Human Reproduction vol.13 no.9 pp.2495–2505, 1998

The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies*

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The impact of demographic, lifestyle, and seminal factors on the sperm chromatin structure assay (SCSA) parameters was evaluated in a population of 277 healthy Danish men. This cohort was established within the framework of a European Concerted Action on occupational hazards to male reproductive capability in order to examine the possible reproductive effects of exposure to styrene or pesticides. The SCSA measures the susceptibility of sperm DNA to in-situ acid-induced denaturation, by multi-parameter flow cytometric analysis after staining with the DNA-specific fluorescent dye acridine orange. The green versus red bivariate cytogram patterns were quite variable among donors, showing a wide heterogeneity of sperm DNA denaturability. Nevertheless, in those cases where we had the possibility to measure two semen samples from the same donor, the cytogram pattern remained stable over time ($0.64 < r < 0.78$). Analysis of variance demonstrated that the SCSA results can be influenced by the age of the donor ($P < 0.0001$), smoking habits ($P < 0.05$), the presence of leukocytes and immature germ forms in the ejaculate ($P < 0.0001$), and the duration of sexual abstinence ($P < 0.0001$). Furthermore, the relationship between the SCSA data and sperm concentration, morphology, and vitality was weak ($-0.22 < r < -0.46$). Therefore, the SCSA provides independent and complementary measurements of semen quality and is thus a useful tool for epidemiological studies, but the effects of some confounders should be accounted for in the survey design and analysis.

*Presented in part at the International Symposium on Environment, Lifestyle & Fertility, Aarhus, 1997

**Asclepios is a BIOMED European Concerted Action Research Project on *Occupational Hazards to Male Reproduction Capability* running in 16 European centres, coordinated by the Steno Institute of Public Health, Department of Occupational Medicine, University of Aarhus, Denmark (see Appendix)

Key words: biological markers/chromatin condensation/epidemiology/flow cytometry/human spermatozoa

Introduction

Environmental risk factors to male reproductive function have generated considerable interest since the reports on reduced sperm counts in men exposed to the nematocide dibromochloropropane were published 20 years ago (Whorton *et al.*, 1977). More than 50 cross-sectional studies aimed at identifying male reproductive toxicants have been undertaken in various occupational settings (Bonde and Giwercman, 1995). Simple seminal characteristics, such as sperm density, morphology, viability and motility have been used as markers of male reproductive function in the vast majority of these studies. Semen analysis is a key component of any field study for evaluating adverse effects on the male reproductive system, with possible implications for fertility potential, embryonic development and hereditary damage (Bonde *et al.*, 1996; Wyrobek *et al.*, 1997). However, it is widely acknowledged that conventional measures of semen quantity and quality have several limitations. In particular, the huge variability of microscopically-based sperm cell features (Schrader *et al.*, 1988) in the human population can hamper the detection of detrimental effects on male reproductive capability ascribable to a particular chemical insult. There is, therefore, a need to develop supplementary objective, sensitive and feasible indicators of male reproductive function and of its possible impairment due to environmental exposure.

As the structural organization of sperm DNA seems vital for the proper functioning of spermatozoa (Ward and Coffey, 1991), methods focusing on the characterization of sperm chromatin condensation and stability have been receiving increasing attention in recent times. The degree of sperm chromatin condensation can be evaluated by: the abundance of phosphotungstic acid deposits in transmission electron microscopy (Francavilla *et al.*, 1996); toluidine or aniline blue staining (Krzanowska, 1982) in optical microscopy; the uptake of DNA-specific fluorescent probes; and posterior analysis using either fluorescence microscopy (Tejada *et al.*, 1984; Bianchi *et al.*, 1993) or flow cytometry (FCM). Precision, objectivity, automation, speed of measurements, and statistical robustness of the results are particular features of FCM, and male germ cell analysis represents one of the most important applications of this technique (Gledhill *et al.*, 1990; Spanò and Evenson, 1991, 1993). A variety of FCM methods have been developed to measure precisely the degree of human sperm condensation and stability by means of several DNA

specific fluorochromes (Evenson *et al.*, 1986), such as DAPI (Otto *et al.*, 1979; Spanò *et al.*, 1984), ethidium bromide/mithramycin (Engh *et al.*, 1992), propidium iodide (Molina *et al.*, 1995), and acridine orange (Evenson *et al.*, 1980).

The method that uses the DNA-specific fluorescent dye acridine orange is known as the sperm chromatin structure assay (SCSA). The SCSA exploits the metachromatic properties of acridine orange to monitor the susceptibility of sperm chromatin DNA to acid-induced denaturation *in situ* (Evenson and Jost, 1994). Abnormal chromatin structure is quantified by FCM measurement of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as green versus red fluorescence cytogram patterns. The low pH treatment apparently causes partial DNA denaturation only in spermatozoa having altered chromatin structure, and a higher proportion of single-stranded DNA (red fluorescence) is expected in structurally altered chromatin than in normally condensed chromatin. As the correlation between conventional andrological parameters and SCSA data is weak (Evenson *et al.*, 1991; Fosså *et al.*, 1997), SCSA has been proposed as a new descriptor of semen quality reflecting semen characteristics complementary to and beyond those of light microscopy assessment. In addition, in the only longitudinal study on a population of normal men published so far, it was shown that SCSA patterns, even though different among individuals, are more stable than other classical semen measures over time for each individual (Evenson *et al.*, 1991). This consistency of acridine orange staining patterns within individuals suggests that SCSA data may provide a reference measurement of semen quality for individuals involved in a longitudinal epidemiological survey under the assumption that deviations from that pattern may reflect the presence of testicular stress.

These premises made SCSA a novel and feasible measure of semen quality and it was considered a good candidate for inclusion in epidemiological and multidisciplinary field studies aimed at evaluating the impact of environmental risk factors to male reproductive function and at the identification of potential reproductive hazards (Bonde *et al.*, 1996; Wyrobek *et al.*, 1997). However, to weigh the role of possible confounding factors, a critical requirement was obviously to clarify how the SCSA results can be influenced by some of the variables contributing to the wide heterogeneity of the human situation. In this paper, the impact on the SCSA parameters of demographic and lifestyle factors as well as the correlation of SCSA data with conventional semen characteristics are described in a population of 277 normal healthy men enrolled in Danish studies to test associations between occupational exposure to pesticides or styrene and alterations of semen quality.

Materials and methods

Population and semen sampling

As a part of a European Concerted Action on occupational hazards to male reproductive capability (Bonde *et al.*, 1996), Danish cohorts of farmers (age range 18–55 years) and reinforced-plastic workers (age range 19–54 years) were established to examine the possible effects of exposure to pesticides and styrene, respectively, in a controlled longitudinal design. Among 789 eligible farmers, 249 (31.6%) were enrolled in this study: 167 farmers used spray pesticides and 82 farmers used biological fertilizers only. The subjects agreed to provide two semen samples, one before and one after the pesticide spraying season, generally running from April to October, but 12 farmers dropped out of the second part. We measured the semen samples twice only for 237 subjects (159 spraying and 78 non-spraying farmers). Among 131 workers hired in four Danish reinforced-plastic factories between October 1994 and February 1995, 28 (21.4%) agreed to participate, were enrolled and provided 57 semen samples (from 21 workers we have multiple samples). All of these workers reported no previous employment in the industry or previous welding exposure.

Altogether 277 men provided 531 semen samples for SCSA analysis. Semen was collected by masturbation and we asked for 2 to 7 days of sexual abstinence prior to the day of collection. For each sample, information on date, time, spillage, occurrence of fever $>$38.5°C, and number of days since last ejaculation was recorded. Information on the reproductive, medical, and occupational history and lifestyle habits were collected using self-completed questionnaires. In particular, the factors taken into consideration for the purposes of this study were: age; body mass index; self-reported history of venereal disease (yes/no), parotitis after puberty (yes/no), genital virus infection (yes/no), fever during the past 3 months, tobacco smoking (never, ex-smoker, smoker), marijuana smoking (yes/no), alcohol consumption, and days of sexual abstinence before sample collection; period of the year when the sample was collected; presence of white blood cells (yes/no); immature germ cells (yes/no) in the ejaculate; and sperm concentration, morphology and vitality.

Laboratory methods

The semen samples were examined in accordance with the guidelines of the World Health Organization (WHO) (Rowe *et al.*, 1993) in a mobile laboratory (Bonde *et al.*, 1996) to determine semen volume, sperm concentration, motility and morphology. Sperm motility was assessed immediately by phase-contrast investigation on a fresh drop of semen placed on a glass slide. Semen smears were prepared for later analysis of sperm morphology and vital scoring. The sample volume was measured in a graduated Falcon tube. The sperm concentration was measured using a Neubauer haemocytometer. Counting was performed using the phase-contrast technique at a magnification of $\times$200. The sample was counted twice and, if more than 10% difference resulted between the two counts, the sample dilution was remixed, and the counting procedure was repeated. The slides for morphology scoring were air-dried, fixed in 96% ethanol and stained using a modification of Papanicolaou's stain. Morphology scoring was carried out according to WHO criteria (all performed by one technician) and included estimation of the proportion of white blood cells and immature germ cells. Furthermore, the smears were also classified according to strict criteria (WHO, 1992) (performed by another technician). The eosin–negrosin colouring technique was used for vital staining.

FCM analysis of SCSA

Aliquots (0.1 ml) of semen were diluted 10-fold with TNE buffer (0.15 M NaCl, 0.01 M Tris–HCl, 1 mM EDTA, pH 7.4) containing 10% glycerol and frozen at –80°C in 1.5 ml microcentrifuge tubes. At the end of the collection period, all samples were coded and shipped on dry ice to the ENEA FCM facility. The SCSA was applied following the procedure described by Evenson *et al.* (1991) and Evenson and Jost (1994), with minor modifications. Individual semen samples, stored at –80°C for several weeks before flow

analysis, were thawed on crushed liquid ice. All samples were properly diluted in TNE buffer in order to work with comparable concentrations. A 0.2 ml aliquot was subjected to acidic denaturation in 0.4 ml of a low pH detergent solution containing 0.1% Triton X-100, 0.15 M NaCl and 0.08 N HCl, pH 1.4. After 30 s, 1.2 ml of a staining solution containing 6 mg/l acridine orange (chromatographically purified; Molecular Probes, Eugene, OR, USA) in 0.2 M Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate (pH 6.0) was added, and the stained sample was placed in the flow cytometer sample chamber. Semen samples used for instrument calibration were from a single, normal human ejaculate sample, not a part of the Danish samples, which was diluted in TNE–glycerol buffer to a concentration of about 2×10^6 spermatozoa/ml, divided into several hundred 0.2 ml aliquots in 1 ml polycarbonate snap-cap tubes, and immediately frozen at –80°C. Calibration aliquots were thawed and measured at each start-up of the flow cytometer and after every 10 samples to ensure standardization and stability of the instrument from sample to sample and from day to day.

Cells were analysed by a Facstar Plus flow cytometer (Becton Dickinson, San Josè, CA, USA), equipped with a 6 W Argon ion laser (Innova 306; Coherent, Santa Clara, CA, USA), tuned at 488 nm and operated at a power output of 300 mW, light mode. Green (530 ± 30 nm) and red (>630 nm) fluorescence values, corresponding to amounts of native and denatured DNA, respectively, were obtained from 5000 cells per sample. The two fluorescences, both processed in peak mode, were separated by a 560 nm dichroic filter. Data were stored in list-mode at 10 bit resolution. All measurements began 3 min after acridine orange staining with a flow rate of approximately 200 cells/s. Scattergram analysis on raw data with each point representing the coordinate of red and green fluorescence intensity values for every individual spermatozoon was carried out using Becton Dickinson standard software (Facstar Plus Research and Lysis). Events accumulated in the lower left corner corresponded to sample debris and were excluded from the analysis. The bivariate data can be conveniently expressed by the function alpha T (αT) which is the ratio of red to total (red plus green) fluorescence intensity (Darzynkiewicz *et al.*, 1975), thus representing the amount of denatured, single-stranded DNA over the total cellular DNA. αT is calculated (ListView; Phoenix Flow Systems, San Diego, CA, USA) for each sperm cell in a sample and the results are expressed as the mean ($\bar{X}$ αT), the standard deviation (SD αT) of the αT distribution, and as the frequency of cells with high αT values, usually called cells outside the main population (COMP αT). αT can range between 0 and 100 but, for practical considerations, it is generally and conveniently reported with values spanning between 0 and 1024 channels of fluorescence. Additionally, another parameter considered in the SCSA analysis is the mean ($\bar{X}$ Green) of the green fluorescence intensity distribution.

Statistical analysis

The between- and within-subject components of variance of SCSA values were computed by conventional methods using the nested procedure of the SAS statistical package (SAS Institute Inc., SAS/STAT User's Guide, Release 6.03; Cary, NC, USA: SAS Institute, 1988). Differences in average SCSA values between groups were examined by analysis of variance (SAS GLM procedure) with adjustment for the effects of age (six 5-year groups), body mass index (high/low), smoking (yes/no), duration of sexual abstinence (days), leukocytospermia (yes/no) and immature sperm cells (yes/no). The analysis of personal characteristics only included the first semen sample, while the analysis of characteristics related to the ejaculate included all semen samples. Models based on repeated semen samples also included a term to indicate the semen sample number. Positive associations which were unravelled by analysis of the first sample were re-examined using the second semen sample. To obtain equality of variance and normality of the residuals, we transformed the dependent variables using the logarithmic function. Violation of the model assumptions was examined by inspection of residual plots and by the testing for normality of the residuals (SAS Univariate procedure with the normal option). Correlation coefficients of SCSA values and conventional measures of semen quality were computed using the non-parametric Spearman's statistics (SAS Corr procedure).

Results

There was no consistent indication that semen quality was altered because of the application of pesticides or by occupational exposure to styrene. Moreover, no difference in the SCSA results emerged among the 167 farmers spraying pesticides, the 82 farmers not spraying pesticides, and the 28 reinforced-plastic workers. Therefore, the SCSA analysis described in this paper includes 277 individuals, irrespective of their occupational exposure status.

Representative data from the SCSA of human sperm samples are reported in Figure 1. The upper panel refers to a sample characterized by a narrow αT distribution whereas the lower panel shows a sample characterized by an increased red fluorescence (denatured DNA) with concomitant loss of green fluorescence (native DNA) describing the extent of denaturation. We have observed that cytogram patterns are quite variable among individuals. Figure 2 displays cytograms from eight different donors selected to show the variety of the different patterns observed. This cytogram variability can be qualitatively classified in terms of the position and shape of the main cell cluster (i.e. narrow red/normal green, narrow red/high green, wide red/normal green, wide red/high green, highly irregular, etc.). In this context, αT and green fluorescence parameters could constitute useful descriptors of the observed inter-individual pattern heterogeneity. We obtained the following mean values (± SD) after SCSA analysis carried out on the 277 subjects: (i) $\bar{X}$ αT = 224.9 ± 23.2 (range: 194.5–381.6); (ii) SD αT = 80.4 ± 27.3 (range: 36.7–185.6); (iii) COMP αT = 15.0 ± 10.6 (range: 2.7–71.1); and (iv) $\bar{X}$ Green = 455.9 ± 38.0 (range: 358.8–641.0). The αT parameters are highly interdependent as demonstrated by the correlation analysis: Spearman's coefficients were 0.89 for COMP αT versus $\bar{X}$ αT; 0.81 for COMP αT versus SD αT; and 0.86 for $\bar{X}$ αT versus SD αT (P was always < 0.0001). On the other hand, $\bar{X}$ Green was not correlated with the αT parameters.

For 258 individuals, we were able to measure at least two semen samples. We have attempted to evaluate the within- and between-variability of the SCSA parameters by pooling the data from the 167 farmers used to spraying pesticides (318 semen samples), the 82 non-spraying farmers (156 semen samples), and the 28 reinforced-plastic workers (57 samples). A summary of the statistical analysis carried out on the SCSA results is shown in Table I. For comparison, the data obtained from the paper on US healthy volunteers (Evenson *et al.*, 1991) have also been reported and an impressive correspondence can be appreciated. The within-subject variation from the first to

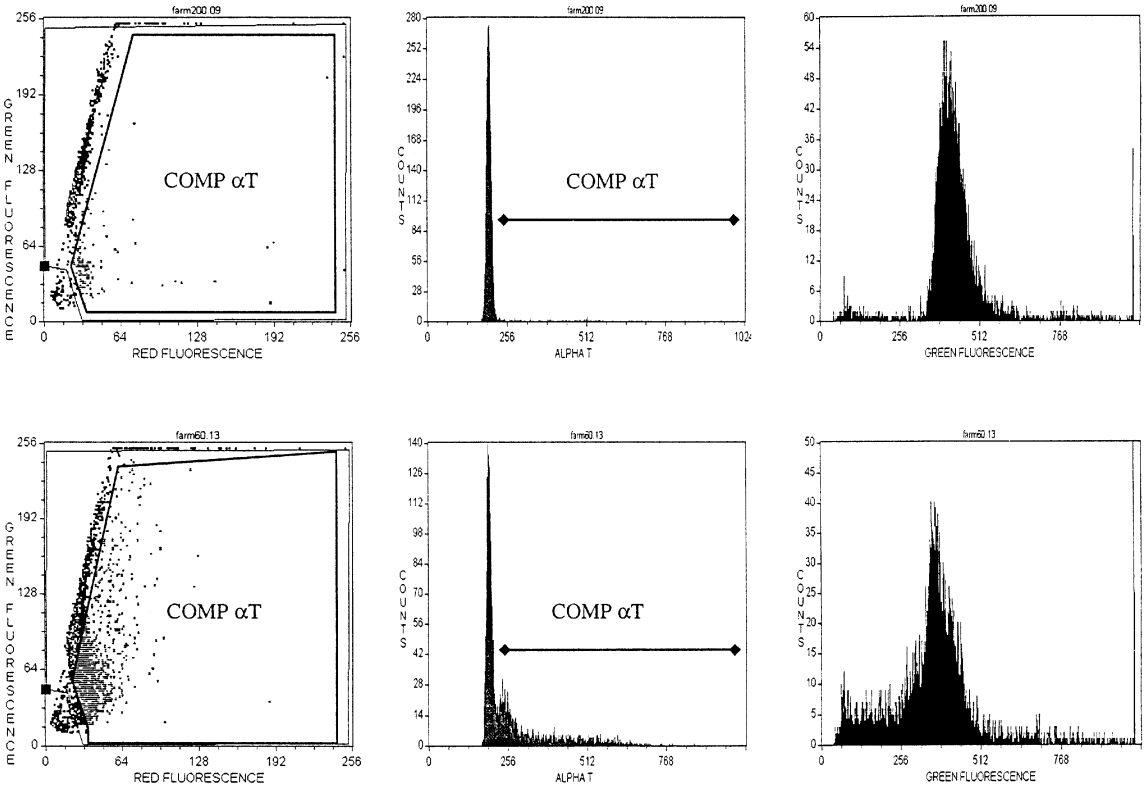

Figure 1. Green versus red fluorescence bivariate cytograms together with corresponding αT and green fluorescence frequency histograms of spermatozoa from two different donors measured by the flow cytometry sperm chromatin structure assay. The fraction of cells with abnormal chromatin (COMP αT) is boxed off in the cytogram and in the corresponding frequency histograms. Upper panel: SD αT = 48.1; COMP αT = 3.5%. Lower panel: SD αT: 104.6, COMP αT = 43.1%.

the second sample was much lower than the between-subject variation indicating that the SCSA values are, as expected, more repeatable within a person than between persons. This is also evident from the high intraclass linear correlation coefficients. We obtained 0.64 for $\bar{X}$ αT, 0.71 for SD αT, 0.77 for COMP αT, and 0.78 for $\bar{X}$ Green (P was always < 0.0001). It should be noted that the small differences between the various Danish groups with respect to αT parameters disappeared when adjusting for differences in age and abstinence period.

The relationships between the SCSA results and demographic and lifestyle factors are reported in Table II. A rather strong relation was found between age and SCSA values. The average COMP αT value almost doubled as age increased from 18 to 55 years and the average $\bar{X}$ αT and SD αT values also increased with age but not in a statistically significant way. It is worthwhile noting that age is also associated with the period of abstinence (see below) and, when the analysis was repeated after adjusting for the abstinence period, the SCSA parameters and age remained strongly associated in all subgroups. Self-reported histories of parotitis, genital infection (gonorrhoea, *Chlamydia*, syphilis, epididymitis), and viral infection were not related to the SCSA results. We found no indication that αT values changed in current tobacco smokers or in ex-smokers. On the other hand, it is worthwhile noting that the parameter $\bar{X}$ Green seems significantly elevated in current smokers compared with that in the group of non-smokers. Furthermore, the average number of cigarettes smoked per day was not related to the SCSA values (data not shown). The same results have been obtained when a cumulative lifetime dose, expressed by the product of years smoking and cigarettes per day, was taken into consideration (data not shown). Only seven men reported smoking marijuana but the SCSA patterns in these men were not unusual. Finally, alcohol drinking was categorized according to the number of alcoholic drinks per week considering the total bottles of beer (33 cl), glasses of wine, spirits and aperitifs. Alcohol consumption was not related to the SCSA results.

For the following variables, the correlations have been tested considering all the 531 semen samples from the 277 donors. The results are illustrated in Table III. The SCSA values in semen samples obtained after a febrile period did not differ from the others even though they were slightly higher than average. The presence of white blood cells in semen (neutrophils and/or lymphocytes) was significantly related to higher SCSA values. Moreover, the presence of immature forms in the ejaculate was significantly correlated with some SCSA parameters, namely COMP αT and $\bar{X}$ Green. A weak but statistically significant association was also reported between αT parameters and the season of sample collection. For example, a higher fraction of COMP αT cells occurs in October–December, while the lowest values were found in January–March. Sexual abstinence periods were divided into 2-day groups. αT parameters showed a trend of deterioration as the period of abstinence increased, at least until about 6 days had elapsed. On the other hand, the parameter $\bar{X}$ Green was unaffected. To further examine the apparent and unexpected

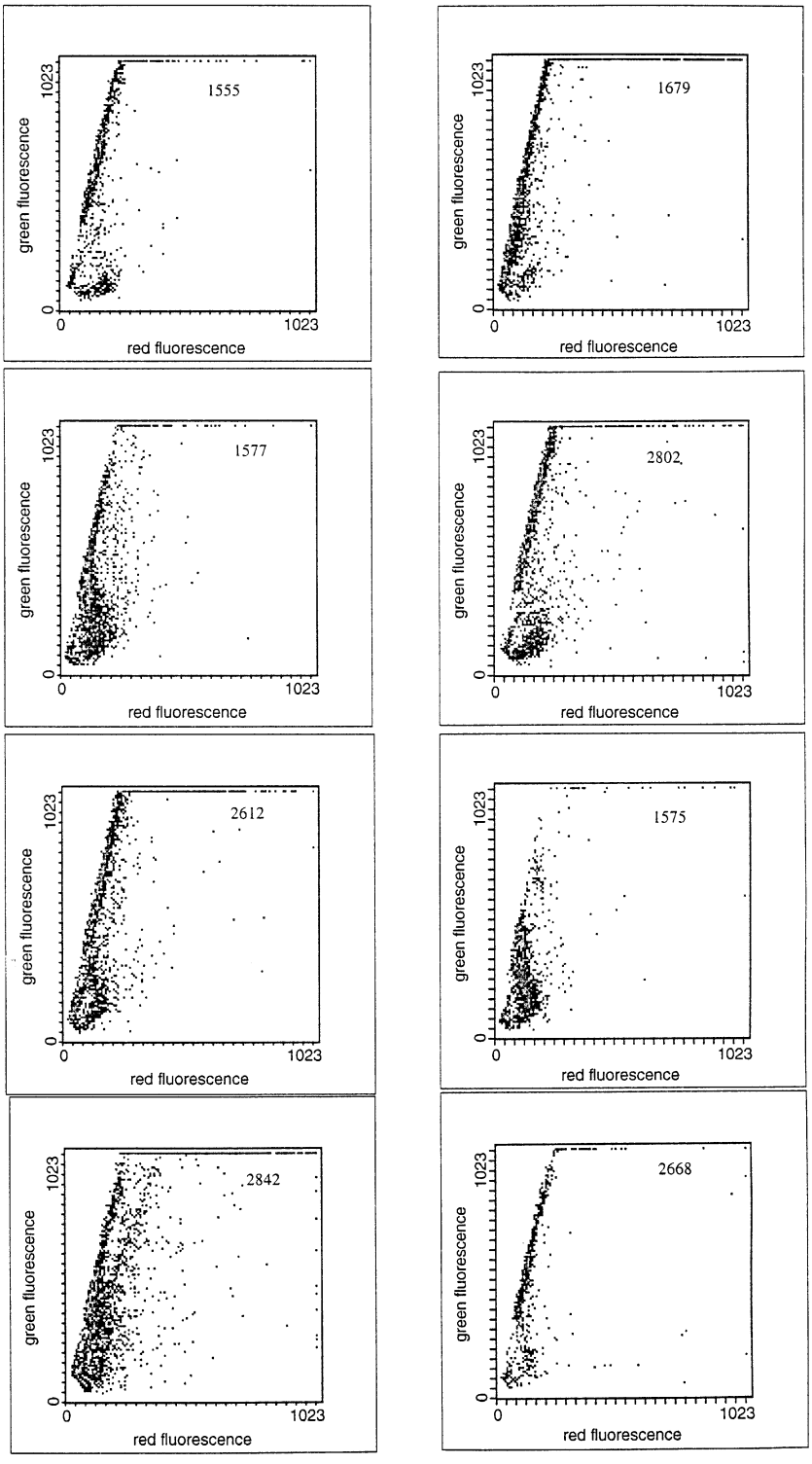

Figure 2. Green versus red fluorescence cytograms of eight representative semen samples illustrating the heterogeneity of the sperm chromatin structure assay patterns observed among donors.

relationship between abstinence and αT parameters, we also outlined the within-person variations of αT parameters in relation to the change of the abstinence period from one sample to the next. Also this longitudinal analysis indicated a highly significant association (Table VI). When the abstinence time increased by one day, the $\bar{X}$ αT increased on average by 1.3 (95% confidence interval 0.6–2.0) and the COMP αT by 0.45% (95% confidence interval 0.15–0.74). It should be noted that, ideally, sexual abstinence period should be expressed in hours in order to take account of diurnal variation. Since it was not possible to do this here, these results should be regarded as preliminary.

The association between the parameters from SCSA and from conventional semen analysis is described in Table V. In

Table I. Descriptive statistics for sperm chromatin structure assay (SCSA) measurements in 277 Danish men

	Mean	SD between	SD within	CV total	CV within
$\overline{X}$ αT					
Pesticide-spraying farmers	222.7	16.2	14.1	9.6	3.9
Non-spraying farmers	228.1	20.8	13.0	10.8	3.7
Plastic workers	219.5	13.6	5.7	6.7	2.0
US study[a]	222.3	38.9	21.8	20	7
SD αT					
Pesticide-spraying farmers	76.8	20.1	15.7	33.2	15.4
Non-spraying farmers	82.5	24.7	12.9	33.7	12.5
Plastic workers	63.2	14.4	10.0	27.6	12.7
US study[a]	155.3	38.7	18.0	28	10
COMP αT					
Pesticide-spraying farmers	14.2	7.6	5.3	65.6	23.8
Non-spraying farmers	16.8	12.1	4.8	78.0	18.7
Plastic workers	11.3	5.5	6.2	73.2	25.3
US study[a]	16.8	7.2	5.0	52	23
$\overline{X}$ Green					
Pesticide-spraying farmers	456.1	30.5	16.2	7.6	2.7
Non-spraying farmers	452.1	24.7	12.9	33.7	2.7
Plastic workers	456.2	36.9	13.6	8.6	2.1
US study[a]	506.2	29.8	18.5	7	3

[a]Data from Evenson *et al.* (1991) referring to a cohort of 45 American healthy volunteers who participated in a longitudinal study including monthly SCSA measurements for 8 consecutive months.
SD between = between-subject standard deviation; SD within = within-subject variation; CV total = total coefficient of variation as percentage of total variation; CV within = average within-donor coefficient of variation as percentage of individual mean.

all cases, low but significant negative (P always < 0.0001) correlations were found. The highest level of correlation, as described by Spearman's correlation coefficients, was observed with the proportion of normal spermatozoa (scored according to the WHO criteria), and very little correlation was found with cell vitality, morphology (scored according to the strict criteria), and sperm count.

Discussion

The purpose of this paper was to describe the variability of the FCM SCSA results across a relatively large normal population and how they could be affected by demographic and lifestyle factors. We think that this information would be useful in future environmental and epidemiological studies aimed at the characterization of a variety of detrimental effects on male reproductive capability. In this study, the assessment of sperm and semen quality has been performed by conventional microscopy scoring and by the FCM SCSA. Notable parameters of the SCSA analysis are $\overline{X}$ Green, which reflects native DNA stainability, and those of αT, which are assumed to reflect abnormalities in mature sperm chromatin structure. We have demonstrated a high level of correlation among the αT parameters which, on the other hand, show no significant associations with $\overline{X}$ Green. Therefore, they can reflect a variety of anomalies that could occur during the complex spermiogenic pathway, eventually leading to incorrect chromatin packaging in the sperm nucleus.

The sperm nuclear condensation process involves a dramatic sequence of events including topological rearrangements, transition of DNA-binding proteins, alteration in transcription, loss of nucleosomal structure, and acquisition of a chromosomal organization consisting almost entirely of condensed chromatin (Ward, 1994; Kramer and Krawetz, 1997). Major participants in this event are protamines, and it has been demonstrated that protamine abnormalities are associated with male sterility (Balhorn *et al.*, 1988; De Yebra *et al.*, 1993). The progressive chromatin packaging normally produces a reduction in DNA stainability relative to round spermatids, and a marked decrease in binding of acridine orange to DNA has also been observed as human spermatozoa traverse the epididymis (Golan *et al.*, 1996). An increased intensity of green fluorescence is perhaps the result of an abnormal exchange of histones for transition proteins and protamines. Lack of appropriate sperm maturation results in an increased DNA stainability (Evenson and Jost, 1994) and an increased number of cells with abnormal staining characteristics have often been reported in semen samples from certain infertility patients (Evenson and Melamed, 1983; Golan *et al.*, 1997). It appeared from our results that samples containing abnormally high fractions of immature germ cells were characterized by higher values of green fluorescence.

The precise timing of chromatin packaging and the events governing and controlling this process in mammalian spermatozoa are, however, not completely understood (Kramer and Krawetz, 1997). It is suspected that progressive sperm chromatin packaging is linked to a complex process of DNA cutting and ligating (McPherson and Longo, 1993; Sakkas *et al.*, 1995), carried out with the participation of endogenous endonucleases. The presence of naturally occurring DNA strand breaks in mature human spermatozoa has been measured by in-situ nick translation (Bianchi *et al.*, 1993), in-situ terminal deoxynucleotidyl transferase driven UTP nick end labelling (TUNEL assay) (Gorczyka *et al.*, 1993; Sailer *et al.*, 1995b; Aravindan *et al.*, 1997), and single-cell gel electrophoresis (COMET assay) (Hughes *et al.*, 1996; Aravindan *et al.*, 1997). A correlation between abnormal sperm chromatin packaging

Table II. Distribution of sperm chromatin structure assay values among 277 Danish men (mean ± SD). Correlation with demographic and lifestyle factors

	%	X αT	SD αT	COMP αT	$\bar{X}$ Green
Age, years					
18–24	3.0	209.4 ± 4.6	59.5 ± 5.2	8.7 ± 2.6	446.6 ± 22.2
25–29	7.5	217.6 ± 11.1	73.8 ± 19.6	10.0 ± 3.8	459.8 ± 37.9
30–34	24.8	224.2 ± 23.8	81.7 ± 30.2	14.0 ± 10.2	464.2 ± 43.1
35–39	26.7	224.7 ± 22.0	81.7 ± 28.4	14.8 ± 10.0	453.0 ± 34.4
40–44	18.1	226.0 ± 28.7	80.5 ± 28.6	16.4 ± 11.1	446.0 ± 28.6
45–55	19.9	227.7 ± 23.3	82.3 ± 27.3	18.0 ± 12.4	450.3 ± 38.0
P-value[a]				< 0.0001	< 0.05
Body mass index, kg/m^2					
< 25	51.9	227.8 ± 27.4	84.3 ± 31.2	16.3 ± 12.4	458.8 ± 42.1
⩾ 25	48.1	220.7 ± 17.3	76.1 ± 21.8	13.6 ± 7.8	449.7 ± 29.7
P-value		< 0.05		< 0.05	
Self-reported venereal disease					
Yes	7.9	226.1 ± 20.8	79.9 ± 28.2	15.1 ± 9.1	455.3 ± 40.5
No	92.1	224.3 ± 23.5	85.9 ± 27.9	15.0 ± 10.6	454.3 ± 36.7
P-value					
Self-reported parotitis after puberty					
Yes	7.1	232.4 ± 25.3	90.7± 31.1	18.8 ± 10.1	452.0 ± 33.4
No	92.9	223.8 ± 23.1	79.5± 27.2	14.7 ± 10.5	454.6 ± 37.2
P-value		< 0.05			
Genital virus infection					
Yes	1.5	214.6 ± 15.2	64.5 ± 24.2	11.3 ± 8.4	426.8 ± 25.5
No	98.5	224.0 ± 23.4	80.6 ± 28.0	15.0 ± 10.6	454.8 ± 36.9
P-value					
Tobacco smoking					
Current smoker	24.4	226.3 ± 18.4	76.1 ± 27.8	12.3 ± 6.7	464.4 ± 40.8
Ex-smoker	22.6	224.4 ± 23.6	79.6 ± 29.2	16.5 ± 13.6	446.5 ± 34.0
Never smoker	53.0	220.4 ± 25.0	82.6 ± 26.6	15.6 ± 10.3	453.2 ± 35.4
P-value					< 0.05
Alcoholic beverages					
Not drinking alcohol	9.4	224.1 ± 18.3	80.2 ± 26.0	14.1 ± 7.9	449.9 ± 32.1
1–7	53.6	225.4 ± 26.6	80.0 ± 30.3	15.3 ± 11.1	455.0 ± 37.3
8–14	20.8	221.6 ± 19.2	78.1 ± 23.6	13.8 ± 8.8	452.3 ± 37.1
⩾15	16.2	225.2 ± 18.9	84.8 ± 24.1	16.3 ± 11.9	458.6 ± 38.9
P-value					

[a]The *P*-value is reported only if < 0.05. All *P*-values according to multiple regression adjusted for the effects of age, body mass index, smoking, season, period of sexual abstinence, leukocytospermia, presence of immature sperm cells.

and the presence of DNA strand breaks has been shown to exist and these anomalies may arise due to flaws in the mechanisms that package and protect the sperm chromatin during spermiogenesis (Bianchi *et al.*, 1993; Gorczyka *et al.*, 1993; Manicardi *et al.*, 1995; Sailer *et al.*, 1995b; Sakkas *et al.*, 1995). In particular, it has been demonstrated recently that DNA strand breaks identified by the COMET assay are strongly correlated with the frequency of COMP αT cells (Aravindan *et al.*, 1997). In addition, it should be mentioned that chromatin alterations, measured by the SCSA, have been found to parallel the pattern of induction of dominant lethal mutations which are most likely a consequence of chromosomal breaks (Estop *et al.*, 1993). Thus, the SCSA appears to detect early stages of chromatin alterations that most likely lead to chromosome breaks.

Our SCSA values from the 277 Danish men were completely within the same range as the data from a series of 45 US men volunteering for a longitudinal study (Evenson *et al.*, 1991), both in the case of inter- and intra-subject variability (Table I) and with respect to the correlation coefficients between SCSA and conventional microscopy-based assessment (Table V). The general agreement between the SCSA values from the two studies is an important aspect for future trials aiming at standardizing the SCSA procedure in different laboratories worldwide, as it seems that sperm DNA denaturation may be much more directly comparable across different populations. This is particularly true for the COMP αT cell fraction. It can be easily and unequivocally identifiable both on the αT frequency histogram and on the bivariate red versus green fluorescence cytogram, making its evaluation quite straightforward. Because of the reciprocal association among the αT parameters and their independence from $\bar{X}$ Green, we propose that essential SCSA variables descriptive of the flow cytometric cytogram relative to a human semen sample should include, at least, COMP αT and $\bar{X}$ Green.

A wide heterogeneity of the SCSA results emerged among the various donors in our study. The same variety of acridine orange staining patterns was also observed in the US study quoted above, especially in terms of the shape and position of the main cell cluster in the bivariate cytograms. Interestingly, a wide variability both in chromatin condensation, evaluated by chromomycin A3 fluorescence, and in the abundance of endogenous nicks in the DNA has also been reported (Sakkas *et al.*, 1995). It should be noted that the human heterogeneity contrasts with the minimal variability of SCSA parameters observed in experimental reproductive experiments carried out

Table III. Distribution of sperm chromatin structure assay (SCSA) values among 277 Danish men[a] (mean ± SD). Correlation with fever, presence of immature and white blood cells in the ejaculate, season, and abstinence time

	%	$\bar{X}$ αT	SD αT	COMP αT	$\bar{X}$ Green
Recent fever					
Yes	8.6	227.8 ± 25.5	82.8 ± 31.8	15.2 ± 11.0	468.7 ± 47.8
No	91.4	223.6 ± 21.6	76.5 ± 25.2	14.6 ± 10.5	453.6 ± 33.7
[b]*P*-value					
Leukocytospermia					
Yes	9.4	238.8 ± 34.2	94.4 ± 31.8	21.3 ± 13.2	480.9 ± 40.7
No	90.6	222.4 ± 19.7	75.3 ± 25.1	14.0 ± 10.0	452.2 ± 33.6
P-value		< 0.0001	< 0.001	< 0.0001	< 0.0001
Immature sperm forms					
Yes	9.2	233.7 ± 34.8	88.8 ± 32.9	19.2 ± 12.8	481.8 ± 41.4
No	90.8	222.9 ± 20.0	75.9 ± 25.3	14.6 ± 10.2	452.2 ± 33.5
P-value				< 0.05	< 0.0001
Season					
Jan.–March	20.7	223.4 ± 22.6	78.3 ± 25.3	13.5 ± 8.7	456.3 ± 35.2
April–June	31.4	224.6 ± 23.0	80.4 ± 28.7	15.7 ± 11.7	455.0 ± 39.3
July–Sept.	42.9	222.5 ± 19.7	73.3 ± 24.2	13.9 ± 9.1	454.8 ± 31.9
Oct.–Dec.	5.0	234.3 ± 28.9	83.8 ± 25.8	19.3 ± 17.9	448.7 ± 38.3
P-value		< 0.01	< 0.01	< 0.05	
Sexual abstinence, days					
0.0–2.0	28.1	218.9 ± 17.2	71.6 ± 23.2	12.5 ± 10.0	453.9 ± 29.7
>2.0–4.0	46.7	222.7 ± 17.8	76.5 ± 24.0	14.0 ± 8.3	456.4 ± 37.7
>4.0–6.0	14.0	233.2 ± 34.6	84.6 ± 30.8	19.2 ± 16.1	451.9 ± 36.1
>6.0	11.2	230.3 ± 23.8	83.8 ± 31.1	17.3 ± 9.8	454.7 ± 37.3
P-value		< 0.0001	< 0.01	< 0.0001	

[a]Values derived from SCSA measurements on 531 semen samples.
[b]The *P*-value is reported only if < 0.05. All *P*-values according to multiple regression adjusted for the effects of age, body mass index, smoking, season, period of sexual abstinence, leukocytospermia, presence of immature sperm cells.

Table IV. The within-person variation of $\bar{X}$ αT and COMP αT relative to changes of the sexual abstinence period from first to second sample

Change in abstinence period, days	%	$\bar{X}$ αT		COMP αT	
		Change	SD	Change	SD
⩽ −3	9.8	−2.02	5.8	−6.74	15
−2	9.4	−0.31	3.9	−3.54	11
−1	16.6	−0.07	5.6	0.49	13
0	29.4	0.47	4.7	1.53	11
+1	12.3	−0.29	5.2	−2.43	14
+2	7.2	2.33	4.7	2.75	13
⩾ +3	15.3	2.03	5.2	4.91	12
[a]*P*-value			0.0036		0.0006

[a]*P*-value after linear regression of change in αT values as a function of change of the abstinence period.

Table V. Spearman's correlation coefficients between some sperm chromatin structure assay (SCSA) values and other semen parameters

	$\bar{X}$ αT	COMP αT	$\bar{X}$ Green
Sperm concentration	−0.29 (−0.26)	−0.31 (−0.29)	−0.37 (0.07)
% normal forms, WHO criteria	−0.38 (−0.29)	−0.38 (−0.36)	−0.46 (−0.03)
% normal forms, strict criteria	−0.22	−0.38	−0.31
% vital sperm	−0.25 (−0.25)	−0.23 (−0.26)	−0.14 (−0.01)

All correlations: $P < 0.0001$.
Data reported in parentheses are from Evenson *et al.* (1991) on 45 US healthy volunteers undergoing monthly SCSA analysis for 8 consecutive months.
WHO = World Health Organization

mainly with inbred strains of rodents. The constancy observed in animal studies was fundamental for the application of SCSA in the field of experimental reproductive toxicology. Damage to the male germ line has been detected after exposure to a variety of environmentally relevant agents (Spanò *et al.*, 1996a,b) and, in many instances, the superior sensitivity of

SCSA, compared with other end-points, has been demonstrated (Sailer *et al.*, 1995a; Foster *et al.*, 1996). Another aspect that came out of our data is the very high degree of repeatability of SCSA data for the same subject. The SCSA cytogram patterns are generally distinctive of a particular donor and consistent within a donor over time, representing a sort of sperm fingerprint, as demonstrated by the high intraclass linear correlation coefficients. This contrasts with other conventional semen parameters and adds to the value of this semen assay in studies of the male reproductive function, corroborating and extending the earlier findings reported by Evenson *et al.* (1991).

This study gave us the possibility to compare the SCSA-derived variables with a number of parameters related to the subject habits and characteristics. We have observed that the average COMP αT value almost doubled as age increased from about 25 years to 55 years. The same trend was also observed in ageing laboratory mice (E. Cordelli, personal communication). The issue of the deterioration of sperm quality with age is quite important in order to verify the alleged link between paternal effects and increase of reproductive failures and birth defects. We have noted that sperm concentration also increased with the age in our population (data not shown). It has been postulated that this unexpected result might reflect a birth cohort effect rather than a real age effect. We can speculate that a birth cohort effect could result from changing pre- or perinatal exposures with implications for fetal testicular development (Sharpe and Skakkebaek, 1993). On the contrary, the deterioration of αT values is, most likely, a genuine age effect not related to prenatal development. This further supports the view that SCSA values and sperm concentration are reflecting different biological phenomena. As far as the relation between the load of genetically abnormal sperm cells and ageing is concerned, controversial results have been obtained so far. FISH analysis has demonstrated that ageing was associated with an increased number of chromosome aneuploidies (Griffin *et al.*, 1995; Robbins *et al.*, 1997) while other studies (Lähdetie *et al.*, 1996) have failed to demonstrate such an association.

Besides the expected correlation of COMP αT and $\bar{X}$ Green with the presence of immature germ cells in the ejaculate, our results demonstrate that the presence of leukocytes in the semen significantly affected both the αT parameters and $\bar{X}$ Green. This observation has also been noticed by other authors (Engh *et al.*, 1992) where the presence of white blood cells in the ejaculate was associated with a general increase in the prevalence of hypocondensed subpopulations of spermatozoa both in men with oligoasthenoteratozoospermia as well as in normozoospermic subjects, emphasizing the effect of inflammatory conditions in the reproductive tract on sperm quality. The results from the analysis of the abstinence period are surprising. We found that the higher the period of abstinence the higher the COMP αT fraction. It seems that, the longer the period the mature spermatozoa stay in the epididymis, the higher is the probability of undergoing a sort of deteriorating process leading to an increased susceptibility of chromatin to acid denaturation. Thus, additional studies specifically addressing this problem would be needed to clarify this observation. In this respect our results do not agree with those obtained by Evenson *et al.* (1991), where, for a single individual, abstinence times from 0.5 to 7 days had no significant effect on αT parameters, COMP αT included.

Among the lifestyle variables considered, while alcohol consumption did not alter any of the SCSA parameters, we have observed that $\bar{X}$ Green increased, weakly but significantly, according to the smoking habits of the subjects. It has been demonstrated that smoking is associated with decreased sperm density, count, and motility but not with nuclear morphometric parameters (Vine *et al.*, 1997). Moreover, in another study carried out by FISH analysis, while alcohol consumption was significantly associated with an increased frequency of sperm aneuploidy and diploidy, only a suggestive association was found between smoking and X aneuploidy (Robbins *et al.*, 1997). It is worthwhile to note that, in order to allocate the donor in the corresponding smoking group, we relied on the answers provided by the questionnaires, and no measurement of cotinine levels, which may provide an objective assessment of tobacco smoke exposure, was attempted.

The weak and negative correlations between the SCSA variables and sperm concentration, per cent sperm head abnormality, and per cent sperm viability are indicative of the fact that SCSA parameters are not necessarily associated with conventional semen measures and may provide additional and independent descriptors of semen quality. SCSA measurements reflect the heterogeneity of the nuclear chromatin conformational organization in the different sperm cells in the ejaculate. Unresolved yet is the degree of heterogeneity that is compatible with fertility. Our results are in agreement with other authors that have demonstrated the independence of the SCSA analysis from the conventional microscopy-based semen assessment (Evenson *et al.*, 1991; Fosså *et al.*, 1997). Thus, the SCSA can complement the results of other analyses carried out in a multidisciplinary context, offering new insights into semen quality assessment and an increased sensitivity to germ cell damage detection, at least at the supra-chromosomal level.

It should not be overlooked that the SCSA is also suited to toxicological field studies as it requires as little as 0.1 ml of semen, the sample can be frozen for several months, and FCM analysis can be carried out in a site geographically distant from the place of sample collection. However, age, period of sexual abstinence, inflammatory status, presence of immature forms in the ejaculate, and smoking habits can affect the SCSA results and should be accounted for in the design and analysis of the epidemiological surveys. Promisingly, the FCM SCSA, like the FISH approach, could constitute an example of a bridging biomarker between human and animal studies and therefore have applications for human genetic risk assessment and for investigations of the reproductive and toxicological factors leading to abnormal reproductive outcomes of paternal origin (Wyrobek, 1993). Associations between increased sperm chromatin defects and decreased fertilization rates have been reported in the context of assisted fertilization techniques (Bianchi *et al.*, 1996; Hoshi *et al.*, 1996; Sakkas *et al.*, 1996). In addition, prior experiments have shown the SCSA to be correlated with fertility rate in domestic animals (Ballachey *et al.*, 1988; Dobriski *et al.*, 1994; Sailer *et al.*,

1996). Therefore, poor quality sperm chromatin structure can be considered highly indicative of male subfertility.

We can envisage that these new types of semen-based tests will be available very soon as tools for the rapid screening of certain conditions of subfertility and infertility in man, as they seem to allow an indirect visualization of sperm cells with protamine-deficient, nicked and partially denatured DNA, regardless of sperm morphology, motility and number. This aspect should be of particular significance in identifying defective samples that appear normal by other semen quality criteria. The importance of the FCM SCSA as a predictor of human fertility will be another issue to be addressed soon, and studies are in progress in our institutions to test the validity of this hypothesis.

Acknowledgements

This work has been partially funded by the EU BIOMED/Concerted Action 'Asclepios - Occupational Hazards to Male Reproductive Capability'.

Authors would like to thank Dr. N.Roeleveld (University of Nijmegen, The Netherlands) for her valuable help during the statistical analysis, Dr. F.Pacchierotti (ENEA, Rome, Italy) for her useful critical comments to the manuscript, Mr. C.Mancini (ENEA) for the artwork, and Mrs. T.N.Sinclair (ENEA) for editorial reviewing of the manuscript.

References

Aravindan, G.R., Bjordahl, J., Jost, L.K. and Evenson, D.P. (1997) Susceptibility of human sperm to *in situ* DNA denatured is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp. Cell. Res*., **236**, 231–237.

Balhorn, R., Reed, S. and Tanphaichitr, N. (1988) Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. *Experientia*, **44**, 52–55.

Ballachey, B.E., Evenson, D.P. and Saacke, R.G. (1988) The sperm chromatin structure assay. Relationship with alternate tests of human semen quality and heterospermic performance of bulls. *J. Androl*., **9**, 109–115.

Bianchi, P.G., Manicardi, G.C., Bizzaro, D. *et al.* (1993) Effect of deoxyribonucleic acid protamination on fluorochrome staining and *in situ* nick-translation of murine and human spermatozoa. *Biol. Reprod*., **49**, 1083–1088.

Bianchi, P.G., Manicardi, G.C., Urner, F. *et al.* (1996) Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. *Mol. Hum. Reprod.*, **2**, 139–144.

Bonde, J.P. and Giwercman, A. (1995) Occupational hazards to male fecundity. *Reprod. Med. Review*, **4**, 59–73.

Bonde, J.P., Giwercman, A., Ernst, E. and Asclepios (1996) Identifying environmental risk to male reproductive function by occupational sperm studies: logistics and design options. *Occup. Environ. Med*., **53**, 511–519.

Darzynckiewicz, Z., Traganos, F., Sharpless, T. and Melamed, M.R. (1975) Thermal denaturation of DNA *in situ* as studied by acridine orange staining and automated cytofluorometry. *Exp. Cell Res.*, **90**, 411–428.

De Yebra, L., Ballesca, J.L., Vanrell, J.A. *et al.* (1993) Complete selective absence of protamine P2 in humans. *J. Biol. Chem*., **268**, 10553–10557.

Dobrinski, I., Hughes, H.P.A. and Barth, A.D. (1994) Flow cytometric and microscopic evaluation and effect on fertility of abnormal chromatin condensation in bovine sperm nuclei. *J. Reprod. Fertil.*, **101**, 531–538.

Engh, E., Clausen, O.P.F., Scholberg, A. *et al.* (1992) Relationship between sperm quality and chromatin condensation measured by sperm DNA fluorescence using flow cytometry. *Int. J. Androl.*, **15**, 407–415.

Estop, A.M., Munné, S., Jost, L.K. and Evenson, D.P. (1993) Studies on sperm chromatin structure alterations and cytogenetic damage of mouse sperm following *in vitro* incubation. Studies on in vitro-incubated mouse sperm. *J. Androl.*, **14**, 282–288.

Evenson, D.P. and Jost, L.K. (1994) Sperm chromatin structure assay: DNA denaturability. In Darzynkiewicz, Z., Robinson, J.P. and Crissman, H.A. (eds.), *Flow Cytometry, Part B*. Second edition, Academic Press, Orlando, pp. 159–175.

Evenson, D.P. and Melamed, M.R. (1983) Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. *J. Histochem. Cytochem*., **31**, 248–253.

Evenson, D.P., Darzyinckiewicz, Z. and Melamed, M.R. (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*, **240**, 1131–1133.

Evenson, D., Darzynkiewicz, Z., Jost, L. *et al.* (1986) Changes in accessibility of DNA to various fluorochromes during spermatogenesis. *Cytometry*, **7**, 45–53.

Evenson, D.P., Jost, L.K., Baer, R.K. *et al.* (1991) Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod. Toxicol*., **5**, 115–125.

Fosså, S.D., De Angelis, P., Kraggerud, S.M. *et al.* (1997) Prediction of posttreatment spermatogenesis in patients with testicular cancer by flow cytometric sperm chromatin structure assay. *Cytometry* (Communications in Clinical Cytometry), **30**, 192–196.

Foster, W.G., MacMahon, A. and Rice, D.C. (1996) Sperm chromatin structure is altered in cynomolgous monkeys with environmentally relevant blood lead levels. *Toxicol. Ind. Health*, **12**, 723–725.

Francavilla, S., Cordeschi, G., Gabriele, A. *et al.* (1996) Chromatin defects in normal and malformed human ejaculated epididymal spermatozoa: a cytochemical ultrastructural study. *J. Reprod. Fertil.*, **106**, 259–268.

Gledhill, B.L., Evenson, D.P. and Pinkel, D. (1990) Flow cytometry and sorting of male germ cells. In Melamed, M.R., Lindmo, T. and Mendelsohn, M.L. (eds.), *Flow Cytometry and Sorting*. Second edition, Wiley-Liss, New York, pp. 531–551.

Golan, R., Cooper, T.G., Oschry, Y. *et al.* (1996) Changes in chromatin condensation of human spermatozoa during epididymal transit as determined by flow cytometry. *Hum. Reprod.*, **11**, 1457–1462.

Golan, R., Shochat, L., Weissenberg, R. *et al.* (1997) Evaluation of chromatin condensation in human spermatozoa: a flow cytometric assay using Acridine Orange staining. *Mol. Hum. Reprod.*, **1**, 47–54.

Gorczyka, W., Traganos, F., Jesionowska, H. and Darzynkiewicz, Z. (1993) Presence of DNA strand breaks and increased sensitivity of DNA *in situ* to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. *Exp. Cell Res.*, **207**, 202–205.

Griffin, D.K., Abruzzo, M.A., Millie, E.A. *et al.* (1995) Non-disjunction studies of human sperm: evidence for a paternal age effect on trisomy. *Hum. Mol. Genet*., **4**, 2227–2232.

Hoshi, K., Katayose, H., Yanagida, K. *et al.* (1996) The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil. Steril.*, **66**, 634–639.

Hughes, C.M., Lewis, S.E.M., McKelvey-Martin, V.J. and Thompson, W. (1996) A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol. Hum. Reprod*., **2**, 613–619.

Kramer, J.A. and Krawetz, S.A. (1997) RNA in spermatozoa: implications for the alternative haploid genome. *Mol. Hum. Reprod.*, **3**, 473–478.

Krzanowska, H. (1982) Toluidine blue staining reveals changes in chromatin stabilization of mouse spermatozoa during epididymal maturation and penetration of ova. *J. Reprod. Fert*., **64**, 97–101.

Lähdetie, J., Ajosenpää-Saari, M. and Mykkänen, J. (1996) Detection of aneuploidy in human spermatozoa of normal semen donors by fluorescence *in situ* hydrization. *Environ. Health Perspect*., **104**, 629–632.

Manicardi, G.C., Bianchi, P.G., Pantano, S. *et al.* (1995) Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biol. Reprod*., **52**, 864–867.

McPherson, S.D. and Longo, F.J. (1993) Chromatin structure-function alterations during mammalian spermatogenesis: DNA nicking and repair in elongating spermatids. *Eur. J. Histochem*., **37**, 109–128.

Molina, J., Castilla, J.A., Gil, T. *et al.* (1995) Influence of incubation on the chromatin condensation and nuclear stability in human spermatozoa by flow cytometry. *Hum. Reprod.*, **10**, 1280–1286.

Otto, F.J., Hacker, U., Zante, J. *et al.* (1979) Flow cytometry of human spermatozoa. *Histochemistry*, **61**, 249–254.

Robbins, W.A., Vine, M.F., Truong, K.Y. and Everson, R.B. (1997) Use of fluorescence *in situ* hybridization (FISH) to assess effects of smoking, caffeine, and alcohol on aneuploidy load in sperm of healthy men. *Environ. Mol. Mutagen*, **30**, 175–183.

Rowe, P.J., Comhaire, F.H., Hargreave, T.B. and Mellows, H.J. (1993*) WHO Manual for the Standardized Investigation and Diagnosis of the Infertile Couple*. Cambridge University Press, Cambridge.

Sailer, B.L., Jost, L.K. and Evenson, D.P. (1995a) Effects of x-irradiation on mouse testicular cells and sperm chromatin structure. *Environ. Mol. Mutagen*, **25**, 23–30.

Sailer, B.L., Jost, L.K., and Evenson, D.P. (1995b) Mammalian sperm DNA susceptibility to *in situ* denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J. Androl.*, **16**, 80–87.

Sailer, B.L., Jost, L.K. and Evenson, D.P. (1996) Bull sperm head morphometry related to abnormal chromatin structure and fertility. *Cytometry*, **24**, 167–173.

Sakkas, D., Manicardi, G., Bianchi, P.G. *et al.* (1995) Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. *Biol. Reprod.*, **52**, 1149–1155.

Sakkas, D., Urner, F., Bianchi, P.G. *et al.* (1996) Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Hum. Reprod.*, **11**, 837–843.

Schrader, S.M., Turner, T.W., Breitenstein, M.J. and Simon, S.D. (1988) Longitudinal study of semen quality of unexposed workers. I. Study overview. *Reprod. Toxicol.*, **2**, 183–190.

Sharpe, R.M. and Skakkebaek, N.E. (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet*, **341**, 1392–1395.

Spanò, M. and Evenson, D.P. (1991) Flow cytometric studies for reproductive toxicology. In Gledhill, B.L. and Mauro, F. (eds.), *New Horizons in Biological Dosimetry*, Wiley-Liss, New York, pp. 497–512.

Spanò, M. and Evenson, D.P. (1993) Flow cytometric analysis for reproductive biology. *Biol. Cell*, **78**, 53–62.

Spanò, M., Calugi, A., Capuano, V. *et al.* (1984) Andrological sperm analysis by flow cytometry and sizing. *Andrologia*, **16**, 367–375.

Spanò, M., Bartoleschi, C., Cordelli, E. *et al.* (1996a) Flow cytometric and histological assessment of 1,2:3,4-diepoxybutane toxicity on mouse spermatogenesis. *J. Toxicol. Environ. Health*, **47**, 101–119.

Spanò, M., Bartoleschi, C., Cordelli, E. *et al.* (1996b) Flow cytometric assessment of trophosphamide toxicity on mouse spermatogenesis. *Cytometry*, **24**, 174–180.

Tejada, R.I., Mitchell, J.C., Norman, A. *et al.* (1984) A test for the practical evaluation of male fertility by acridine orange fluorescence. *Fertil. Steril.*, **42**, 87–91.

Vine, M.F., Setzer, R.W., Everson, R.B. and Wyrobek, A.J. (1997) Human sperm morphometry and smoking, caffeine, and alcohol consumption. *Reprod. Toxicol.*, **11**, 179–184.

Ward, W.S. (1994) The structure of the sleeping genome: implications of sperm DNA organization for somatic cells. *J. Cell Biochem.*, **55**, 77–82.

Ward, W.S. and Coffey, D.S. (1991) DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol. Reprod.*, **44**, 569–574.

Whorton, M.D., Krauss, R.M., Marshall, S. and Milby, T.H. (1977) Infertility in male pesticide workers. *Lancet*, **2**, 1259–1260.

World Health Organization (1992) *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction.* Cambridge University Press, Cambridge.

Wyrobek, A.J. (1993) Methods and concepts in detecting abnormal reproductive outcomes of paternal origin. *Reprod. Toxicol.*, **7**, 3–16.

Wyrobek, A.J., Schrader, S.M., Perreault, S.D. *et al.* (1997) Assessment of reproductive disorders and birth defects in communities near hazardous chemical sites. III. Guidelines for field studies of male reproductive disorders. *Reprod. Toxicol.*, **11**, 243–259.

Received on December 17, 1997; accepted on June 3, 1998

Appendix

Asclepios is an EU Biomedical Concerted Action research project dedicated to Occupational Hazards to Male Reproductive Capability, coordinated by the Steno Institute of Public Health, University of Aarhus, Denmark, with the following participants: **Aarhus, Denmark:** A.Abell, J.P.Bonde, S.B.Larsen, G.Danscher, E.Ernst, H.Kolstad**; Bruscia, Italy:** P.Apostoli, S.Porru; **Copenhagen, Denmark:** A.Giwercman; **Erlangen, Germany:** K.H.Schaller, W.Zschiesche; **Ghent, Belgium:** P.Kiss, A.Mahmud, M.Vanhhorne, H.Verstraelen; **Helsinki, Finland:** M.-L.Lindbohm, H.Taskinen, M.Sallmen; **Lodz, Poland:** K.Sitarek; **London, UK:** A.Dale, M.Joffe, N.Shah; **Milan, Italy:** L.Bisanti; **Nijmegen, The Netherlands:** N.Roeleveld, H.Thuis, G.A.Zielhuis; **Paris, France:** P.Jouannet, P.Thonneau; **Pietrasanta, Italy:** L.Lastrucci; **Rome, Italy:** M.Spanò; **Strasbourg, France:** A.Clavert; **Turku, Finland:** J.Lähdetie; **Zeist, The Netherlands:** W. de Kort.