SciMedCentral

JSM Invitro Fertilization

Review Article

Sperm Chromatin Structure Assay (SCSA®): The Clinical Utility of Measuring Sperm DNA Damage and its Potential Improvement with Supplemental Antioxidants

Donald Evenson[1]*, Parviz Gharagozloo[2], and Robert John Aitken[3]

[1]*SCSA Diagnostics, South Dakota State University, USA*
[2]*CellOxess Biotechnology, USA*
[3]*Priority Research Centre for Reproductive Biology, University of Newcastle, Australia*

***Corresponding author**
Donald Evenson, SCSA Diagnostics, South Dakota State University, 302 6th St W, Suite B, Brookings, SD, Tel: 605-592-9071; Fax: 605-592-9021; Email: don@scsatest.com

Submitted: 20 November 2016

Accepted: 07 December 2016

Published: 02 January 2017

Keywords
- Infertility
- SCSA
- Sperm DNA damage
- Antioxidant
- Fertilix

Abstract

The Sperm Chromatin Structure Assay (SCSA®) is the pioneering sperm DNA fragmentation assay that precisely measures the percent of sperm in a semen sample that have Sperm DNA Damage (SDD), a negative factor for successful pregnancy. The SCSA® is a rapid, dual parameter, computer driven assay with diligent unbiased flow cytometer measurements on five to ten thousand sperm per sample providing rigorous statistics. The SCSA® is the Gold Standard assay and is the most used SDD assay world-wide for human and animal sperm with hundreds of thousands research and clinical samples measured. As such, the SCSA® is the only internationally standardized SDD assay that is validated for a clinically established threshold, with precise and repeatable measures in the human clinic. Frozen clinical samples may be sent on dry ice or in liquid nitrogen dry shippers internationally by FEDEX, or equivalent, to a SCSA Diagnostic Center that results in clinical reports within 1-3 days. One-fourth of men attending an infertility clinic have high levels of sperm DNA fragmentation that need clinical counsel on Assisted Reproductive Technology (ART) such as use of intrauterine insemination (IUI) to *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The threshold for *in vivo* and IUI fertilization that suggests moving to ICSI is 25% of sperm in an ejaculate with SDD (25 %DFI). Sperm samples with severe DNA fragmentation have greatly increased statistical risk for lack of pregnancy or miscarriage. A major emphasis for male factor infertility patients is to reduce the level of SDD by changes in life style and the use of antioxidant supplements. One such supplement with great promise shown by pre-clinical experiments is Fertilix®, a scientifically formulated product now in clinical trials.

ABBREVIATIONS

SDD: Sperm DNA Damage; SCSA®: Sperm Chromatin Structure Assay; AO: Acridine Orange; OR: Odds Ratio; DFI: DNA Fragmentation Index; ART: Assisted Reproduction Techniques; TUNEL: Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labelling Assay; TdT: Terminal deoxynucleotidyl Transferase; IUI: Intrauterine Insemination; ICSI: Intracytoplasmic Sperm Injection

INTRODUCTION

Male factor infertility is now recognized as causative in approximately 50% of infertile couples. According to a large recent study [1], one-fourth of men attending an infertility clinic have high levels of SDD that need clinical counsel on Assisted Reproductive Technology (ART) such as use of intrauterine insemination (IUI) to *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). This study has indicated that % DFI values can be used to decide between IVF or ICSI; samples with DFI > 20% compromise pregnancy outcomes. Sperm DNA Damage (SDD), often resulting from oxidative stress [2] and impacting the male reproductive tract, is now recognized as one of the most serious defects towards achieving a healthy pregnancy and the health of the progeny. It is therefore not surprising that antioxidants or antioxidant formulations are frequently consumed by patients to reduce the level of oxidative DNA damage in spermatozoa. However, surveys of physicians have shown that current treatments are recommended haphazardly, without proper scientific consideration of their mode of action, safety or proof of therapeutic benefits [3].

As a significant male infertility factor, the detection of SDD and its optimum resolution requires clarity as a matter of urgency. Towards this, three significant conditions need to be met; 1) Implementation of standard evaluation and treatment protocols for large populations of couples seeking infertility treatment, 2) A standardized and proven laboratory protocol that measures sperm DNA damage prior to and following antioxidant treatment and 3) a scientifically designed antioxidant to be consumed over the course of at least one spermatogenic cycle (about 3 months).

In 1980, Evenson and colleagues pioneered the concept that sperm DNA integrity, measured in situ, could be a valuable indicator of male factor infertility [4]. A sperm DNA fragmentation test, called the Sperm Chromatin Structure Assay (SCSA®) was invented. SCSA® is now the most extensively used sperm DNA fragmentation test worldwide and has specific, unique characteristics which define it as the reference point in this field. An initial publication in Science [4] provided data showing that the SCSA® test was valuable for both human and animal infertility diagnoses. Further carefully controlled animal fertility and toxicology studies showed that the SCSA® test is a high precision test which yields low CV's for repeat measurements [5-8]. Data were also obtained from bull and boar studs where thousands of inseminations of semen, from a dozen or so males, showed excellent correlations between SCSA® data and fertility outcomes with R-values in the range of 0.6 - 0.9 (P<0.01- 0.001) [9-11]. Additionally, sperm from toxicant-treated mice had highly significant dose-response values with %DFI [12-16]. In a longitudinal study [17] of men exposed to soft coal smoke air pollution (presumably c-PAHs), SCSA® was the only test to detect dramatic damage to sperm DNA despite standard semen parameters not changing. Interestingly, this study also revealed a statistically significant association between glutathione-S-transferase M1 (GSTM1-) null genotype and increased %DFI (beta= 0.309; 95% CI: 0.129, 0.731) as defined by SCSA®. This provided novel evidence for a gene-environmental interaction between GSTM1 and air pollution [18]. Early data on human fertility showed OR's of 6-7X greater probability of pregnancy when less than 25% of sperm had measurable DNA strand breaks as measured by the SCSA® [19-21]. Furthermore, and quite significantly, the SCSA® assay is sufficiently sensitive to predict which ART pathway i.e., IUI, IVF or IVF-ICSI, should be clinically utilized in the treatment of infertile couple [1]. For example, samples with DFI > 20% already compromise pregnancy outcomes [1].

At a practical level, the conduct of the SCSA® test is a rapid, technician friendly test that produces high machine precision, dual parameter measures. "Dual parameter" means that each sperm, represented as a dot on the recording oscilloscope (scattergram, see figures below) has a value for both green fluorescence (native DNA) and red fluorescence (broken DNA). For example, a single sperm may be characterized by a green value of 540/1000 channels and red value of 140/1000 channels. These dual parameters report not only on the % of sperm with broken DNA but also the extent of sperm DNA fragmentation. The SCSA® test also records the HDS (High DNA Stainable) sperm population, i.e., the maturity level of the sperm with respect to its nuclear protein composition [22]. If excess sperm nuclear histones above the approximate 15% histones in human sperm, are not replaced by protamines, the green fluorescence will be increased since acridine orange (AO) stained histone-complexed DNA stains 2.3 X more than protamine complexed DNA [23]. In a study of 182 human semen samples, the %HDS population was significantly correlated with and % CMA3 stained sperm(r=0.610, P < 0.0001) [24], the latter known to stain histones. The relationship of the level of HDS to reproductive outcome is not clear; however, some data suggest that it is related to increased miscarriages [20]. The rational proposed is that abnormal chromatin structure may cause an abnormal read out of genes that are required for proper embryo growth.

The SCSA® DNA fragmentation test is a robust and reproducible test if the published protocol is followed precisely. Any variation of the published protocol runs the risk of erroneous results for the SCSA® test. The SCSA® protocol has been detailed [6]; however, in brief, it is performed as follows:

SCSA® Protocol

1) Frozen raw semen is thawed in a 37°C water bath until the ice just disappears and then diluted with TNE buffer

2) Acid (pH 1.20) denaturation of DNA for 30s at sites of existing DNA strand breaks

3) AO staining of ss (red) and ds (green) DNA

4) Measurement 5 x 10^3 sperm by flow cytometry

In brief, AO is a small planar molecule (MW= 265) that intercalates into intact, double stranded DNA and fluoresces green (515-530nm) when excited by blue light (usually a 488nm laser beam). AO stacks on ssDNA and this complex collapses into a crystal that when exposed to blue light has a metachromatic shift to red fluorescence (> 630-640 nm) [22]. To differentially stain intact DNA and DNA with single or double strand breaks, the sites of strand breaks need to be opened, i.e. denatured, prior to staining. This has been accomplished by two means: 1) heating the sperm at 100°C for 5 min. [4], and 2) treating the sperm with a buffer (pH 1.20) for 30 sec [5]. It is noted that the two methods yield the same result. Comparisons of the different DNA fragmentation tests have suggested to some that the TUNEL assay directly measures sperm DNA strand breaks while SCSA® measures 'potential' DNA strand breaks. However, since neither the heat nor the acid treatments used in SCSA are severe enough to break the DNA phosphodiester bonds, it has been concluded that the SCSA® test does in fact also measure existing DNA strand breaks [7]. In closer comparison of SCSA and TUNEL, it has been estimated that TUNEL measurements consistently report about one-third less sperm with DNA strand breaks than the SCSA® test [7]. This phenomenon is likely due to the TUNEL assay utilizing a large protein (TdT) to tag DNA strand breaks with inherently limited access to the highly-condensed sperm chromatin, while the SCSA® assay uses the small AO molecule which can effectively penetrate condensed sperm chromatin [7].

Clinical printouts of patient samples are shown below. Figure (1) (left) shows SCSA® data on human semen with good DNA integrity (left panel). Figure (2) (right panel) shows clinical results on semen sample with very poor DNA integrity. Note the extremely low (> 0.0) SD of both measures of %DFI.

The clinical threshold for SCSA® in the context of human SDD has been shown for natural or IUI fertilization to be ~25% DFI for predicting a longer time to pregnancy, higher incidence of miscarriage or the failure to achieve pregnancy [19]. Bungum et al., showed that at 25% DFI (as measured by SCSA®), pregnancy rates for IUI fell to ~2% [26]. The obvious question these studies raise is that if only 25% of the sperm have measurable damaged DNA, why do the remaining 75% of spermatozoa not present a high probability for achieving a pregnancy? This question is also

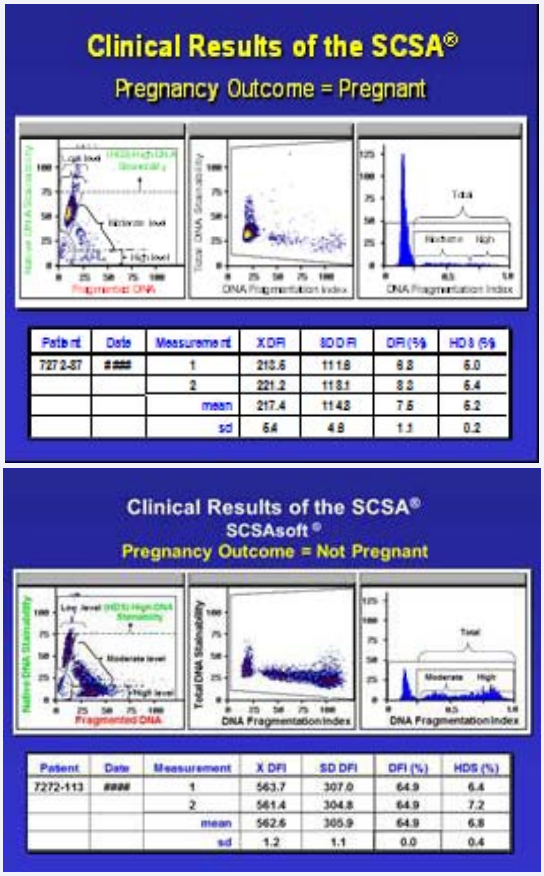

Figure 1,2 Left panel. Raw data from flow cytometer showing each of 5000 sperm as a dot on a scattergram. Y axis= Green fluorescence with 1024 gradations (channels) of DNA stainability. X axis= Red fluorescence with 1024 gradations of red fluorescence (ss DNA). Axes are 1024/10. Dotted line at Y =75 marks the upper boundary of DNA staining of normal sperm chromatin; above that line are sperm (dots) with abnormal sperm chromatin allowing more DNA stainability. Three levels of sperm DNA integrity: Normal, Moderate and High. Bottom left corner shows gating out of seminal debris.

Middle panel. Raw data from left panel are converted by SCSAsoft® software (or equivalent) to red/ red + green fluorescence [22]. This transforms the angled normal sperm display in left panel to a vertical pattern that is critical for accurately delineating the percent of sperm with fragmented DNA and other levels of broken 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 DFI and High DFI (moderate + high DFI = total %DFI).

dramatically highlighted by field trials in pigs; namely, the DFI threshold has repeatedly been shown to be 6% as measured by the SCSA® [10]. In this instance, why do the 94% of spermatozoa, which supposedly have "good sperm DNA integrity", fail to generate a normal pregnancy rate? The answer is hypothesized to lie in what we call the" iceberg effect" [22]. To elaborate, while 6-25% sperm above the "waterline" may be measured as defective, the remaining 75-94% of cells below such thresholds are either in an early transition state of DNA fragmentation (pre-fragmentation) or carry other types of structural defects not measured by current assays, but are nevertheless detrimental to achieving pregnancy.

As previously alluded to, the primary cause of sperm DNA damage is oxidative stress. It should therefore come as no surprise that the use of antioxidants in ameliorating oxidative stress has received much attention from scientists, physicians and the nutraceutical industry. Indeed, many scientific publications including several review papers report the beneficial use of antioxidants in promoting male fertility [3,27-29]. As a result, over the last decade, a small industry has boomed around the use of antioxidant nutraceutical formulations to treat male infertility with at least 20 such formulations now available in the USA alone. The majority of them substantially differ in the variety of antioxidant ingredients and doses used. Little or no credible *in vivo* or human clinical data are reported for any of them. Worryingly, many of these formulations combine a large number of antioxidants with aggressive dosing. This arbitrary practice raises the strong possibility of over-supplementation resulting in 'reductive stress' potentially depleting the physiological levels of reactive oxygen species known to be critical for normal sperm function [3,30-32]. Lack of personalization of these supplements means that men with low sperm DNA damage are treated in the same way as those with severe levels thus adding to the risks associated with over-supplementation. Two key questions remain. Which antioxidants and which doses may represent an "optimum combination" for men with varying degrees of oxidative stress? And, would such a combination be efficacious in reducing not only sperm DNA oxidation but also sperm DNA fragmentation?

Based on the principles of medicinal chemistry and drug design, our group of scientists recently reported such a personalized combination of antioxidant ingredients [33]. Trademarked Fertilix®, this product was examined in extensive preclinical studies by two independent laboratories in France and Spain. The results from both laboratories provide strong evidence that Fertilix® restores the normal redox balance within the male reproductive tract, reduces sperm oxidative DNA damage and increases pregnancy rates in mouse models of oxidative stress induced male infertility [33]. Specifically, oral administration of Fertilix® completely prevented sperm oxidative DNA damage in glutathione peroxidase 5 (Gpx5), knockout mice relative to the wild type and restored pregnancy rates to near-normal levels in mice subjected to scrotal heat shock [33]. The data observed in preclinical studies are highly promising as natural antioxidant molecules are involved in similar biochemical activities in cells across most mammalian species, including humans. The preclinical data provide strong evidence that using a scientifically validated antioxidant supplement pre-conceptually may significantly improve the chances of a successful pregnancy. A large international randomized double-blind placebo-controlled cross-over clinical trial is now underway to explore the efficacy of Fertilix® against sperm DNA fragmentation and a variety of primary and secondary reproductive outcomes.

CONFLICT OF INTEREST

D. Evenson is CEO of SCSA Diagnostics; P. Gharagozloo is CEO of CellOxess.

REFERENCES

1. Oleszczuk K, Giwercman A, Bungum M. Sperm chromatin structure assay in prediction of in vitro fertilization outcome. Andrology. 2016. 4: 290-296.
2. Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. Reprod Fertil Dev. 2016; 28: 1-10.
3. Gharagozloo P, Aitken RJ. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Hum Reprod. 2011; 26: 1628-1640.
4. Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. Science. 1980; 210: 1131-1133.
5. Evenson D. Sperm Chromatin Structure Assay (SCSA): 30 years' experience with the SCSA. In: Agarwal A, Zini A, editors. Sperm DNA and Male Infertility and ART. Springer Publishers. 2011; 125-149.
6. Evenson D. Sperm DNA fragmentation analysis using the sperm chromatin structure assay. In: Carroll D, Aston K, editors. Methods in Molecular Biology. Springer Publishers. 2012.
7. Evenson DP. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Animal Reprod Science. 2016; 169: 56-75.
8. Evenson DP, Jost LK, Baer RK, Turner TW, Schrader SM. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. Reprod Toxicol. 1991; 5: 115-125.
9. Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol Reprod. 1987; 36: 915-925.
10. Didion BA, Kasperson KM, Wixon RL, Evenson DP. Boar fertility and sperm chromatin structure status: a retrospective report. J Androl. 2009; 30: 655-660.
11. Ballachey BE, Saacke RG, Evenson DP. The sperm chromatin structure assay: Relationship with alternate tests of sperm quality and heterospermic performance of bulls. J Andrology. 1988; 9: l09-115.
12. Evenson DP, Baer RK, Jost LK. Long term effects of triethylenemelamine exposure on mouse testis cells and sperm chromatin structure assayed by flow cytometry. Environ and Molecular Mutagenesis. 1989; 14: 79-89.
13. Evenson DP, Wixon R. Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA). Toxicol Appl Pharmacol. 2005; 207: 532-537.
14. Sailer BL, Sarkar LJ, Jost, LK, Bjordahl J, Evenson DP. Effects of heat stress on mouse testicular cells and sperm chromatin structure as measured by flow cytometry. J Androl. 1997; 18: 294-301.
15. Evenson DP, Jost LK, Baer RK. Effects of methyl methanesulfonate on mouse sperm chromatin structure and testicular cell kinetics. Environ Mol Mutagen. 1993; 21: 144-153.
16. Evenson DP, Baer RK, Jost LK. Long term effects of triethylenemelamine exposure on mouse testis cells and sperm chromatin structure assayed by flow cytometry. Environ and Molecular Mutagenesis. 1989; 14: 79-89.
17. Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, et al. Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. Hum Reprod. 2005; 20: 2776-2783.
18. Rubes J, Selevan SG, Sram RJ, Evenson DP, Perreault SD. GSTM1 genotype influences the susceptibility of men to sperm DNA damage associated with exposure to air pollution. Mutat Res. 2007; 625: 20-28.
19. Evenson D, Wixon R. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. Reprod Biomed Online. 2006; 12: 466-472.
20. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, et al. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod. 1999; 14: 1039-1049.
21. Spano M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil Steril. 2000; 73: 43-50.
22. Evenson DP, Larson K, Jost LK. The sperm chromatin structure assay (SCSA TM): clinical use for detecting sperm DNA fragmentation related to male infertility and comparisons with other techniques. Andrology Lab Corner. J Andrology. 2002; 23: 25-43.
23. Evenson D, Darzynkiewicz Z, Jost L, Janca F, Ballachey B. Changes in accessibility of DNA to various fluorochromes during spermatogenesis. Cytometry. 1986; 7: 45-53.
24. Jeffay SC, Strader LF, Buus RM, Evenson DP, Olshan AF, Herring AH, et al. Relationships among semen endpoints used as indicators of sperm nuclear integrity. Am SocAndrol. 2006.
25. Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR. Thermal denaturation of DNA in situ as studied by acridine orange staining and automated cytofluorometry. Exp Cell Res. 1975; 90: 411-428.
26. Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, et al. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. Hum Reprod. 2007; 22: 174-179.
27. Tremellen K, Miari G, Froiland D, Thompson J. A randomized control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment. Aust N Z J Obstet Gynaecol. 2007; 47: 216-221.
28. Ross C, Morriss A, Khairy M, Khalaf Y, Braude P, Coomarasamy A, et al. A systematic review of the effect of oral antioxidants on male infertility. Reprod Biomed Online. 2010; 20: 711-723.
29. Showell MG, Mackenzie-Proctor R, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. Antioxidants for male subfertility. Cochrane Database Syst Rev. 2014; CD007411.
30. Chen S, Allam JP, Duan YG, Haidl G. Influence of reactive oxygen species on human sperm functions and fertilizing capacity including therapeutical approaches. Arch Gynecol Obstet. 2013; 288: 191-199.
31. Ghyczy M, Boros M. Electrophilic methyl groups present in the diet ameliorate pathological states induced by reductive and oxidative stress; a hypothesis. Br J Nutr. 2001; 85: 409-414.
32. Brewer AC, Mustafi SB, Murray TVA, Rajasekaran NS, Benjamin IJ. Reductive stress linked to small HSPs, G6PD, and Nrf2 pathways in heart disease. Antioxid Redox Signal. 2013; 18: 1114-1127.
33. Gharagozloo P, Gutiérrez-Adán A, Champroux A, Noblanc A, Kocer A, Calle A, et al. A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: promising preclinical evidence from animal models. Hum Reprod. 2016; 31: 252-262.

Cite this article

Evenson D, Gharagozloo P, Aitken RJ (2017) Sperm Chromatin Structure Assay (SCSA®): The Clinical Utility of Measuring Sperm DNA Damage and its Potential Improvement with Supplemental Antioxidants. JSM Invitro Fertil 2(1): 1008.