



A Sperm Chromatin Structure Assay (scsa): Background Analysis Of Azoospermic Semen, Validity Of Snap Freezing Semen Prior To Assay, And The Between-ejaculate Variability Seen With Donors

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Abstract

The sperm chromatin structure assay (SCSA) was applied to human sperm, and factors affecting its routine operation investigated. The findings indicated that (a) non-sperm cells in semen can contribute to the results of the SCSA, (b) snap freezing in liquid nitrogen does not affect the result of the SCSA and is a good way of storing semen prior to testing, (c) there is good reproducibility when snap freezing several straws suggesting that they remain representative of the original sample, (d) two donors produced semen samples over many weeks which invariably gave the same clinical information, and (e) there was a significant correlation between the DNA fragmentation index and the number of sperm in the ejaculate.

Introduction

The sperm chromatin structure assay (SCSA) is a diagnostic test that indicates the degree of DNA fragmentation by denaturing sperm DNA at the sites of DNA breaks [6]. The SCSA is gaining popularity in specialist andrology laboratories and appears to provide useful information on the probability of continued embryonic development and the establishment of pregnancy after fertilization [2], and yet the recent WHO manual [15] still regards the SCSA as a "research procedure". As with any other diagnostic test, validation of the assay is important [9] and some technical and biological factors that affect the results obtained with the SCSA have been examined previously [1, 4]. The present study aimed to develop further an understanding of the SCSA applied to human sperm under routine conditions by (a) assessing the contribution of non-sperm samples by performing the SCSA on semen from a vasectomised man, (b) investigating the validity of storing samples prior to assay, and (c) describing the biological variability seen when two donors produced weekly

semen samples over a 10 week period.

Methods

All semen was collected by masturbation and analysed within 1 hr for volume, sperm concentration, morphology and motility [15]. Semen samples were collected and processed for the different parts of the study as follows:

1. *Post- vasectomy semen.* A semen sample from a vasectomised man was processed and analysed within 2 hours of production without freezing.
2. *Validation of snap freezing.* Five men, whose partner had failed to conceive after 12 months and were undergoing fertility investigations, produced samples with 2-5 days sexual abstinence. The SCSA was performed on an aliquot of fresh semen within 1 hour of production. An aliquot of the semen was also loaded in to 0.3ml CBS™ High Security straws, snap frozen in liquid nitrogen and then thawed for assay in the same batch as the fresh semen.
3. *Replicate straws.* Semen was produced after 3 days sexual abstinence by a patient with a known elevated DFI, snap frozen within 1 hour in ten 0.3ml CBS™ High Security straws, and stored in liquid nitrogen for subsequent batch analysis with the SCSA of all the straws.
4. *Biological variability of donors.* Two sperm donors produced semen once per week for 10 consecutive weeks with no restriction on their sexual activity. Aliquots of semen were snap frozen and analysed subsequently with the SCSA in a single batch to minimize between-assay variability.

Frozen samples were thawed at 37°C for 30 secs and placed in an ice bath for 5 mins before running through the SCSA, whilst fresh semen was simply placed directly in the ice bath for 5 mins before performing the SCSA. The SCSA sperm preparation was performed as described previously [5], with the stained sperm analysed using a Becton Dickinson FACSCalibur flow

cytometer and BD CellQuest™ Pro acquisition and analysis software (BD Biosciences, San Jose, CA, USA). A total of 5,000 sperm were counted each time after staining with acridine orange (Cat no. A3568; Invitrogen Australia Pty Ltd, Mulgrave, Victoria 3170, Australia). Acridine orange has a 488nm excitation wavelength with green emission collected using a 530/30nm band pass filter and red emission collected with a 670 nm long pass filter.

The results were plotted on a graph with the ordinate axis being the single stranded DNA staining, and the abscissa being the double stranded DNA staining. The plot was divided into the following regions as shown in Illustration 1:

- a) Region 1 (R1) – the complete population of sperm being the sum of R2, R3 and R4,
- b) Region 2 (R2) – the normal population of sperm with an acceptably low amount of single stranded DNA,
- c) Region 3 (R3) – the population of sperm with an unacceptable amount of fragmented or single stranded DNA. This equates to the COMP_t described previously [5], and the ratio of R3:R1 as a percentage is termed the DNA fragmentation index (DFI), and
- d) Region 4 (R4) – the population of sperm exhibiting a high amount of staining associated with double stranded DNA. The ratio of R4:R1 as a percentage is termed the high DNA stainability (HDS).

Results

A semen sample from a vasectomised man was analysed with the SCSA, and gave a DFI of 69.3% and an HDS of 26.5%, confirming that non-sperm cells are detected by this method.

Plunging semen into liquid nitrogen and analysing after thawing gave a DFI (8.8% ± 2.2%) that was similar to the corresponding fresh sample (8.2% ± 2.1%), showing an excellent correlation between the fresh and frozen semen ($r=0.977$, p

The two donors collecting over a 10 week period always gave samples with DFI results in the normal range (2.3%-7.0% and 3.4%-7.0%). However, the results seemed linked to the semen quality as there was a significant correlation between the DFI and the total number of sperm in the ejaculate for both donors as shown in Illustration 2.

Discussion

Analysis of a semen sample from a vasectomised man

showed clearly that cells other than sperm can be detected by the SCSA. Cells that stain with acridine orange following acid denaturation include lymphocytes [3] which are present in semen albeit in very low numbers [8]. Presumably, the contribution of these occasional cells to the DFI calculation will be most significant in severely oligozoospermic samples.

The running of the SCSA using a flow cytometer requires many quality checks and can be time consuming and costly to start-up and run each day. Depending upon the number of samples received by the laboratory, it may prove more efficient to batch analyse samples or send them elsewhere for analysis. Based upon previous reports showing the stability of DNA when frozen [11, 14], two strategies can be seen to be in operation in clinics namely (a) stockpiling samples for batch analysis by either snap freezing semen in liquid nitrogen [13], cooling in nitrogen vapour [1], and freezing at -80°C, or (b) the storage of samples on dry ice for shipment to a central laboratory with [7] or without [10] cryoprotectant. Interestingly, calibration samples have been frozen immediately at -100°C by some workers but who used cryoprotectant in their clinical samples [7]. The current study has confirmed that the snap freezing of sperm in liquid nitrogen does not affect the DFI as measured with the SCSA, and that the reproducibility of results when frozen in a number of straws shows that each straw remains representative of the original sample.

The present study showed good reproducibility by the two donors. The reproducibility of results from donors followed for 8 months has been reported previously [7], with individual donors showing remarkable consistency. However, it should be acknowledged that changes in the health of a man, e.g. alterations in accessory gland function [12], can change things. An interesting finding in the present study was the positive correlation with total sperm content of the ejaculate (Illustration 2). Given that abstinence is a major factor in influencing semen quality of any individual man, and that the two donors in this study had no restrictions on sexual activity, it is feasible that the two parameters (sperm numbers and DFI) were both influenced by the same variable, namely sexual abstinence. A positive correlation between the DFI and abstinence has been reported elsewhere [12].

It can be concluded that (a) non-sperm cells in semen can contribute to the results of the SCSA, (b) snap freezing in liquid nitrogen does not affect the result of the SCSA and is a good way of storing semen prior to testing, (c) there is good reproducibility when snap freezing several straws suggesting that they remain representative of the original sample, (d) two donors

produced semen samples over many weeks which invariably gave the same clinical information, and (e) there was a significant correlation between the DNA fragmentation index and the number of sperm in the ejaculate.

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Illustrations

Illustration 1

A typical cytogram of a fertile man obtained with the sperm chromatin structure assay (SCSA).

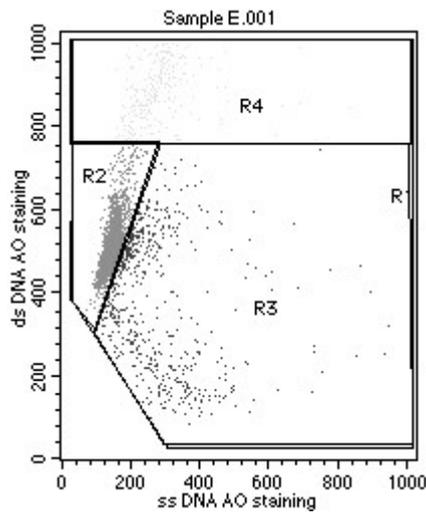
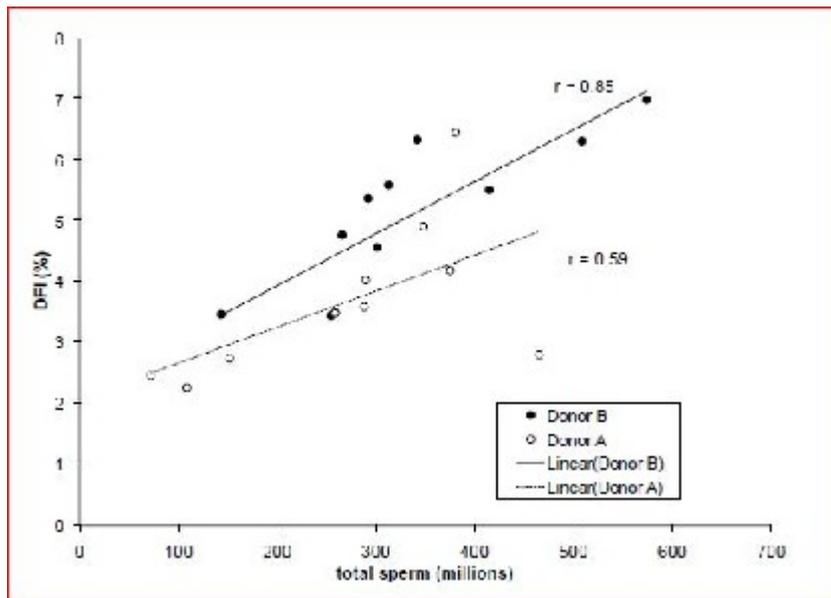


Illustration 2

Correlation of the DNA fragmentation index (DFI) and total sperm in the ejaculate for two donors.



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