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Variability and Laboratory Factors Affecting the Sperm Chromatin Structure Assay in Human Semen

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ABSTRACT: During the past decade, the sperm chromatin structure assay (SCSA) has become an important tool for assessing semen quality in the human andrology laboratory. The SCSA uses the metachromatic properties of the fluorescent dye acridine orange (AO) in combination with flow cytometry to determine the sperm DNA susceptibility to denaturation in situ. The objective of this study was to evaluate laboratory factors affecting the SCSA and the variation between replicates. Semen ejaculates from 3 healthy volunteers were analyzed using the SCSA protocol as described by Evenson and Jost (2000), determining the X-mean, Y-mean, DNA fragmentation index (DFI), standard deviation of DFI (SD-DFI), and high DNA stainability (HDS). In experiment 1, the effects of thawing time, time of day, day, laboratory technician, donor, and incubation period before analysis were investigated. In experiment 2, the effects of sheath fluid, AO equilibration buffer, day, laboratory technician, donor, and incubation period before analysis were investigated. A significant difference was found between the 3 donors with respect to the X-mean, Y-mean, DFI, SD-DFI, and HDS. It was shown that incubation of the semen samples on ice postthaw had a significant effect on the X-mean, Y-mean, DFI, and SD-DFI. The laboratory technician conducting the analysis accounted for up to 15.4% for the variation of the SCSA measurements. The time of day affected the variation for the Y-mean (23.5% of the total variation of the Y-mean), and the day affected the variation for the X-mean (82.8% of the total variation of the X-mean). Incubation on ice for 5 to 25 minutes postthaw had a significant effect on the DFI and SD-DFI in both experiments. This study shows that several protocol steps in the SCSA affect the results obtained from the assay. Precise protocol description and standardization of the SCSA are therefore essential to achieve high agreement within and between different laboratories.

Key words: Flow cytometry, DNA integrity, spermatozoa, acridine orange.

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During the past 2 decades, there has been an increasing interest in reliable assays for assessing semen quality in the human fertility clinic. The conventional methods used are limited to microscopic determination of sperm concentration using a hemocytometer (Jorgensen et al, 1997) and evaluation of sperm motility and morphology (Keel et al, 2002). These methods usually involve a subjective assessment of a few hundred sperm, and quality assurance is rarely implemented in the laboratories performing such analysis. Flow cytometry is a technique that is superior to conventional light microscopy techniques in terms of objectivity, number of cells measured, speed, and precision (Spano and Evenson, 1993). The technique has been used on human sperm to determine a number of factors, including membrane integrity, mitochondrial function, acrosome status, and multiparameter measurement (Garrido et al, 2002).

The structure of the sperm chromatin and the stability of the DNA in relation to the fertility potential have been widely studied. Various assays that assess different aspects of the chromatin structure and DNA integrity of sperm cells that use specific and complex interactions between compounds and DNA have been developed. These include the single-cell gel electrophoresis assay (Comet assay) (Haines et al, 1998), terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) (Sailer et al, 1995), in situ nick translation (NT) (Manicardi et al, 1995), and the sperm chromatin structure assay (SCSA) (Evenson and Jost, 2000). In the 1980s, Evenson et al (1980) carried out the pioneering work that described the flow cytometric assessment of sperm chromatin structure. The SCSA protocol has since been refined by Evenson et al (2002). This method uses the metachromatic properties of the dye acridine orange (AO) to detect the susceptibility of sperm DNA to acid–induced denaturation in situ. The SCSA protocol uses flow cytometry to detect green fluorescence from AO when intercalated into the double-stranded DNA helix and red fluorescence when the dye is bound to single-stranded nucleic acids (Darzynkiewicz et al, 1975). Although Evenson and Jost

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(2000) described the protocol for the SCSA in great detail, several authors have since introduced changes in the analytic procedure (Tejada et al, 1984; Golan et al, 1997; Giwercman et al, 1999; Spano et al, 1999; Acevedo et al, 2002). In most cases, these changes have not been validated against the protocol described by Evenson and Jost (2000).

Several reports emphasize the need for improvement in overall quality of semen testing within and between laboratories (Neuwinger et al, 1990; Jorgensen et al, 1997; Keel et al, 2000). However, the subjective nature of conventional semen analyses, combined with their relatively low precision due to the low number of cells assessed, leads to poor intra- and interlaboratory reproducibility; therefore, the introduction of standardized or quality controlled procedures will probably have a limited effect. The conventional analyses are used to determine whether parameters obtained from an ejaculate are within the range characterized by fertile men, and these methods can therefore provide only unclear cut-off values when used for the prediction of fertility status. Many of the advantages that accrue when using flow cytometry may, when applied to assessment of sperm cells, help overcome some of the mentioned problems found in conventional semen analysis. The SCSA is objective, fast, and precise, and the data obtained from human (Evenson et al, 1999; Larson-Cook et al, 2003) and animal (Ballachey et al, 1987; Evenson et al, 1994) studies have shown that the fragmentation of sperm DNA can be detrimental to achieving and sustaining a pregnancy. Statistical thresholds have been established for fertility prognosis when using SCSA procedures in humans (Evenson et al, 2002). The different measures obtained when using SCSA procedures have also been shown to be independent from conventional semen quality measures, and the assay therefore makes a contribution to the semen analysis profile (Evenson et al, 1991).

In the field of semen analysis, validation of a method is important because it is essential to have specific, precise, objective, and accurate laboratory tests to establish a correlation of the data with fertility or to determine the fertility potential of a semen sample correctly (Amann, 1989). Precision of a laboratory test is of great concern to the andrologist in the fertility clinic, since the results of the semen analysis are often used to advise a patient about his fertility and the prognosis for the treatment of the couple. To use established cut-off values and ensure uniform diagnosis, within and between laboratory variations should be determined and followed closely. The precision of a laboratory test is influenced by a number of factors, including the number of cells assessed, but also the human error involved in running the test and the performance or variation of the instruments used (Amann, 1989; World Health Organization, 1999). Increasing attention to the details of standard procedures and protocols should therefore increase the precision of results and reduce variation (Keel et al, 2000), both between and within laboratories.

During our first trials with SCSA protocol, described by Evenson and Jost (2000), smaller disagreements between duplicate measurements of the same sample were not an uncommon phenomenon, and it was observed that different factors in the SCSA protocol could affect the SCSA measures. The objective of the present study was to evaluate specific factors affecting the measurements obtained from the SCSA in human semen.

Materials and Methods

Semen Sample Preparation

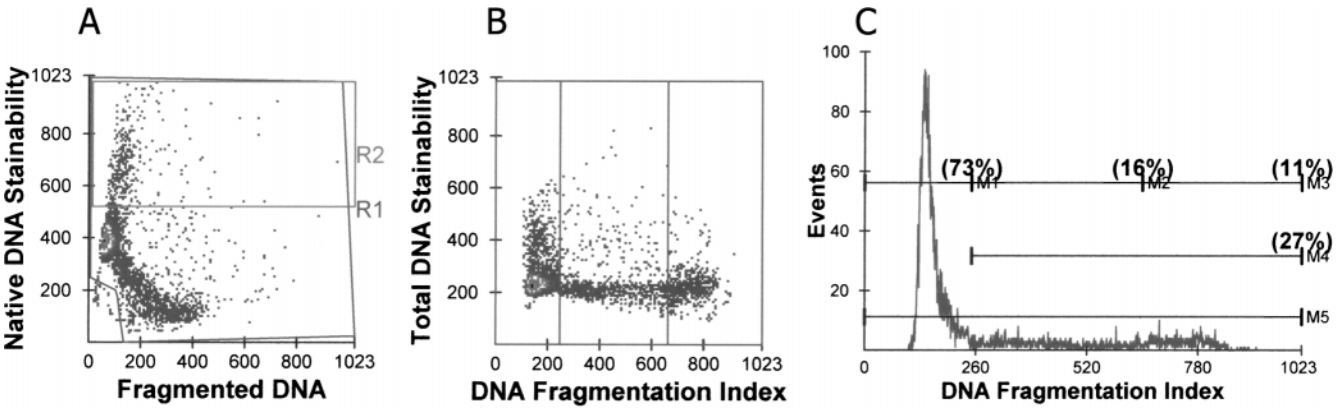
The study included a single ejaculate from each of 3 healthy volunteers. Prior fertility information was not available. Sperm concentration was determined using a Makler chamber (SEFI Medical Instruments, Haifa, Israel), and the ejaculates were then diluted with TNE buffer (0.01 M TrisCl, 0.15 M NaCl, 1 mM EDTA disodium, pH 7.4) to a concentration of 20×10^6 sperm/mL and packed in 0.23-mL straws (IMV Technologies, L'Aigle Cedex, France). The straws were sealed, frozen in nitrogen vapor, and subsequently stored in liquid nitrogen.

Fluorescent Staining

The SCSA was performed according to the procedure previously described (Evenson and Jost, 2000; Evenson et al, 2002). After thawing, aliquots of the thawed semen were diluted to a concentration of 2×10^6 sperm/mL with TNE buffer to a total volume of 200 μL in a 5-mL Falcon tube (BD Biosciences, San Jose, Calif). Immediately, 400 μL of an acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% [vol/vol] Triton X-100, pH 1.2) was added, and a stopwatch was started. After exactly 30 seconds, 1.20 mL of AO staining solution was added containing 6 μg of AO (chromatographically purified; Polysciences Inc, Warrington, Pa) per milliliter of buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0).

Flow Cytometric Measurements

The samples were analyzed using a FACScan (BD Biosciences) flow cytometer with an air-cooled argon orthogonal laser operated at 488 nm at 15 mW of power. After transiting a 560-nm short-pass dichroic mirror, the green fluorescence (FL1) was collected through a 515- to 545-nm band-pass filter. After transiting a 640-nm long-pass filter, the red fluorescence (FL3) was collected through a 650-nm long-pass filter. The FACScan has been shown to be capable of successfully measuring mammalian and avian sperm using the SCSA (Evenson et al, 1995). The sheath/sample was set on ''low'' and adjusted to a flow rate of 200 events per second when analyzing a sample with a concentration of 2×10^6 sperm/mL. Immediately after the addition of the AO staining solution, the sample was placed in the flow cytometer and was run through the system. Data acquisition of 5000 events



A representative example from the sperm chromatin structure assay (SCSA) of a human semen sample. **(A)** Native DNA vs fragmented DNA cytogram, where each point represents the coordinate of red (FL3) and green (FL1) fluorescence. Region 2 (R2) marks high DNA stainability (HDS). **(B)** Total DNA stainability vs DNA fragmentation index (DFI) scattergram. **(C)** DFI frequency histogram with calculation of total (M4), moderate (M2), and high (M3) DFI. The 3 diagrams are obtained by the SCSASoft software program.

was begun exactly 3 minutes after the initiation of acid detergent treatment and was collected in list mode using BD CellQuest Pro version 4.0 software (BD Biosciences). The laboratory technician manually recorded the X-mean (red fluorescence) and the Y-mean (green fluorescence) values for each sample.

Data Analysis

List mode data were analyzed with the software program SCSASoft (SCSA Diagnostics Inc, Brookings, SD). From the list mode data, SCSASoft automatically calculated the percentage of sperm with an abnormally high DNA stainability (HDS), the level of DNA fragmentation index (DFI), and the standard deviation of the DFI (SD-DFI). Note that SCSA terminology has recently been changed; DFI was formerly termed COMPα_t (cells outside the main peak of α_t); SD-DFI was formerly termed SDα_t; and what is now known as HDS was termed HIGRN (high green fluorescence) (Evenson et al, 2002). An example of an SCSA report generated from a human semen sample is shown in the Figure.

Experimental Design

Experiment 1—To determine the effects that thawing time, incubation time on ice, and variation caused by laboratory technician, day, and time of day had on the samples, a factorial design was performed repeatedly for 4 days with semen from 3 donors.

Semen straws were thawed in a water bath at 37°C for 7 or 30 seconds and then incubated on ice. After 0, 5, 10, 15, 20, and 25 minutes of incubation, an aliquot of the samples was diluted, stained, and analyzed. This was done in the morning and in the afternoon in random order by 2 laboratory technicians. A sample of AO equilibration buffer (400 μL of an acid detergent solution and 1.20 mL of an AO staining solution) was run through the flow cytometer for at least 60 seconds between every analysis. The same sheath fluid was used throughout the study (0.05% [vol/vol] Triton X-100). For each of the 3 donors, a total of 16 straws were analyzed; 4 straws per donor were analyzed each day.

Experiment 2—To determine the effects that sheath fluid, incubation time on ice, AO equilibration buffer, and variation caused by laboratory technician and day had on the samples, a factorial design was performed repeatedly for 4 days with semen from 3 donors.

Semen straws were thawed in a water bath at 37°C for 30 seconds and then incubated on ice. After 0, 5, 10, 15, 20, and 25 minutes of incubation, an aliquot of the samples was diluted, stained, and analyzed. The samples were analyzed in random order by the same 2 laboratory technicians as in experiment 1. A sample containing AO equilibration buffer was run between all analyses for 60 seconds or only before the first analysis (0 minutes). The sheath fluid used in this study was either 0.05% (vol/vol) Triton X-100 or FACSFlow (BD Biosciences). For each of the 3 donors, a total of 16 straws were analyzed; 4 straws per donor were analyzed each day.

Statistical Analysis

The statistical analyses were performed using SAS, version 8.2 (Statistical Analysis Systems Institute, Cary, NC). The effect of laboratory factors was evaluated in the 2 experiments by an analysis of variance using a mixed model. The outcome variables were the X-mean, Y-mean, DFI, SD-DFI, and HDS. In the first experiment, laboratory technician, day, and time of day were included as random effects in the analyses. The fixed effects of laboratory factors evaluated were the donor, thawing time, and incubation time on ice. In the second experiment, the laboratory technician and day were included as random effects. The fixed effects of laboratory factors evaluated were the donor, sheath fluid, incubation time on ice, and AO equilibration buffer method. Two-way interactions between the fixed effects were included. A backward elimination of nonsignificant interactions and factors was used. The assumption of normality was evaluated for each outcome by the Shapiro-Wilks test for normality. The assumption of equal variances was evaluated by visual inspection of residual plots. To fulfill the assumption of a normal distribution, DFI was transformed using the ARSIN transformation. A 5% significance level was used throughout the study.

Table 1. *Descriptive analysis of the different measures in the sperm chromatin structure assay in experiment 1, given as mean (±SD); the effects of donor, time on ice, and thawing are shown**

	X-mean	Y-mean	DFI	SD-DFI	HDS
Donor†					
A	96.45 B‡	382.17 B	10.63 B	105.60 B	4.07 C
	(3.11)	(3.36)	(0.97)	(4.52)	(0.32)
B	122.67 A	365.83 C	25.97 A	196.16 A	6.49 A
	(3.67)	(3.54)	(1.03)	(4.56)	(0.41)
C	77.98 C	392.66 A	4.38 C	75.78 C	5.53 B
	(3.01)	(2.53)	(0.48)	(3.72)	(0.38)
Time on ice§					
0	100.13 A	380.19	13.95 A	127.94 A	5.40
	(19.02)	(12.68)	(9.17)	(52.70)	(1.04)
5	98.65 B	380.90	13.61 B	124.98 B	5.46
	(18.72)	(11.16)	(9.20)	(51.42)	(1.09)
10	99.21 B	380.25	13.72 AB	125.89 B	5.36
	(18.94)	(11.83)	(9.22)	(52.12)	(1.08)
15	99.08 B	380.10	13.64 B	125.95 B	5.37
	(19.07)	(11.44)	(9.28)	(52.20)	(1.11)
20	98.56 B	380.08	13.50 B	124.84 B	5.31
	(18.47)	(11.35)	(9.22)	(51.55)	(1.00)
25	98.56 B	379.79	13.52 B	125.47 B	5.29
	(18.62)	(11.04)	(9.14)	(51.37)	(1.11)
Thawing‖					
30	99.02	380.04	13.52	125.58	5.30 B
	(18.45)	(11.28)	(8.96)	(51.20)	(1.04)
7	99.04	380.40	13.80	126.11	5.43 A
	(18.92)	(11.75)	(9.31)	(51.88)	(1.09)

* SCSA indicates sperm chromatin structure assay; DFI, DNA fragmentation index; SD-DFI, standard deviation of DFI; and HDS, high DNA stainability.
† Donors (A, B, and C).
‡ Different letters indicate significant differences ($P < .05$).
§ Incubation time on ice (0, 5, 10, 15, 20, and 25 minutes).
‖ Thawing procedure (37°C for 30 and 7 seconds).

Results

Descriptive data of the different measures in the SCSA for the 2 experiments are shown in Tables 1 and 2, respectively. The 3 donors used in the experiments were significantly different with respect to the X-mean, Y-mean, DFI, SD-DFI, and HDS. Donor A had a mean DFI value of 10.63% and 8.93% for experiments 1 and 2, respectively. Donor C had a mean value of 4.38% and 3.74%, whereas donor B had a mean DFI value of 25.97% and 24.60% for experiments 1 and 2, respectively. The SCSA results place donors A and C within the category ''excellent'' (<15% DFI), while donor B is in the ''fair'' category (25%–30% DFI) (Evenson et al, 2002).

Experiment 1

X-mean and Y-mean—The X-mean for the samples with no incubation on ice (0 minutes) was significantly different from that of the 5 samples that were incubated on ice (5, 10, 15, 20, and 25 minutes) for each of the 5 comparisons ($P < .05$). There were no significant differences between the samples that were incubated on ice ($P > .05$). There was also no difference between the 2 thawing methods. For the Y-mean, no significant difference was found between the 5 incubation times, and no difference was found between the 2 thawing methods.

DNA Fragmentation Index—The statistical analysis of the ARSIN-transformed data showed that incubation time (5, 15, 20, and 25 minutes) had a significant effect on DFI when compared with no incubation on ice (0 minutes) ($P < .01$), but at the 10-minute incubation, there was no significant difference ($P = .08$). There were no significant differences in DFI between the samples that were incubated on ice. The 2 thawing methods did not differ significantly in DFI.

Standard Deviation of DFI—Analysis of SD-DFI showed that incubation on ice (5, 10, 15, 20, and 25 minutes) had a significant effect when compared to no incubation on ice (0 minutes) ($P < .01$). There were no significant differences in SD-DFI between the samples that were incubated on ice. There was no significant difference between the 2 thawing methods with respect to SD-DFI.

High DNA Stainability—Analysis of HDS showed that

Table 2. *Descriptive analysis of the different measures in the sperm chromatin structure assay in experiment 2, given as mean (±SD); the effects of donor, time on ice, sheath fluid, and AO method are shown; interactions are shown in Table 3**

	X-mean	Y-mean	DFI	SD-DFI	HDS
Donor†					
A	92.16	395.24	8.93	103.15 B‡	4.77
	(4.19)	(4.97)	(0.71)	(4.86)	(0.33)
B	121.99	372.16	24.60	197.36 A	7.11
	(5.65)	(6.47)	(1.02)	(4.96)	(0.47)
C	73.73	394.52	3.74	71.41 C	5.95
	(3.59)	(7.11)	(0.35)	(3.84)	(0.45)
Time on ice§					
0	97.98 A	387.40	12.82 A	126.66 A	5.99
	(21.16)	(14.12)	(9.17)	(55.96)	(1.05)
5	96.10 B	388.69	12.36 BC	123.97 B	6.15
	(20.68)	(11.90)	(8.96)	(53.93)	(1.09)
10	95.65 B	387.42	12.35 BC	124.04 B	5.99
	(20.31)	(12.59)	(8.93)	(53.80)	(0.98)
15	95.83 B	386.98	12.46 B	123.94 B	5.84
	(20.57)	(12.50)	(8.97)	(54.01)	(1.02)
20	94.90 B	386.77	12.25 C	122.11 C	5.82
	(20.21)	(11.87)	(8.97)	(53.60)	(1.05)
25	95.29 B	386.58	12.30 BC	123.12 BC	5.87
	(20.58)	(11.87)	(8.94)	(54.04)	(1.08)
Sheath fluid‖					
I	94.24	382.12	12.22	122.40 B	5.80
	(19.99)	(11.58)	(8.86)	(53.87)	(0.99)
II	97.67	392.49	12.63	125.55 A	6.09
	(20.79)	(10.99)	(8.99)	(53.82)	(1.08)
AO method¶					
I	96.55 A	386.18	12.46	124.47 A	5.91
	(20.23)	(12.54)	(8.83)	(53.39)	(1.02)
II	95.37 B	388.43	12.39	123.48 B	5.98
	(20.69)	(12.21)	(9.03)	(54.33)	(1.07)

* SCSA indicates sperm chromatin structure assay; AO, acridine orange; DFI, DNA fragmentation index; SD-DFI, standard deviation of DFI; and HDS, high DNA stainability.
† Donors (A, B, and C).
‡ Different letters indicate significant differences ($P < .05$).
§ Incubation time on ice (0, 5, 10, 15, 20, and 25 minutes).
‖ Sheath fluid (I: 0.05% [vol/vol] Triton X-100 and II: FASCFlow, BD).
¶ AO equilibration buffer method (I: between all samples and II: only before the first sample).

there was no significant difference between the 6 time points ($P > .05$). There was a significant difference between the 2 thawing methods ($P = .003$).

Experiment 2

X-mean and Y-mean—The X-mean for the samples not incubated on ice (0 minutes) was significantly different from that for the samples incubated on ice (5, 10, 15, 20, and 25 minutes). There was a significant difference between the 2 AO equilibration buffer methods used with respect to the X-mean ($P = .001$). There was no significant difference for the X-mean between the 2 sheath fluids ($P = .47$), but an effect caused by the interaction between sheath fluid and donor ($P < .001$) was observed, which is shown in Table 3. For the Y-mean, there was a significant difference between sheath fluids ($P < .001$), incubation on ice ($P = .002$) and AO equilibration buffer method ($P < .001$). Interactions between sheath fluid and donor ($P < .001$), donor and incubation time ($P < .001$), and AO equilibration buffer method and incubation time ($P < .001$) were detected (Table 3).

DNA Fragmentation Index—Analysis of DFI showed that the DFI at the time point immediately postthaw (0 minutes) differed significantly from that obtained for the samples incubated on ice (5, 10, 15, 20, and 25 minutes) ($P < .01$). The only other time points that differed with respect to DFI were 15 and 20 minutes ($P = .020$). The 2 types of sheath fluid were not significantly different ($P = .15$) with respect to DFI. An interaction between the AO equilibration buffer method and donor was found ($P = .028$) (Table 3).

Standard Deviation of DFI—The analysis of SD-DFI

Table 3. *Descriptive analysis of the interactions between different measures in the sperm chromatin structure assay in experiment 2, given as mean; the significant interactions between donor, time on ice, sheath fluid, and AO method are shown**

Donor†	Time on Ice‡	Y-mean	HDS
A	0	396.19 A§	4.88 AB
	5	396.06 A	4.89 AB
	10	395.81 A	4.95 A
	15	395.06 AB	4.66 BC
	20	394.44 AB	4.70 ABC
	25	393.88 B	4.56 BC
B	0	369.88 C	7.14 B
	5	374.25 A	7.39 A
	10	372.06 B	7.11 B
	15	371.88 B	6.91 B
	20	372.81 AB	7.05 B
	25	372.06 B	7.05 B
C	0	396.13 A	5.95 ABC
	5	395.75 AB	6.17 A
	10	394.38 CB	5.90 BC
	15	394.00 C	5.96 AB
	20	393.06 C	5.72 C
	25	393.81 C	6.01 AB

Donor†	Sheath Fluid¶	X-mean	Y-mean
A	I	90.83	391.21B
	II	119.36	399.21B
B	I	72.54	366.79B
	II	93.42	377.52A
C	I	124.77	388.36B
	II	74.83	400.68A

Donor†	AO method¶	DFI
A	I	9.07 A
	II	8.76 B
B	I	24.47
	II	24.72
C	I	3.79
	II	3.66

AO Method¶	Time on Ice‡	Y-mean
I	0	387.63 A
	5	387.25 AB
	10	385.88 C
	15	385.96 BC
	20	385.08 C
	25	385.29 C
II	0	387.17 B
	5	390.13 A
	10	388.96 A
	15	388.00 B
	20	388.46 B
	25	387.88 B

Table 3. *Continued*

AO Method‖	Sheath Fluid‖	HDS
I	I	5.83
	II	6.00
II	I	5.78 B
	II	6.18 A

* Abbreviations are explained in the first footnote to Table 2.
† Donors (A, B, and C).
‡ Incubation time on ice (0, 5, 10, 15, 20, and 25 minutes).
§ Different letters indicate significant differences ($P < .05$).
‖ Sheath fluid (I: 0.05% [vol/vol] Triton X-100 and II: FASCFlow, BD).
¶ AO equilibration buffer method (I: between all samples and II: only before the first sample).

showed that there was a significant difference between the 2 used sheath fluids ($P = .044$). The SD-DFI immediately postthaw (0 minutes) differed significantly from that obtained from the samples incubated on ice (5, 10, 15, 20, and 25 minutes) ($P < .001$). There was also a significant difference between the samples incubated at 5 and 20 minutes ($P = .020$), 10 and 20 minutes ($P = .016$), and 15 and 20 minutes ($P = .021$). There was a significant effect caused by the AO equilibration buffer method ($P = .036$).

High DNA Stainability—Analysis of HDS showed that there was no significant difference between the 2 AO equilibration buffer methods ($P = .24$). Significant differences were detected between the 2 types of sheath fluid ($P < .001$) and incubation time ($P < .001$). Interactions between the donor and incubation time ($P = .030$), and the AO equilibration buffer method and sheath fluid were found ($P = .040$) (Table 3).

Random Effects—The variations due to random effects in the 2 experiments are shown in Tables 4 and 5. The DFI was affected by day in both experiments, but laboratory technician and time of day had little or no effect on this measure. For the SD-DFI, both laboratory technician and day had an effect, but time of day had no effect. For the HDS, both day and laboratory technician had an effect in experiment 1, but this was not the case in experiment 2.

Table 4. *Variation in percentage due to laboratory technician, day, time of day, and residual for the different measures in the sperm chromatin structure assay in experiment 1**

Variable	X-mean	Y-mean	DFI	SD-DFI	HDS
Laboratory technician	0.00	0.00	0.00	15.44	7.26
Day	47.05	0.00	4.22	7.06	1.81
Time of day	0.00	23.49	0.00	0.00	0.00
Residual	52.95	76.51	95.78	77.49	90.92

* SCSA indicates sperm chromatin structure assay; DFI, DNA fragmentation index; SD-DFI, standard deviation of DFI; and HDS, high DNA stainability.

Table 5. *Variation in percentage due to laboratory technician, day, and residual for the different measures in the sperm chromatin structure assay in experiment 2**

Variable	X-mean	Y-mean	DFI	SD-DFI	HDS
Laboratory technician	0.00	0.00	0.04	3.02	0.00
Day	82.76	26.48	13.39	12.24	0.00
Residual	17.23	73.52	86.57	84.74	100.00

* Abbreviations are explained in the footnote to Table 4.

Discussion

Today, the SCSA is used in many laboratories all over the world to assess human semen quality. The method is objective, and it is possible to analyze a large number of cells in a short time. The SCSA measures have been shown to be independent from traditional semen measures, and the DFI has been shown to correlate with fertility.

There are few data available in the literature concerning variation related to laboratory procedure and repeatability and reproducibility between replicates of the SCSA. In one study, duplicate measurements from an individual semen sample were shown to be highly repeatable, with correlations of 0.988 for COMPα_t, 0.991 for SDα_t, 0.985 for HIGRN, 0.982 for the X-mean, and 0.973 for the Y-mean (Evenson et al, 1999). In another study, it was found that for an SCSA method that was slightly modified, the intra-assay coefficients of variation for the COMPα_t varied between 0.8% and 16.8%, and the corresponding value for the SDα_t varied between 1.3% and 5.8% (Giwercman et al, 1999). In a recently published paper, the correlation between replicates measured by the SCSA was determined to be 0.87 for SDα_t and 0.98 for COMPα_t (Acevedo et al, 2002). One study of 2 laboratories performed a quality control exercise in conjunction with a study designed to determine if there was a correlation between sperm motility and the SCSA demonstrated an interlaboratory, Pearson correlation of $r = 0.90$ (Giwercman et al, 2003). The 2 laboratories used 2 slightly different SCSA protocols.

In the present study, we found that a number of laboratory factors affected the outcome of the SCSA. It was shown that even a short (5 minutes) incubation time on ice postthaw had an effect on the measures X-mean, DFI, and SD-DFI in both experiments; therefore, careful protocol description should be practiced when measuring the same frozen-thawed semen sample twice in order to achieve a measurement in duplicate. It was also shown that thawing method had a significant effect on HDS. Interactions between sheath fluid and donor for the X-mean, Y-mean, and HDS and between the AO equilibration buffer method and donor for DFI were shown. According to the protocol by Evenson and Jost (2000), samples for the SCSA should be thawed in a water bath at 37°C until the last remnant of ice has disappeared, and the samples should be analyzed immediately thereafter. The remainder of the sample should be stored on ice, and a repeated staining and measurement should be performed directly after completing the first analysis. In our study, we found a significantly higher DFI and SD-DFI in the samples analyzed directly postthaw than in the same samples stored on ice for up to 25 minutes. This could be caused by a thermally induced increase of fluorescence intensity of AO-stained cells. The fluorescence intensity of AO has been described to be temperature dependent at higher temperatures (Darzynkiewicz et al, 1975). An alternative and far-fetched explanation for the decrease in DNA denaturation following incubation on ice is that the storing or thawing procedure may enhance the sperm DNA integrity in the short term. Unfortunately, a comparison of these data with SCSA results obtained from the fresh semen, before freezing, was not possible due to unavailability of sufficient amounts of data. With regard to the thawing procedure (7 vs 30 seconds), the only effect was observed for HDS, where HDS was significantly higher for the shorter thawing time. The sperm in the HDS region are characterized by increased DNA stainability and are excluded from the calculation of the DFI and SD-DFI. This population of HDS is supposedly composed of immature cells that lack chromatin condensation (Evenson et al, 1999) but may also represent doublets. The significantly higher HDS found for the 7-second thawing procedure may therefore be explained by a higher number of doublets due to clumping. The random effect of laboratory technician was also lower in experiment 2 where the thawing time was 30 seconds throughout the experiment. The recommended method of thawing semen frozen in 0.23-mL straws is therefore 37°C for 30 seconds.

We also found that sheath fluid consisting of 0.05% Triton X-100 should be used and that a sample containing an AO equilibration buffer should be run through the flow cytometer for about 60 seconds between stained aliquots to ensure saturation of the tubes in the flow cytometer, as recommended by Evenson and coworkers (Evenson and Jost, 2000). The combined use of an AO equilibration buffer and 0.05% (vol/vol) Triton X-100 resulted in the most stable results for the SCSA measurements. The interactions shown for some of the variables between donor sheath fluid and the donor and AO equilibration buffer method show the weakness of having included only 3 donors in the study. If more donors with a larger variation between the SCSA measurements had been included, perhaps the results from this part of the study would have been clearer.

It has previously been recommended that ‘‘reference samples’’ be used to establish instrument settings and

when running a series of samples, after about every 5 samples. In this way, the reference samples should ensure stability of the instrument and quality control over all measurements (Evenson and Jost, 2000). The mean red (X) and green (Y) fluorescence values should fall within plus or minus 5 channels of an established laboratory standard, and the flow cytometer should be adjusted to accomplish this (Evenson and Jost, 2000). In the present study, it was not possible to adjust the flow cytometer so that the X- and Y-mean values stayed within plus or minus 5 channels by measuring just a single reference sample in duplicate. A larger number of reference samples would be required to obtain the settings for the X- and Y-mean values; then, adjustments should be made accordingly.

The variation due to time of day was shown to be 23.5% for the Y-mean measurement in experiment 1. This change in green fluorescence during the day has previously been observed to occur in another type of instrument when working with the SCSA (Evenson and Jost, 2000). In the present study, we found that for the 3 variables DFI, SD-DFI, and HDS, the variation due to time of day was close to 0%. We therefore agree that reference samples should be used to establish daily instrument settings and measurements between test samples. However, we cannot recommend adjusting the flow cytometer on the basis of the results determined from a single reference sample obtained while analyzing a row of samples. The flow cytometer should be adjusted according to the reference sample before running a row of test samples, but these established instrument settings should be used throughout the day without any further adjustment. If any slight deviations occur during the day, they will be recorded in the X-mean, Y-mean, DFI, SD-DFI, and HDS, for the reference samples analyzed in duplicate for every 5 test samples.

Our experience is that, even with objective flow cytometric techniques, the individual running the assay often has an influence on the outcome of the assay. In the present study, the laboratory technician accounted for up to 15% of the variation in the SCSA variables DFI, SD-DFI, and HDS. In comparison, the variations due to day and time of day were smaller for each of these 3 variables. By adopting a standardized training program in how to handle samples in a correct and uniform way in cell preparation, staining, and measuring, the effect of the laboratory technician may be markedly reduced. Useful tools that can be used when introducing a new technique into a laboratory should include careful standardization and a detailed protocol description of the technique, an extensive training program, and a follow-up in the form of an intralaboratory quality control program. For a widely used method such as the SCSA, after having established an intralaboratory quality control program, interlaboratory variation should be considered. As previously suggested, when managing interlaboratory variation for the SCSA, it may be useful to establish a quality control reference center (Evenson et al, 2002).

This variation study was performed as part of determining the immediate source of laboratory variation for the SCSA. This type of study is an important part of introducing a new technique to a laboratory before commencing further experiments or when beginning to use it in routine clinical analysis. In this study, the most stable results when using the SCSA for the analysis of human semen were achieved by thawing samples frozen in 0.23-mL straws at 37°C for 30 seconds and placing them on ice for 5 minutes before the first aliquot was diluted, stained, and analyzed. The subsequent repeated measurement of a sample (10 or 15 minutes of incubation on ice postthaw) will give a more precise estimate of the DFI and SD-DFI. Sheath fluid consisting of 0.05% (vol/vol) Triton X-100 should be used, and a sample with an AO equilibration buffer should be run through the tubes of the flow cytometer between every analysis.

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