# Comparison of the Halosperm® test kit with the Sperm Chromatin Structure Assay (SCSA®) infertility test in relation to patient diagnosis and prognosis

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The Halosperm® test kit, the latest of the sperm DNA fragmentation tests, is considered by Fernández et al. to be a suitable replacement for the Sperm Chromatin Structure Assay (SCSA®) test. Although the test is ingenious and interesting, the data lack the statistical rigor of the SCSA® test. (Fertil Steril® 2005;84:846–9. ©2005 by American Society for Reproductive Medicine.)

The focal point of this Controversy section is the usefulness of sperm DNA fragmentation tests in the human infertility clinic. The issues are: [1] Is sperm DNA fragmentation of clinical importance? [2] If so, what are the criteria for clinically acceptable tests, including the Halosperm® test kit? [3] Do these tests provide an established clinical threshold for patient diagnosis and prognosis?

This is the silver anniversary year of the Sperm Chromatin Structure Assay (SCSA®) (1), the first of the current assays and the one that has been used in an attempt here to validate the Halosperm® test. Since the final revision and naming of the SCSA protocol was accomplished 20 years ago, our laboratory has measured at least a hundred thousand animal and human sperm samples in *exactly* the same way, providing a huge data base on the relevance of this assay, including the many factors that can contribute to artifacts.

Details of the SCSA test have previously been reviewed (2). However, in the context of comparison of the SCSA and the Halosperm® kit paper, some remarks are included here regarding the validation of the SCSA. The SCSA has been validated as highly repeatable, dose responsive to genotoxic agents, and biologically stable and meaningful in several dozen published articles that clearly documented these points. The SCSA data on bulls that had individually sired thousands of offspring ($r = -0.65$, $P<.01$) (3) as well as sensitive heterospermic insemination trials ($r = -0.94$, $P<.01$) established the SCSA as a predictor of male sub/infertility (4). The SCSA data identified boars with a lower fertility rate ($r = -0.69$, $P<.002$) and lower piglets/litter ($r = -0.57$, $P<.02$), most likely due to early embryo death attributed to male genome failure (Didion and Evenson, unpublished data). Genotoxic agents known to cause dominant lethal mutations produced SCSA measurable DNA fragmentation 5 days before the occurrence of early embryo death suggesting that SCSA was detecting the early molecular events (DNA strand breaks) that later led to nonfunctional paternal genes and embryo death.

The robust power of flow cytometry and the SCSA test were shown in measurements of eight monthly consecutive semen samples from 45 men comparing SCSA data with classically measured semen quality (5). The SCSA scattergrams consisting of 5,000 dots, each representing a single sperm, were dramatically similar from month to month for each man, although they differed in varying degrees between men. The statistics were equally dramatic, showing a much smaller coefficient of variation (CV) (10%) of SCSA values over time compared to the classic semen quality measures (44% within-subject sperm count). Recognizing that any new test of semen quality is of value only if it measures an independent parameter, it was of importance to note the weak correlations between SCSA parameters and sperm count, motility, and morphology were −0.27, −0.30, and 0.21, respectively (2). A number of studies done in our laboratory and those of other investigators have shown variations in correlations between SCSA data and classic semen parameters. The highest seen has been −0.60 between motility and the DNA Fragmentation Index (DFI), which has been rationalized as potential reactive oxygen species (ROS) activity, damaging both sperm cell membranes, including mitochondrial membranes and DNA.

In the Georgetown male factor infertility study (2), male partners of 200 presumably fertile couples provided a semen sample at each of the first 3 months of a 12-month study. The SCSA data clearly showed a highly significant relationship between the percentage of sperm with fragmented DNA

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(% DFI), formerly termed cells outside the main population (% COMP), and time to conception. Considering the mean %DFI values from couples conceiving within the first 3 months as having "excellent fertility potential," those conceiving within the next 9 months had a significantly higher DFI value ($P<.01$) and those not conceiving by the end of month 12 had ever higher DFI values ($P<.001$). It is from this study that the statistical categories of ≤15% DFI = excellent fertility potential; >15% to <30% DFI = good fertility potential; ≥30% DFI = fair to poor fertility potential, were derived. Of concern, it was stated in this study that no couples (6) conceived in the first 3 months with a DFI value of >30%; however, this statement was unfortunately misconstrued by some as "no conceptions will ever occur when the DFI value is above 30%." It is of note that the data suggested 39% of miscarriages were related to a DFI >30%. The odds ratio of achieving pregnancy were 6.5 times if the DFI was <30%.

The SCSA data obtained by Spano et al. (7) on 215 first pregnancy of Danish couples showed an odds ratio of 10 times greater probability of pregnancy by natural intercourse if the DFI values were <40%.

A review of the SCSA and other DNA fragmentation tests and early clinical results (7) was written at the request of the Section Editor with very short notice that included a telephone survey from New York City during 9/11–14/2001. The survey suggested a value of about 10% pregnancies when the DFI was >30%. Unfortunately, this was misprinted as 1% but not noticed by the authors until months later. Hundreds of reprints were sent out with the corrected survey number, but a formal correction was not published.

Bungum et al. (8) found that patients undergoing IUI were 8.7 times more likely to deliver a baby with an SCSA-defined DFI ≤27% ($P=.01$); these data have been recently confirmed by this same group with a larger data set (Bungum, personal communication). Although the predictivity of the SCSA for in vivo and IUI fertilizations are clear for reduced pregnancy odds, the results from IVF and intracytoplasmic sperm injection (ICSI) are less predictable (9). Odds ratios from several IVF/ICSI studies ranged from 1.5 to 3.3 times the pregnancy rate (PR) if the %DFI was <30 (significance range: $P=$not signicant to .0001) or less than 27%. Larson et al. (10) showed no ICSI pregnancies with %DFI >27%, whereas Bungum et al. (8) found the results of ICSI were significantly better than those of routine IVF in the >27% group. Possible reasons for the lack of clarity in these reports are the small number of patients in many studies, lack of distinction between routine IVF and ICSI, length of time between SCSA testing and pregnancy attempts, and, most importantly, the success rate of different ART clinics. It is noted that Henkel et al. (11), using the Tunel assay, found that with >36% Tunel-positive sperm that the fertilization rate was the same but the PR decreased from 34.65% to 19.05% ($P=.03$).

A meta-analysis of three studies (n = 1,575) (2, 7, 8) was conducted to investigate the relationship of sperm DNA fragmentation on pregnancy outcome using in vivo and IUI procedures. Using the Cochran-Mantel-Haenszel statistic, the meta-analysis indicated that patients were 7.3 times more likely to achieve a pregnancy/delivery if the DNA fragmentation Index (DFI) <30% (p=0.0001). The Breslow-Day Test, which tests the homogeneity of all the odds ratios used in the meta-analysis, showed that the odds ratio for all studies tested was not significantly different (p=0.96) and showed similar trends.

In six other studies (n = 1950) (2, 7, 8, 11–13) that included routine IVF fertilization as well as in vivo and IUI procedures, the meta-analysis indicated that patients were ~3.0 times more likely to achieve a pregnancy/delivery if the DFI was <30% ($P=.0001$). The odds ratio for all studies tested was not significantly different ($\chi^2 = 11.7$, $P=.07$) and showed similar trends.

A meta-analysis of five studies using IVF/ICSI (n = 216) (8, 10, 12, 14, 15) showed that the patients were 1.7 times more likely to achieve a pregnancy/delivery if the DFI was <30% ($P=.11$). With the Breslow-Day test, the odds ratio for all studies tested was significantly different ($\chi^2 = 10.3$, $P=.04$). Patients using IVF or ICSI with DFI values >30% showed a trend for increased spontaneous abortions (9).

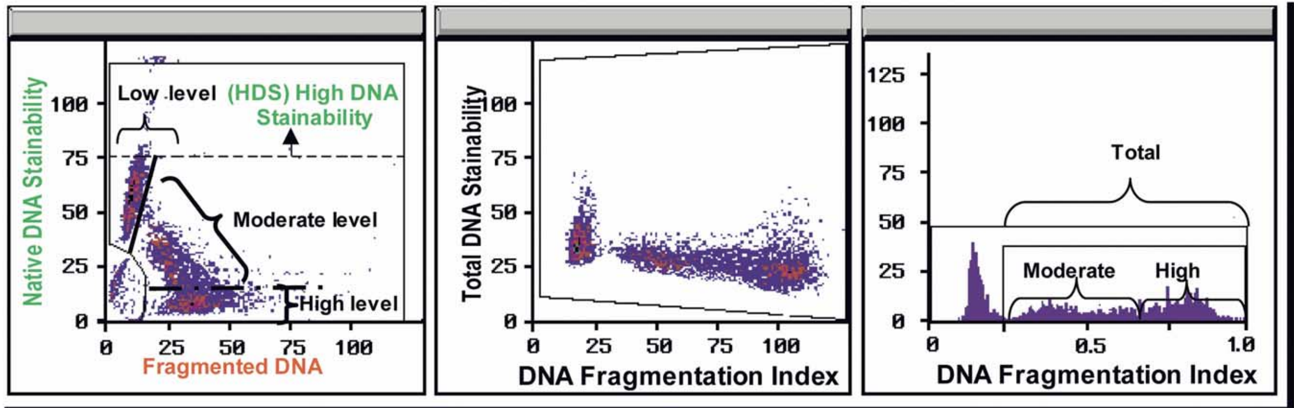
These meta-analyses show that the SCSA infertility test is significantly predictive for reduced pregnancy success using natural intercourse, IUI, and to a lesser extent routine IVF and ICSI. These data suggest that if a man has a DFI of >30% that IUI should probably not be considered and that the couple move to routine IVF or ICSI.

The SCSA cytograms show four different cell populations: percent total (the percentage of sperm with moderate and high levels of DNA fragmentation), percent moderate (percentage of sperm with moderate levels of DNA fragmentation), percent high (percentage of sperm with high levels of DNA fragmentation), and high DNA stainability. High DNA stainability measures the percentage of sperm with immature chromatin. High DNA stainability sperm have less chromatin condensation, which leads to increased DNA stainability. Patients with >15% high DNA stainability take a longer time to in vivo and IUI pregnancies (2, 8).

It is significantly noted here that SCSA has another parameter that has a higher correlation with a fertility index as seen for cattle and for toxicant damage to mouse sperm. This factor is the SD of the DFI (DFI expressed as channels 0–1,024) in 5,000 individual sperm with the calculated parameter of red/red + green. For bulls, the correlation between fertility and %DFI was −0.40 ($P<.01$), whereas it was −0.58 ($P<.01$) for the SD of DFI. In the more sensitive bull heterospermic insemination experiments that rule out interfemale factors, the correlations were −0.74 ($P<.05$) and −0.94 ($P<.01$) between %DFI and SD DFI, respectively (Fig. 1). These factors are now being studied in more detail for human fertility.

**FIGURE 1**

SCSA Clinical Report. *Left panel*: SCSA cytogram of red fluorescence (fragmented DNA) vs. green fluorescence (native DNA stainability). *Middle panel*: SCSAsoft (SCSA Diagnostics, Brookings, SD) software reconfigured data from SCSA cytogram. Y = total DNA stainability, X = DNA Fragmentation Index. *Right panel*: Frequency histogram of DNA Fragmentation Index from data in middle panel. *Note*: The DFI histogram can only be derived from the calculated parameters shown in the middle panel and NOT from the cytogram in the left panel. Note the incredibly high repeatability between measurements of two separate aliquots of the same semen sample. HDS = High DNA Stainability.




| Patient | Date | Measurement | mean DFI | SD DFI | Total DFI(%) | HDS(%) |
|---|---|---|---|---|---|---|
| 7272-113 | #### | 1 | 563.7 | 307.0 | 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 | 0.4 |

*Evenson. Comparison between Halosperm® kit and SCSA. Fertil Steril 2005.*

The orientation of the cytograms placement pattern is uniquely advantageous to help decipher the pathology of the sample. The mean and SD of the DFI data are based on all sperm analyzed and are independent of any computer operator gating of raw data. In some cases, the mean and SD of DFI provides insights into the fertility diagnosis that are not available from the %DFI alone. The table columns of mean DFI and SD DFI seen in Fig. 1 provide quantitative and qualitative values that are free from human intervention for derived SCSA numerical output.

In comparison of the two assays, the Halosperm kit measures only ~500 sperm on a light microscope slide in contrast to 5,000 sperm for the SCSA test.

The results shown in Fernández et al. (16) illustrate that there are substantial differences in numerical values between the results of the two tests, and furthermore, that they diverge more widely for samples with higher SCSA DFI values. For example, a sample with a SCSA DFI of 30%–35% could have a Sperm Chromatin Dispersion result ranging between 20% and 50% (i.e., −10% to +15%). Moreover, given that the interobserver error can be up to 12%, the real biological variability could be either negligible or almost double what is shown in that figure. We believe that the use of SD rather than SEM would have better shown the differences between the two tests. The following apparent 95% ranges have been back-calculated from the results of Fernández et al.:

- normozoospermic men (n = 46): the stated mean 16.3 and SEM ± 6.0 leads to a SD of 40.69 and a 95% CI from −65.9 to +98.5.
- OAT group (n = 23): the stated mean 47.3 ± SEM 17.3 leads to a SD of 83.0 and a 95% CI from −124.8 to +219.4.

We note what appears to be a lack of significance ($\chi^2$ analysis) regarding the mean sperm cells with fragmented DNA observed among the four technicians. Because of the very high percentage (6%–12%) in the intraobserver CV it is understandable that there was no significant difference found due to the high variability within technician scoring. The power of flow cytometry provides a CV of SCSA values in our laboratory below 3% for sample replicates.

In summary, the Halosperm kit is measuring sperm DNA strand breaks, and the data provided support that view with regard to the SCSA test. The primary difference between the two tests is the power of flow cytometry with the SCSA test, which is of great importance for andrology laboratories.

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