatible to measure with exacting results for the two sperm DNA fragmentation assays that use flow cytometry, namely, the SCSA and TUNEL tests. For the SCSA test, it has long been known that measurements on different flow cytometers produce the same results when using the SCSAsoft®, or equivalent, software for clinical output. In 1995, Evenson and ten collaborators in seven centers on two continents made comparative SCSA %DFI measurements of aliquots of the same frozen semen aliquots from human, mouse, rat, turkey, bull, ram, boar, and stallion [46]. Both epi-illumination and orthogonal optic flow cytometers were compared. Even with the great difference in the shape of the cytograms between FCMs with orthogonal vs. epi-illumination optics, using software equivalent to SCSAsoft showed the near exact same level of %DFI (26% and 25% DFI) (Table 4.3).

Of great importance, the overall %DFI values for the total 132 samples had correlations of 0.9886 ($P < 0.001$). This number solidly demonstrates that the crucial SCSA measurements around the world on very different flow cytometers produced with SCSAsoft (or equivalent red/red + green fluorescence) the near exact same results.

Table 4.3 Correlations between the same SCSA variables measured on the PCP22A and Cytofluorograf 30 FCM

Species							
	Bull ($n = 23$)	Rams ($n = 18$)	Boars ($n = 28$)	Stallions ($n = 39$)	Mice ($n = 14$)	Humans ($n = 10$)	Overall ($n = 132$)
%DFI	0.9788	0.9816	0.9952	0.9864	0.9961	0.9833	0.9871
SD DFI	0.9902	0.9934	0.9983	0.9909	0.9998	0.9241	0.9886

4.4.2.3 Comparisons Between Measurements of Aliquots of Human Patient Semen Samples on Three Continents

A near exact level of reproducibility is seen (Fig. 4.10) with aliquots of human semen samples shared between SCSA Diagnostics, Inc. and SCSA certified laboratories in Denmark and India. Similar correlations between international labs using the same FCM and the TUNEL assay have been reported [47].

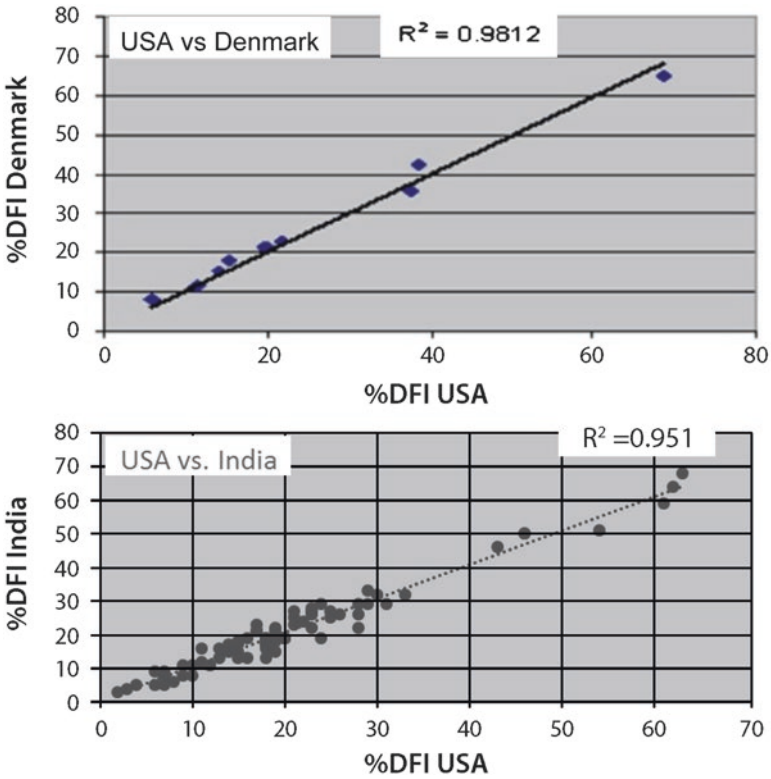


Fig. 4.10 Correlations between SCSA data obtained on three continents. Upper box. Correlation between SCSA %DFI on ten frozen/thawed human samples on two different brands of flow cytometers (Cytofluorograf 30; Ortho Diagnostics) at SCSA Diagnostics, Inc. in South Dakota. USA and (FACScan, Beckton Dickenson) at the University of Copenhagen, Denmark. (Correlation: R2 = 0.961). Lower box. Correlation between SCSA %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: R2 = 0.9812)

4.5 SCSA Data as Related to Male Age, a Very Important Infertility Issue

While the age of females seeking pregnancy has received vast amounts of coverage in medical and laymen publications, very little has been said about the effects of the man's age on male factor infertility. Data in Fig. 4.11 show that above age 45, the man's sperm DNA integrity deteriorates more rapidly with increasing %DFI.

Both healthy donors [48] and men attending infertility clinics (n = 3026) [49] show a significant increase of %DFI at about age >45 and a decreasing %HDS. These data have been hypothesized to relate to the data in a Swedish study that followed the consequences on offspring of fathers conceiving a child after the age of 40 [49]. Sperm DNA fragmentation becoming significantly elevated at >40 age is consistent with the significantly elevated psychiatric birth defects of offspring [50].

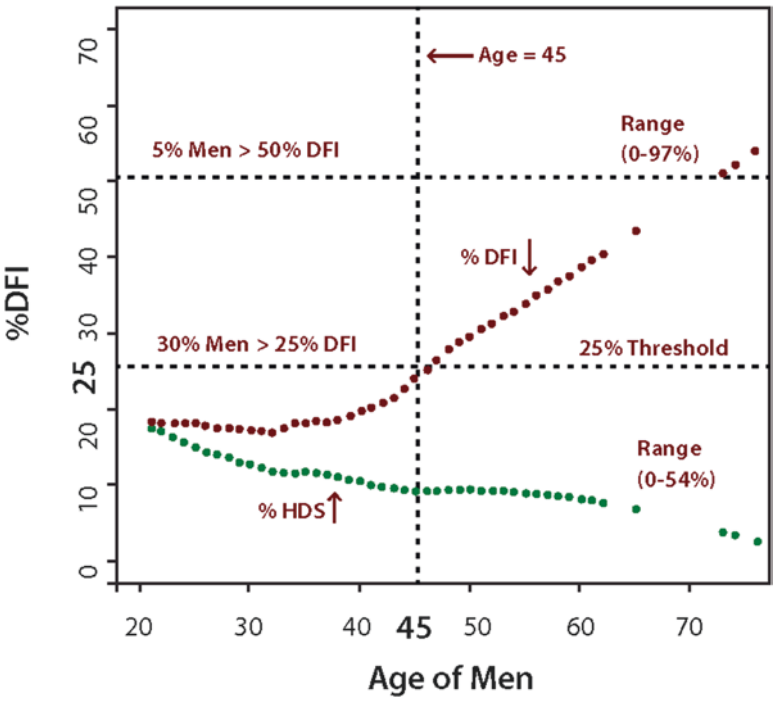


Fig. 4.11 Data on 3026 men attending fertility clinics and sending semen samples to SCSA Diagnostics showing %DFI and %HDS

4.6 SCSA Data Are Predictive of Male Infertility Via Intercourse, IUI, IVF, and ICSI

4.6.1 Pioneer In Vivo Male Factor Study

The 1980 *Science* paper [4] showed the first flow cytometric comparison of sperm DNA fragmentation between known fertile men and men attending an infertility clinic. The mean sperm DNA fragmentation score for men seeking their fertility status was nearly twice as high as the score for men of known fertility [4]. These human data were complemented with clear results (as seen above) of data on bulls and boars of known varying fertility.

The first well-executed in vivo study correlating sperm DNA integrity with pregnancy outcome was done in collaboration with Mike Zinaman at Georgetown University [8]. The SCSA test was used to measure human semen samples from 165 presumably fertile couples wishing to achieve pregnancy over 12 menstrual cycles. Any woman with female infertility factors was excluded. SCSA data from the male partners of 73 couples (group 1) achieved pregnancy during months 1–3 were compatible with "high fertility." These SCSA values were significantly different from those of 40 couples (group 3) achieving pregnancy in months 4–12 ($P < 0.01$) and of those male partners of 31 couples (group 4) not achieving pregnancy ($P < 0.001$). Group 2 contained couples who had a miscarriage. "Based on logistic regression, the level of %DFI was the best predictor for whether a couple would not achieve pregnancy." Some 84% of males in Group 1 had <15% DFI; no couples achieved pregnancy in Group 1 with >30% DFI. Using selected cutoff values for chromatin integrity, the SCSA data predicted 7 of 18 miscarriages (39%).

Shortly after the above publication, Spano et al. [27] published a time to natural pregnancy on 215 "Danish first pregnancy planners" with no previous knowledge of their fertility status. Data was obtained on 1301 cycles (838 cycles, months 1–6; 463 cycles, months 7–24). The probability of pregnancy in a menstrual cycle across the entire range of SCSA values obtained from the initial semen samples is incorporated into the drawing in Fig. 4.7 that also includes pregnancy estimates from IUI and IVF/ICSI studies [8, 27, 28, 51–54].

At 20% DFI, fecundability started dropping and became very small for values of 30–40%. Thus, the probability of producing a healthy pregnancy via intercourse sharply declined beginning at 20% DFI and was negligible when this fraction added up to 40%. As stated by the authors, "this level 'makes this individual a good candidate' not to conceive." The results of both above studies [8, 27] are consistent with the finding that sperm chromatin structure is reflective of fertility potential, which significantly deteriorates when %DFI is >30%. As stated, SCSA data is highly indicative of male subfertility, regardless of the number, the motility, and the morphology of the spermatozoa [27].

The publications of the two above studies remained for many years as the only two papers showing odds ratios (ORs) via intercourse on semen samples measured by the SCSA. These ORs of 7–8 were confirmed by independent meta-analysis [51, 52].

In contrast to the data presented in these two above studies, the 2006 (and subsequent years) American Society for Reproductive Medicine Compendium of Practice Report found no significant effects of elevated sperm DNA fragmentation by using a 30% DNA fragmentation index (DFI) threshold for natural fertilization and SCSA data (odds ratio, 1.07; 95% confidence interval, 0.39–2.93) [51]. In an independent meta-analysis [51, 52], it was shown that these two in vivo studies showed significant odds ratios of 6.54 (95% confidence interval, 1.71, 24.91) and 7.58 (95% confidence interval, 2.54, 22.67), which resulted in the conclusion that the pregnancy rates are statistically significantly higher for the group with DFI below the thresholds of 30% and 40%, respectively.

4.6.2 ART Clinic

4.6.2.1 IUI

A SCSA study including IUI couples was done by Bungum et al. [54] in 2007. Of great interest was the observation that when the SCSA %DFI value was greater than 30%, the pregnancy rate was a dramatically low 1.5% in contrast to those with <30% that had a successful pregnancy rate of 19.0%. These data strongly suggested that men with a DFI of >30% had a very low chance with both natural and IUI conception and should move to ICSI. These IUI data are also incorporated into the clinical interventions as seen in Fig. 4.7.

Figure 4.7 shows the very significant drop in successful pregnancies as the %DFI falls from the 20 % to 30%. The threshold for IUI and natural fertility has been set at ~25% DFI [8, 27, 54].

4.6.2.2 IVF/ICSI

Bungum et al. [54] analyzed a total of 998 cycles (387 IUI, 388 IVF, and 223 ICSI). No statistical difference between the outcomes of IVF versus ICSI was observed in the group with DFI ≤27%. In the DFI >27% group, however, the results of ICSI were significantly better than those of IVF. Comparing ICSI with IVF, the OR (95% CI) for BP was 26 (1.9–350). The IVF and ICSI fertilization rates were not statistically different between high- and low-DFI groups. More men with >15% HDS had lower (<25% and <50%) IVF fertilization rates. Men with >30% DFI were at risk for low blastocyst rates (<30%) and no ongoing pregnancies. Thus, the authors proposed that "all infertile men should be tested with SCSA as a supplement to the standard semen analysis. When DFI exceeds 30%, ICSI should be the method of choice."

A recent study by Oleszczuk et al. [28] was based on 1633 IVF or ICSI cycles. DFI values were categorized into four intervals: DFI ≤ 10% (reference group), 10% < DFI ≤ 20%, 20% < DFI ≤ 30%, and DFI > 30%. 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 pickup (OPU), odds ratios (ORs) for at least one good quality embryo (GQE) were significantly lower in the standard IVF group if DFI > 20%. OR for live birth calculated per OPU was significantly lower in standard IVF group if DFI > 20% (OR 0.61; 95% 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 was statistically significantly higher for DFI > 20% (OR 1.7; 95% CI: 1.0–2.9; $p = 0.05$). OR for miscarriage was significantly increased for DFI > 40% (OR 3.8; 95% CI: 1.2–12; $p = 0.02$). *The results suggest that ICSI might be a preferred method of* in vitro *treatment in cases with high DFI.*

4.7 Conclusions

Now after nearly four decades of basic and clinical research with an estimated > ~150,000 animal and human sperm samples measured by the SCSA, it can be stated with confidence that the *SCSA test is well suited for testing in the human clinic. Specifically:*

- A 0–20% DFI is considered excellent DNA integrity. However, for the man with one or more abnormal WHO semen parameters, the OR significantly decreases for a successful pregnancy.
- From 20% to 30% DFI, a continuous falling off odds for a successful pregnancy by in vivo and IUI.
- HDS >25% [22–26] and certainly >35% leads to very poor embryo development, few blastocysts, and embryos arresting at about eight-cell stage.
- When SCSA %DFI is above 20–30%, there are data to support moving from standard IVF treatment to ICSI.
- Above 40–50% DFI, the odds for pregnancy are very low by any means of fertilization and with increased odds for miscarriages. Consideration may be made to use testicular sperm/ICSI (TESE) [55].
- Men above the age of 45 seeking to father a child should have sperm analyzed by SCSA since these men are at increased risk of sperm DNA damage and this is the point of age at which the mean %DFI is indicative of poorer pregnancy outcomes.
- It is a small cost, relative to many other male and female infertility tests, to take a SCSA test that may indicate the male as the prime factor in lack of a pregnancy. Such SCSA reports become highly valuable to *both* the patient and the clinic's interests.
- A recent review by Agarwal et al. [56] outlined the evolution of sperm DNA fragmentation (SDF] tests from their origin to current utility in the urology and infertility clinics and recognize that SDF has been generally acknowledged as a

valuable tool for male fertility evaluation. These authors [56] note that the latest American Urological Association (AUA) and the European Association of Urology (EWAU) have acknowledged the importance of DNA fragmentation in sperm as guidelines on male infertility. The authors conclude their review with the statement: "SDF testing should be included in the evaluation of male factor fertility along with the standard semen analysis. Any couple that fails to obtain a pregnancy within a year would gain a valuable insight into the potential that couple infertility may be due to sperm DNA fragmentation and, if so, to proceed with the recommendation to reduce SDF by lifestyle changes or select an ART procedure in part determined by the results of a SDF test."

- SCSA testing can be done at any lab that follows the precise published protocol on all known flow cytometers when using SCSAsoft, or equivalent, software for clinical reports; alternatively, most continents have labs with commercial SCSA testing, including North America (www.scsatest.com), London (www.tdlpathology.com), India (www.andrologycenter.in), Brazil (www.androscience.com), and Sweden (www.med.lu.se), and other sites may become available.

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