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Heterogeneity of Sperm Nuclear Chromatin Structure and Its Relationship to Bull Fertility[1]

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ABSTRACT

The relationship between sperm nuclear chromatin structure and fertility was evaluated in two groups of Holstein bulls: Group 1, 49 mature bulls, and Group 2, 18 young bulls. Fertility ratings had been estimated for Group 1 and nonreturn rates were known for Group 2. Semen samples were measured by the sperm chromatin structure assay (SCSA): sperm were treated to induce partial in situ DNA denaturation, stained with acridine orange, and evaluated by flow cytometry. Acridine orange intercalated into double-stranded DNA emits green fluorescence upon excitation with 488 nm light, and red fluorescence when associated with single-stranded DNA. An index of DNA denaturation per cell is provided by alpha-t [α_t = red/(red + green) fluorescence]. The standard deviation ($SD\alpha_t$), coefficient of variation ($CV\alpha_t$) and proportion of cells outside the main population ($COMP\alpha_t$) of the α_t distribution quantify the extent of denaturation for a sample. Intraclass correlations of the α_t values were high (>0.70), based on four collections obtained over several years from Group 1 bulls. Negative correlations were obtained between fertility ratings and both $SD\alpha_t$ (-0.58, $p<0.01$) and $COMP\alpha_t$ (-0.40, $p<0.01$) in Group 1, and between nonreturn rates and both $SD\alpha_t$ (-0.65, $p<0.01$) and $COMP\alpha_t$ (-0.53, $p<0.05$) in Group 2. These data suggest that the SCSA will be of value for identification of low fertility sires and poor quality semen samples.

INTRODUCTION

Semen quality and its relationship to fertility are of major concern in animal production. Quality tests are routinely used to determine acceptability of extended semen for breeding purposes, and thus the accuracy of the measurement is extremely important. Although techniques for semen evaluation have been extensively investigated, a need still exists for improved methods to predict semen quality and, ultimately, sire fertility (Saacke, 1983, 1984).

Flow cytometry (FCM) provides a powerful technique for measurement of sperm cells (Gledhill et al., 1979). Recent applications of FCM have included determination of ratios of X- and Y-chromosome-bearing sperm (Garner et al., 1983; Pinkel et al., 1985), and evaluation of spermatozoal function (Evenson et al., 1982; Matyus et al., 1984; Garner et al., 1986). The sperm chromatin structure assay (SCSA), developed by Evenson and coworkers (1980a, 1985, 1986) uses FCM to measure extent of in situ denaturation of nuclear DNA in sperm. Increased susceptibility to denaturation corresponds to heterogeneity of chromatin structure. Studies on humans (Evenson and Melamed, 1983; Evenson et al., 1984a,b), mice (Evenson et al., 1985, 1986; Ballachey et al., 1986a) and bulls (Evenson et al., 1980a; Ballachey et al., 1986b) indicate that increased heterogeneity of chromatin structure is associated with disturbances of spermatogenesis, high proportions of morphologically abnormal sperm, and infertility.

The primary objectives of this study were: 1) to estimate repeatabilities, computed as interclass and

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intraclass correlations, of the SCSA on sperm samples collected over a several-year period, and 2) to evaluate the relationship between bull fertility and heterogeneity of sperm chromatin, as determined by the SCSA. A related objective was to compare results of acid vs. heat for induction of DNA denaturation in situ.

MATERIALS AND METHODS

Semen Samples

Frozen semen from Holstein bulls was supplied by Eastern Artificial Insemination Cooperative, Inc. (EAIC), Ithaca, NY. Samples were processed by routine procedures, using a milk-based extender, and frozen in 0.5 ml polyvinylchloride straws in liquid nitrogen until prepared for FCM analysis. Sperm from two groups of bulls were evaluated.

Group 1 (G1) consisted of forty-nine mature bulls representing a cross-section of sires on a regular semen collection schedule at EAIC. The number of collections available per bull varied from one to eight, with a total of 191 collections on all bulls. Several straws were obtained from each collection, enabling repeat measurements. Based on post-thaw motility, all collections were acceptable for routine use in artificial insemination. Fertility ratings, based on nonreturn rates adjusted for various environmental effects and expressed as a deviation from zero, had been estimated for EAIC bulls by Everett and Bean (1986). Fertility ratings of G1 bulls ranged from −13.05 to +4.72; higher values were associated with increased fertility. Standard errors of the fertility ratings had also been computed, and only bulls with standard errors less than 1 were included in this analysis.

In order to estimate repeatabilities, four separate collections were obtained from 31 of the G1 bulls. The first and second samples were collected within a one-month period, and the third and fourth also within a one-month period. The time period between collections of the second and third samples ranged from a minimum of 3 months to several years.

Group 2 (G2) consisted of eighteen bulls collected about 15 months of age, as sampling sires (i.e., young sires being progeny tested prior to extensive use in artificial insemination), and again at 4 years of age. Nonreturn rates (measured at 59 days and based on 350 first services) were known for these bulls at the younger age.

Cell Preparation and Staining

Samples were measured by the sperm chromatin structure assay, which uses acridine orange (AO) dye. Either acid (SCSA/acid; Evenson and Melamed, 1983; Evenson et al., 1985; Evenson, 1986) or heat (SCSA/thermal; Evenson et al., 1980a, 1984a.b, 1985; Evenson, 1986) was used to potentially induce in situ denaturation of DNA prior to staining. In previous publications, the SCSA/acid has also been referred to as the two-step acridine orange method (TSAO; Darzynkiewicz et al., 1976), and the SCSA/thermal as the thermal denaturation (TD) method.

The utility of AO is based on its metachromatic emission of fluorescence upon excitation. AO intercalated into double-stranded (ds) nucleic acids fluoresces green, whereas when bound to single-stranded (ss) nucleic acids, red fluorescence is emitted. Since mature sperm are essentially devoid of RNA (Monesi, 1971) the green and red fluorescence intensities of AO-stained sperm are indicative of the amount of stainable ds-DNA and ss-DNA, respectively.

The SCSA/thermal method, initially developed to measure heterogeneity of chromatin structure (Evenson et al., 1980a), involves heat treatment of isolated fixed sperm nuclei and is relatively time-consuming. In contrast, the SCSA/acid generally is done on freshly thawed sperm in extender, although it can also be done on isolated nuclei (fresh or fixed). Either technique detects increased susceptibility of chromatin to denaturation.

Freshly thawed whole sperm cells from all collections on G1 and G2 bulls were measured by the SCSA/acid. Additionally, from each of 29 G1 bulls, sperm nuclei from a single, randomly chosen collection were isolated and fixed, and subsequently measured by both the SCSA/acid and SCSA/thermal.

For the SCSA/acid, straws were removed from liquid nitrogen and placed on ice for 5–7 min. Comparison of semen thawed by this technique vs. that thawed for 30 s in a 37°C water bath and then placed on ice has demonstrated that thawing procedure has no effect on results of the SCSA. Semen was then extruded into a test tube and diluted with TNE buffer (0.01 M tris(hydroxymethyl)aminomethane [Tris]-HCl, 0.15 M NaCl and 1 mM disodium ethylenediaminetetraacetate [EDTA], pH 7.4) to obtain a concentration of $1-2 \times 10^6$ cells per ml. A 0.2-ml aliquot was then mixed with 0.4 ml of 0.1% (v/v) Triton X-100, 0.08 N HCl and 0.15 M

NaCl. This solution permealizes the cell membrane, allowing uptake of the dye, and may induce partial denaturation of the DNA if chromatin structure of the cell is abnormal. Thirty seconds later, 1.2 ml of AO staining solution (0.2 M Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate, pH 6.0; with 6.0 μg/ml AO) were added. The sample was immediately placed in the flow cytometer for measurement 3 to 5 min later.

For the SCSA/thermal, purified and fixed sperm nuclei were used. To obtain nuclei, freshly thawed samples were washed in 6 ml TNE, pelleted by centrifugation (6000 × *g* for 10 min), then resuspended in 1.5 ml TNE. Cells were transferred to a Falcon plastic test tube (#3033), placed in an ice water slurry and sonicated with a Bronwill Biosonik IV sonicator (VWR Scientific, Minneapolis, MN) at a setting of 50-low for a total of 60 s (30 s each: sonication, cooling, sonication). Three ml of TNE and 1.5 ml of 60% sucrose (w/w) in 0.01 M Tris-HCl and 2 mM disodium EDTA (pH 7.4) were added to the sonicate, which was then layered over 9 ml of the same sucrose solution in a 16-ml Sepcor (Separation Science Corporation, Stratford, CT) polycarbonate test tube. Samples were spun in a Sorvall HB4 rotor for 1 h at 25,000 × *g*. The pellet, containing isolated nuclei, was suspended in 1 ml of "resuspension buffer" (0.15 M NaCl, 5 mM $MgCl_2$, and 0.02 M Tris-HCl, pH 7.4), forcefully pipetted into 9 ml of 1:1 70% ethanol:acetone in a glass test tube, and stored at −20°C. A minimum of overnight storage was allowed for fixation. (Samples have been stored for periods up to a year with no apparent detectable alterations.)

For FCM measurements the fixed nuclei were pelleted, resuspended in 3 ml of "heating buffer" (2 mM sodium cacodylate, 0.1 mM disodium EDTA, and 40% ethanol, pH 6.0) and allowed to equilibrate for 30 min. Nuclei were centrifuged for 10 min at 10,000 × *g* in a Sorvall HB4 rotor and the resulting pellet was resuspended in 1 ml of heating buffer. For analysis of an unheated aliquot, a 0.2-ml suspension of nuclei was stained with 2.0 ml of AO staining buffer (0.15 M NaCl, 5 mM $MgCl_2$, 0.02 M Tris-HCl, pH 7.4; with 8.0 μg/ml AO) and measured by FCM after 3 min. A second aliquot of 0.4 ml (twice the volume, to compensate for loss of volume due to heating and nuclei adhering to the test tubes) was transferred to a plastic test tube, placed in a boiling water bath for 5 min, then cooled in an ice-water slurry for 15 s. Two ml of the same AO staining solution were added and the nuclei measured by FCM 3 min later. Thus, for each sample, a measurement was obtained on an unheated and a heated aliquot. After nuclei were measured by the SCSA/thermal, an additional aliquot was measured by the SCSA/acid. For both the SCSA/thermal and SCSA/acid, all procedures (except heating) were done at 4°C.

Fluorescence Measurements

Flow cytometric determinations used a Cytofluorograf II equipped with a Lexel 100 mW argon ion laser and interfaced to a 2150 computer system (Ortho Diagnostic Systems, Inc., Westwood, MA). The green (515–530 nm) and red (>600 nm) fluorescence emitted by each cell after laser beam excitation (35 mW, 488 nm) was directed through photomultiplier tubes and quantified. In order to standardize instrument settings for sperm samples evaluated on different days, fluorescent polystyrene beads (1/8 bright; Coulter Electronics, Hialeah, FL) were added to the first sample evaluated each day, and electronic gains adjusted so that coordinate position of the red and green signals of the beads was the same from day to day.

Alpha-t (α_t), defined as the ratio of red to total (red + green) fluorescence (Darzynkiewicz et al., 1975), was computed for each cell or nucleus by computer protocols, and the distribution of the α_t values determined for each sample. Although the theoretical distribution of α_t values is continuous from 0 to 1, the measurements were made over 1000 channels (levels) of fluorescence; thus values presented here are expressed on a scale of 1 to 1000. Five thousand cells were measured for each sample.

Based on the distribution of α_t values, the following statistics were computed for each sample: 1) mean channel of the α_t distribution ($\bar{X}\alpha_t$); 2) standard deviation of the α_t distribution ($SD\alpha_t$); 3) coefficient of variation of the α_t distribution ($CV\alpha_t$); and 4) proportion of cells outside the main α_t peak ($COMP\alpha_t$) (refer to Figure 1 for clarification). The last value, $COMP\alpha_t$, was obtained by visually inspecting the α_t distribution on the computer screen and setting a region to enumerate the percentage of cells falling to the upper side of the main α_t peak. This is essentially equal to the percentage of cells that have increased susceptibility to denaturation, relative to the "normal" cells in the sample. All four α_t values are useful to

quantify differences in susceptibility of the DNA in chromatin to denaturation; higher values are associated with increased ss-DNA content and chromatin heterogeneity.

Microscopic Evaluation

Percentage of morphologically abnormal sperm heads (% abnormal) was evaluated on the same 29 samples from G1 bulls that were also measured by SCSA/acid on fresh cells and both SCSA/acid and SCSA/thermal on isolated fixed nuclei. For G2 bulls, % abnormal was evaluated for samples collected when bulls were 15 months old. Sperm were stained in suspension with 0.5% Eosin Y for 30 min and then smeared onto glass slides. After air drying, the slides were quickly rinsed in methanol, dried and coverslipped using Permount. Two hundred cells per bull were examined for morphological abnormalities by light microscopy, using a 100X oil immersion objective lens for a total magnification of 1250X. Sperm heads were classified only as normal or abnormal.

Data Analysis

Statistical analyses were done with programs available through the Statistical Analysis System (SAS, 1984). Repeatabilities within straw and within collection were calculated as interclass (i.e., product-moment or simple) correlations. Repeatabilities among collections were computed as intraclass correlations, using expected mean squares from the nested analysis of variance (Kempthorne, 1957).

RESULTS

Means and standard errors of α_t variables on sperm stained by the SCSA/acid, as well as % abnormal, fertility ratings, and nonreturn rates are listed in Table 1 to provide an indication of the magnitude of these values, and the extent of variation associated with them.

In Figure 1 are cytograms (A and C) and corresponding α_t frequency histograms (B and D) of two G1 bulls, presented to illustrate the type of data obtained on sperm prepared by the SCSA/acid, and the variation in staining pattern possible among bulls. The horizontal and vertical axes on the cytograms represent relative stainability of ss-DNA and ds-DNA, respectively, measured by levels of red and green fluorescence. The elongated pattern of the signal seen in the cytograms is characteristic of sperm cells measured in a flow cytometer with orthogonal axes of laser illumination, sample stream flow, and fluorescence detection. Because sperm nuclei are asymmetrical and have a high refractive index, the fluorescence detected is dependent upon orientation of the nucleus when intersected by the laser beam. Varying orientations lead to the elongated signal (Gledhill et al., 1976). Since α_t is measured as a ratio of red to total fluorescence, it is independent of the effect of nuclear orientation.

Note the greater proportion of sperm with increased levels of red fluorescence in cytogram C as compared to A, and the resulting shift in the distribution of α_t values (shoulder on the right side of peak) in the histograms. Alpha-t values for histogram B

TABLE 1. Mean values of variables for each group of bulls.[a]

Variable	Group 1	Group 2	
	n = 49	15 Months (n = 18)	4 Years (n = 18)
1) $\bar{X}\alpha_t$[b]	222.4 ± 3.4	206.7 ± 4.1	212.2 ± 5.9
2) $SD\alpha_t$	43.6 ± 1.6	52.5 ± 3.3	50.8 ± 3.7
3) $CV\alpha_t$	19.6 ± .7	25.1 ± 1.2	23.5 ± 1.0
4) $COMP\alpha_t$	12.6 ± 1.1	15.1 ± 2.4	14.7 ± 2.8
5) % Abnormal[c]	6.6 ± 1.0[c]	13.9 ± 3.4	
6) Fertility rating	0.2 ± 0.4		
7) Nonreturn rate[d]		65.6 ± 2.3	

[a]Values are mean ± SEM.

[b]Alpha-t variables from the sperm chromatin structure assay; value for each bull computed as the average of all measurements made on all collections of that bull. $\bar{X}\alpha_t$, $SD\alpha_t$, $CV\alpha_t$, and $COMP\alpha_t$ refer to the mean, standard deviation, coefficient of variation, and cells outside the main peak, respectively, of the α_t distribution.

[c]Percentage of morphologically abnormal sperm heads, scored by light microscopy; n = 29.

[d]Nonreturn rate at 59 days, based on 350 1st services.

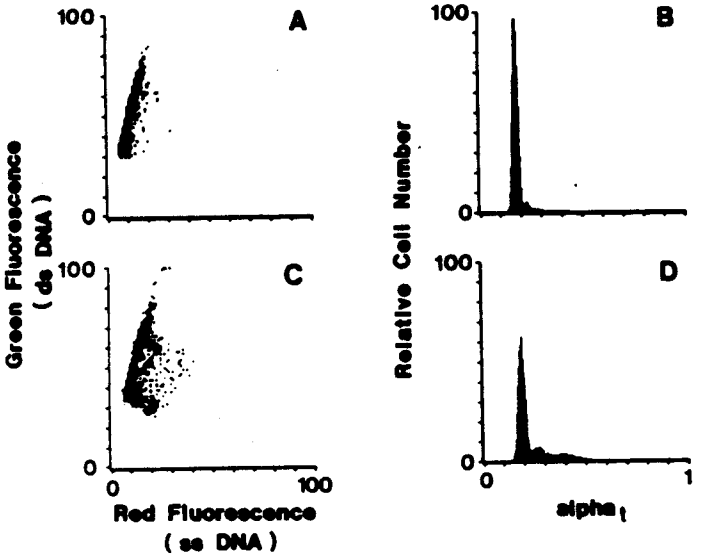

FIG. 1. Green vs. red fluorescence cytograms and corresponding alpha-t (α_t) frequency histograms of sperm prepared by the sperm chromatin structure assay and measured by flow cytometry from two G1 bulls. Each *point* in the cytograms represents a sperm cell; levels of green and red fluorescence indicate the relative content of double-stranded (ds)- and single-stranded (ss)-DNA, respectively, in the nucleus. Alpha-t is defined as the ratio of red to total (red + green) fluorescence; higher values of α_t correspond to a greater degree of DNA denaturation in sperm.

were 184, 35.9, 19.5 and 8%, and for histogram D were 226, 80., 35.4 and 26% for $\bar{X}\alpha_t$, $SD\alpha_t$, $CV\alpha_t$ and $COMP\alpha_t$, respectively.

Repeatability estimates of α_t values on sperm prepared by SCSA/acid are presented in Table 2. Extremely high values (0.91 to 0.99) were noted for all traits when measuring within an ejaculate (same straw or duplicate straws, 1 and 2, respectively, Table 2) measured on one day. When measurements were made on duplicate straws on different days (3, Table 2), repeatabilities were lower for $\bar{X}\alpha_t$, $SD\alpha_t$ and $CV\alpha_t$ (0.31, 0.79, and 0.67, respectively), whereas for $COMP\alpha_t$ the value remained extremely high (0.98). Repeatabilities 1, 2, and 3 were computed as interclass (simple) correlations. Repeatabilities of α_t values from two collections obtained within one month of each other, computed as intraclass correlations (4, Table 2), were slightly higher than, but not significantly different from, values computed across four collections (5, Table 2) made over several years; all were high and positive (>0.70).

For G1 sires, correlation coefficients between sperm α_t variables, and between those variables and fertility ratings, are presented in Table 3. Negative correlations ($p<0.01$) of −0.58, −0.53 and −0.40 were obtained for fertility ratings with $SD\alpha_t$, $CV\alpha_t$, and $COMP\alpha_t$, respectively. These values do not differ significantly from each other. The relationships between fertility ratings and both $SD\alpha_t$ and $COMP\alpha_t$, including values of the linear regression, are presented in Figure 2.

Correlation coefficients among α_t values obtained on sperm (or isolated fixed nuclei) from a single collection of each of 29 G1 bulls, measured by both the SCSA/acid and SCSA/thermal methods, are presented in Table 4. Since the $CV\alpha_t$ and $SD\alpha_t$ are closely correlated, and provide much of the same information, $CV\alpha_t$ values were not included in the table.

TABLE 2. Repeatabilities of alpha-t values of sperm analyzed by the sperm chromatin structure assay from Group 1 bulls.

Repeatability[a]	Value[b]			
	$\bar{X}\alpha_t$	$SD\alpha_t$	$CV\alpha_t$	$COMP\alpha_t$
1) Between 2 measurements on a single straw, measured on the same day (68 pairs)	0.92	0.96	0.97	0.99
2) Between 2 straws of the same collection, measured on the same day (40 pairs)	0.91	0.93	0.94	0.99
3) Between 2 straws of the same collection, measured on different days (60 pairs)	0.31	0.79	0.67	0.98
4) Between 2 collections made within a 1-month period, measured on the same day (62 pairs, 31 bulls)	0.90	0.83	0.92	0.76
5) Among 4 collections made over a several-year period, measured on the same day (31 bulls)	0.89	0.71	0.83	0.70

[a]Repeatabilities 1, 2, and 3 computed by interclass correlation, 4 and 5 by intraclass correlation; all computed across bulls.

[b]$\bar{X}\alpha_t$, $SD\alpha_t$, $CV\alpha_t$, and $COMP\alpha_t$ refer to the mean, standard deviation, coefficient of variation, and cells outside the main peak, respectively, of the α_t distribution.

TABLE 3. Correlation coefficients between alpha-t variables[a] of sperm and fertility ratings of Group 1 bulls.[b]

	Variable			
Variable	$SD\alpha_t$	$CV\alpha_t$	$COMP\alpha_t$	Fertility rating
$\bar{X}\alpha_t$	0.32*	−0.10	0.09	−0.17
$SD\alpha_t$		0.91**	0.82**	−0.58**
$CV\alpha_t$			0.82**	−0.53**
$COMP\alpha_t$				−0.40**

[a]Alpha-t (α_t) variables from the sperm chromatin structure assay; numbers of collections per bull ranged from 1 to 8, and values for each bull were obtained by averaging all measurements made on all collections of each bull. $\bar{X}\alpha_t$, $SD\alpha_t$, $CV\alpha_t$, and $COMP\alpha_t$ refer to the mean, standard deviation, coefficient of variation, and cells outside the main peak, respectively of the α_t distribution.

[b]n = 49 bulls.

*Significantly different ($p<0.05$) from zero.

**Significantly different ($p<0.01$) from zero.

The $SD\alpha_t$ and $COMP\alpha_t$ from the SCSA/acid method are also closely correlated, whether obtained on freshly thawed intact sperm (0.83, $p<0.01$) or on isolated fixed nuclei (0.82, $p<0.01$). The $X\alpha_t$ from the SCSA/acid on whole sperm was not closely correlated with any of the other variables. However, for the SCSA/acid on fixed nuclei, $X\alpha_t$ was positively correlated with $SD\alpha_t$ and $CV\alpha_t$. The $COMP\alpha_t$ values from the SCSA/acid on whole sperm vs. fixed nuclei were very highly correlated (0.97, $p<0.01$), indicating that factors contributing to the increased red fluorescence were inherent in the nuclear chromatin.

No significant correlations were obtained between α_t values from the unheated aliquot of the SCSA/thermal with any of the α_t values from the SCSA/acid, but the $SD\alpha_t$ values of the unheated and heated aliquots, SCSA/thermal, were positively correlated (0.72, $p<0.01$). For the heated aliquot of the SCSA/thermal, the $SD\alpha_t$ was positively correlated with the $SD\alpha_t$ (0.42, $p<0.05$) and $COMP\alpha_t$ (0.36, $p<0.05$) from the SCSA/acid on whole sperm, and with $COMP\alpha_t$ (0.34, $p<0.05$) from SCSA/acid on fixed nuclei.

The $SD\alpha_t$ from the SCSA/acid on whole sperm or fixed nuclei, and from the SCSA/thermal (heated aliquot), all were correlated with fertility ratings, and the r values obtained (−0.68, −0.57, $p<0.01$; −0.41, $p<0.05$) were not significantly different from each other (Table 4). In addition, $COMP\alpha_t$ from the SCSA/acid on whole sperm or fixed nuclei and $X\alpha_t$, SCSA/acid on fixed nuclei, were negatively correlated with fertility ratings. These correlations of α_t values with fertility ratings, based on a single collection from each of 29 bulls, did not vary significantly from correlations obtained when using the average α_t values on all available collections of 49 bulls (Table 3).

TABLE 4. Correlation coefficients between sperm alpha-t variables,[a] proportion of morphologically abnormal sperm heads, and fertility ratings of Group 1 bulls.[b]

		SCSA/acid					SCSA/thermal					
		Whole cells		Fixed nuclei			Unheated		Heated		% Abnormal[d]	Fertility rating
Method	Variable[c]	2	3	4	5	6	7	8	9	10	11	12
SCSA/acid on whole cells	1) $\bar{X}\alpha_t$	0.23	0.03	−0.09	0.10	0.07	0.09	0.09	−0.15	0.12	0.10	0.00
	2) $SD\alpha_t$		0.83**	0.79**	0.88**	0.81**	−0.24	0.11	0.14	0.42*	0.31	−0.68*
	3) $COMP\alpha_t$			0.77**	0.75**	0.97**	−0.25	0.06	0.22	0.36*	0.28	−0.41
SCSA/acid on fixed nuclei	4) $\bar{X}\alpha_t$				0.90**	0.80**	−0.19	−0.02	0.15	0.11	0.32*	−0.45**
	5) $SD\alpha_t$					0.82**	−0.23	0.11	0.24	0.29	0.31	−0.57**
	6) $COMP\alpha_t$						−0.25	0.08	0.18	0.34*	0.32*	−0.39*
SCSA/thermal, unheated on fixed nuclei	7) $\bar{X}\alpha_t$							−0.02	0.14	−0.17	−0.09	−0.03
	8) $SD\alpha_t$								0.28	0.72**	0.25	−0.12
SCSA/thermal, heated on fixed nuclei	9) $\bar{X}\alpha_t$									0.41*	−0.11	−0.16
	10) $SD\alpha_t$										0.23	−0.41*
	11) % abnormal											−0.37*

[a]The sperm chromatin structure assay/acid method was used on whole sperm and on isolated fixed nuclei, and the sperm chromatin structure assay/thermal method on isolated fixed nuclei.

[b]n = 29; All measurements were on sperm from a single collection of each bull.

[c]$\bar{X}\alpha_t$, $SD\alpha_t$, $CV\alpha_t$, and $COMP\alpha_t$ refer to the mean, standard deviation, coefficient of variation, and cells outside the main peak, respectively, of the α_t distribution.

[d]Proportion of morphologically abnormal sperm heads, scored by light microscopy.

*Significantly different ($p<0.05$) from zero.

**Significantly different ($p<0.01$) from zero.

TABLE 5. Correlation coefficients between values obtained on Group 2 bulls.[a]

Variable[bc]	15 months old					4 years old			
	$SD\alpha_t$	$CV\alpha_t$	$COMP\alpha_t$	% Abnormal	Nonreturn rate	$\bar{X}\alpha_t$	$SD\alpha_t$	$CV\alpha_t$	$COMP\alpha_t$
1) $\bar{X}\alpha_t$	0.83**	0.64**	0.89**	0.56**	−0.41*	0.25	0.38	0.49*	0.42*
2) $SD\alpha_t$		0.96**	0.89**	0.66**	−0.65**	0.27	0.41*	0.52*	0.45*
3) $CV\alpha_t$			0.77**	0.61**	−0.68**	0.27	0.38	0.46*	0.40*
4) $COMP\alpha_t$				0.70**	−0.53*	0.17	0.33	0.45*	0.40*
5) % Abnormal[d]					−0.48*	0.32	0.47*	0.58**	0.47*
6) Nonreturn rate[e]						−0.29	−0.51*	−0.50*	−0.50*
7) $\bar{X}\alpha_t$-4 yr							0.96**	0.89**	0.89**
8) $SD\alpha_t$-4 yr								0.98**	0.95**
9) $CV\alpha_t$-4 yr									0.93**

[a] n = 18.

[b] Variables 1–6 measured on 15-month-old bulls, variables 7–10 on 4-year-old bulls.

[c] Alpha-t variables obtained by the sperm chromatin structure assay. $\bar{X}\alpha_t$, $SD\alpha_t$, $CV\alpha_t$, and $COMP\alpha_t$ refer to the mean, standard deviation, coefficient of variation, and cells outside the main peak, respectively, of the α_t distribution.

[d] Proportion of morphologically abnormal sperm heads (ABN), scored by light microscopy.

[e] Nonreturn rate at 59 days, based on 350 1st services.

*Significantly different ($p<0.05$) from zero.

**Significantly different ($p<0.01$) from zero.

Generally, correlations between α_t values and % abnormal were positive but not strong (Table 4), although for both $X\alpha_t$ and $COMP\alpha_t$ (SCSA/acid on fixed nuclei) with % abnormal, the r values were significant (0.32, $p<0.05$). Percentage abnormal was negatively correlated (−0.37, $p<0.05$) with fertility rating.

Correlations between variables measured on G2 sires are listed in Table 5. Alpha-t values (SCSA/acid) and % abnormal of samples collected from 15-month-old sires were all negatively correlated with nonreturn rate of the young sires; the strongest correlation was −0.68 ($p<0.01$) between $CV\alpha_t$ and nonreturn rate. Positive correlations ($p<0.01$) were seen between α_t values and ABN.

The interclass correlation between α_t on samples collected at 15 months and again at 4 years of age gives an estimate of the repeatability of the measure-

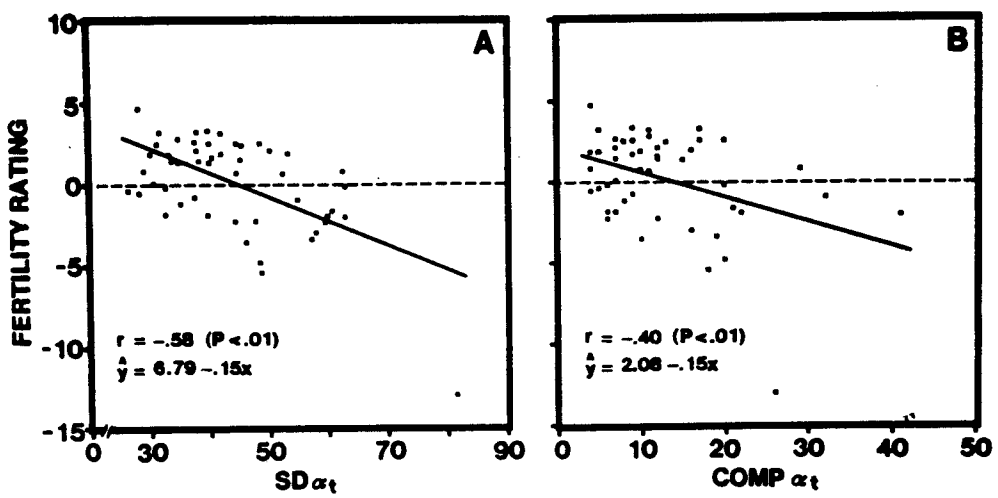

FIG. 2. Relationships between fertility rating and *A*) the standard deviation of alpha-t ($SD\alpha_t$) and *B*) cells outside the main peak of alpha-t ($COMP\alpha_t$) for G1 bulls. Alpha-t values, from the sperm chromatin structure assay, were based on the average of all available collections per bull; n = 49 bulls.

ment. The $SD\alpha_t$, $CV\alpha_t$, and $COMP\alpha_t$ values were all repeatable ($p<0.05$), with correlations of 0.41, 0.46, and 0.40, respectively, between the two ages.

DISCUSSION

Considerable variation in α_t values was observed in G1 and G2 bulls, as indicated in Figures 1 and 2. Values for $COMP\alpha_t$ in both groups ranged from less than 5% to about 40%. Similar variation has been found in other studies on bulls (Evenson et al., 1980; Ballachey et al., 1986b) and rams (Ballachey, unpublished results) and $COMP\alpha_t$ values for human samples have approached 100% (Evenson et al., 1984b).

If the SCSA is to be useful for evaluation of semen quality and potentially for prediction of sire fertility, replicate FCM measurements on straws from a single collection must be repeatable. In this study, measurements (SCSA/acid) on G1 bulls were made over a period of eight months, with all collections of a bull being measured on the same day. Generally, two bulls were evaluated per day. During this time, minor modifications were implemented in the SCSA/acid technique, and these, combined with the normal day-to-day variation associated with the flow cytometer, would be expected to contribute to decreased repeatability estimates for duplicate straws measured on different days. As seen in Table 2, the within-collection repeatabilities of $X\alpha_t$ were much higher when measurements were made on the same day than when measurements were made on different days. However, for $COMP\alpha_t$ in particular, and also for $SD\alpha_t$ and $CV\alpha_t$ repeatabilities were very high, regardless of whether the two straws were measured on the same day or different days. Thus, it appears that these α_t values may be more reliable than $X\alpha_t$ when comparing samples that are not all measured at the same time. The extremely high repeatability (0.98) of $COMP\alpha_t$ across days demonstrates that the response of cells to the staining conditions is uniform, and that a single straw is representative of a collection. It is felt that a large part of the variation in the other α_t variables results from minor fluctuations in instrument settings and sample handling, and should not be attributed to inherent differences in the samples themselves. In consideration of experience gained over the last two years in the methodology, it is possible that across-day repeatabilities of $X\alpha_t$, $SD\alpha_t$ and $CV\alpha_t$ would improve in future measurements.

The high repeatabilities (≥ 0.70), based on either 2 or 4 collections per bull, indicate that variation between bulls is greater than within bulls. Generally, the four samples collected from a bull were quite similar (within a 10% range) in $COMP\alpha_t$ values. In a few cases, however, marked differences were noted in α_t values of the four samples, and two sires had $COMP\alpha_t$ values varying by approximately 25% between the two collections made within a one-month period. No association was seen between sire fertility and variation of the α_t values in the four collections.

Factors causing variation in α_t values between ejaculates are not well understood. Although all bulls were apparently in good health and on a regular collection schedule, subclinical disease and stress factors could affect results of the SCSA. Other sperm samples measured in this laboratory (data not presented) have indicated that illness of the bull can be associated with higher α_t values of sperm. Differences in handling and processing of samples following collection do not appear to have a major effect on α_t values.

The $X\alpha_t$, $SD\alpha_t$, $CV\alpha_t$, and $COMP\alpha_t$ are all obtained on the same sample and are not independent, as indicated by positive correlations between them (Tables 3 and 4). However, in some cases, it appears that each may provide unique information, and so all four values are included. For example, $COMP\alpha_t$ describes the proportion of cells with defective chromatin, but provides no information as to the extent of abnormality of these sperm. At present, it is not known if the mean α_t value of the $COMP\alpha_t$ subpopulation is related to semen quality. However, $SD\alpha_t$, which is influenced by both the proportion of sperm with increased denaturation (i.e., $COMP\alpha_t$) and the degree of denaturation of these cells, appears to have a stronger correlation with fertility ratings than does $COMP\alpha_t$.

The fertility rating (−13.05) of one bull was considerably lower than values of other G1 bulls, and inspection of the graphs in Figure 2 shows that the point for this bull falls apart from the points for the other bulls. Since it was felt that the fertility rating and α_t measurements accurately represented the actual values (i.e., there was not an error in measurement or recording of the data), this point was not considered to be a true outlier and it was included in the statistical analyses. However, correlations were also computed with values for this bull deleted from the data set, and although the resulting r values were decreased, they were still significant: −0.41 ($p<0.01$)

between fertility rating and $SD\alpha_t$, and -0.32 ($p<0.02$) between fertility rating and $COMP\alpha_t$.

In consideration of several factors, the significant correlations of α_t values with fertility ratings were especially encouraging for the potential application of the SCSA as a measure of bull fertility. First, correlations of a measure of semen quality with fertility may be affected by the range of fertility present in the data set. Linford et al. (1976) estimated correlations of semen quality tests with nonreturn rate in two groups of sires, one with a limited range and the other with a wide range in nonreturn rates. Correlations between semen evaluation tests and nonreturn rates were largely nonsignificant for the first group and highly significant for the second group. In the present study, G1 sires were generally of acceptable fertility for use in artificial insemination, with an approximate range in nonreturn rates (of which fertility ratings were a function) from 60% to 75%, relatively narrow in terms of detecting semen quality differences.

Second, accurate fertility data in any species are difficult to obtain. Although estimations of fertility ratings were based on 2×10^6 total services (Everett and Bean, 1986), and therefore should be very good estimates of relative fertility of bulls, a standard error was associated with each fertility rating. The magnitude of this error depended upon the number of inseminations per bull. To increase accuracy, only bulls with standard errors less than 1 were included in G1. Nevertheless, standard errors varied considerably (from 0.16 to 0.99), a fact that may have contributed to decreased correlation coefficients in the present results.

Third, as discussed by Elliot (1978), there may be a threshold level for a semen quality trait above which no corresponding increase in fertility will result, even with improvement of that trait. Possibly, correlations with fertility would be obtained only if sperm concentrations were below a certain "critical" number. Since the fertility ratings in the present study did not result from a fertility trial using critical numbers of sperm, but rather from routine inseminations, sperm concentrations could well be above a theoretical critical level, a fact that complicates detection of correlations with fertility.

Finally, estimates of fertility ratings of G1 were based on inseminations representing a large number of collections per bull, in some cases obtained over a period of many years. In contrast, α_t values were based on only a few collections (Table 3) or a single collection (Table 4) per bull. Continuing studies are now underway to determine correlations of α_t values with nonreturn rates from fertility trials where inseminations were done using critical numbers of sperm, and only a single collection per bull was used to evaluate fertility. Since samples from these fertility trials have already been measured by routine methods of semen evaluation, this will also provide the opportunity to compare the SCSA to these other methods.

The nonsignificant correlations of $X\alpha_t$, SCSA/acid on whole sperm, with any other variables (Table 4) may in part be attributed to the higher across-day variation seen for $X\alpha_t$. In contrast, the SCSA/acid measurements on fixed nuclei were made on three days, under more uniform conditions, and in this case, $X\alpha_t$ was correlated not only with $SD\alpha_t$ and $COMP\alpha_t$ (SCSA/acid, fixed nuclei), but also with fertility ratings.

Based on the positive correlations between $SD\alpha_t$ (heated aliquot, SCSA/thermal) and either $SD\alpha_t$ or $COMP\alpha_t$ (SCSA/acid whole sperm), and the negative correlation between $SD\alpha_t$ (heated aliquot, SCSA/thermal) and fertility ratings (Table 4), it appears that both techniques are detecting the same factors contributing to heterogeneity of chromatin structure. Positive associations have also been noted between α_t values of mouse sperm measured by the SCSA/acid and the SCSA/thermal (Evenson et al., 1985). Because the SCSA/acid is far more rapid and convenient, it is now being used almost exclusively in continuing studies.

The results obtained on G2 sires generally corroborate those obtained on G1. All samples were run on a single day, thus eliminating across-day instrument variation in the measurements. As suggested above, this may have influenced the positive correlations seen for $X\alpha_t$ with the other α_t values. The negative correlations between all α_t values and nonreturn rates agreed well with those obtained for G1 bulls. For example, the correlation between $SD\alpha_t$ and nonreturn rate was -0.65 ($p<0.01$) in G2, and -0.58 ($p<0.01$) between $SD\alpha_t$ and fertility rating in G1 bulls. Repeatabilities of α_t values tended to be lower than those obtained for G1 sires, and may have been influenced by several factors. Age differences between the two collections could be more important in G2, relative to the mature G1 sires. The average time interval for collection of the four samples from G1 sires was shorter than the time interval (approximately three years for all bulls) in G2. At time of collection, G2 sires were not all on a regular collection schedule, a fact that may have affected semen quality. Finally,

across-day variation in measurements may have inflated between sire variation in G1, but was not present in estimates of repeatabilities for G2. Nevertheless, the repeatabilities of $CV\alpha_t$ and $COMP\alpha_t$ are significant, and indicate that the SCSA may be useful for predicting future semen quality, and thus could be a criterion for culling young bulls.

In the present work, no attempt was made to characterize types of morphological abnormalities, but it seems likely that certain abnormalities would be associated with alteration of chromatin structure and increased α_t values, whereas others may show no relationship. Furthermore, as with other measures of semen quality, the strength of the correlations will be dependent upon the variation present in the semen samples being evaluated. The correlations between α_t values and % abnormal for G1 were not as strong as those obtained from G2 sires. In G1, the average % abnormal value was low (approximately 7%), but in the 15-month-old bulls of G2, the average was higher (approximately 14%), and there were two samples with high (45% and 57%) % abnormal, which increased the mean and variance. Both these samples had high α_t values as well, and thus strongly influenced the positive correlations seen between % abnormal and α_t values in G2. Previous work on beef bulls (Ballachey et al., 1986b) has also shown positive correlations between abnormal morphology and $CV\alpha_t$ of sperm collected from the vas deferens.

In mice, induction of sperm head abnormalities by chemical treatment is associated with increased α_t values (Evenson et al., 1985, 1986). In contrast, inbred mice with naturally occurring high proportions of sperm morphological abnormalities may nevertheless have normal chromatin structure, as measured by the SCSA/acid (Ballachey et al., 1986a). These inbred mice are known to be highly fertile. To date, no fertility data have been obtained on the chemically treated mice.

Variation in fertility observed in bulls cannot be adequately explained by conventional methods of sperm quality evaluation, which has led to speculation that nonmorphological defects in the sperm genome may be implicated in reduced fertility (Bishop, 1964; Gledhill, 1970; Salisbury et al., 1977). In studies on humans, Bedford et al. (1973) and Evenson et al. (1980b) detected large variation in nuclear decondensation of morphologically normal sperm. Gledhill (1970) suggested that chromatin defects associated with infertility may actually be abnormalities in structure or binding of the nuclear proteins rather than defects in the DNA itself. Evenson et al. (1985) discussed structural alterations of the chromatin that could potentially result in higher α_t values of sperm measured by the SCSA. Although it is likely that the increased α_t values are due to abnormalities of DNA-protein interaction, leading to a decreased resistance of the DNA to denaturation in situ, the specific structural factors involved, and the contribution of these factors to reduced fertilizing ability of sperm and/or early embryonic death, have not been elucidated. Regardless of the structural nature of the defects, it is evident that bovine semen samples vary widely in stability of sperm nuclear chromatin, and that this variation is associated with differences in fertility.

In conclusion, the SCSA measures structural stability in sperm nuclear chromatin and provides a unique measure of sperm cell quality, which in many cases may be independent of methods currently used for semen evaluation. The present results suggest that the SCSA may be valuable both for identification of bulls of suboptimal fertility, and as a routine analysis for evaluation of semen from bulls in regular production.

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REFERENCES

Ballachey BE, Hohenboken WD, Evenson DP, 1986a. Sperm head morphology and nuclear chromatin structure evaluated by flow cytometry in a diallel cross in mice. Can J Genet Cytol 28:954–66

Ballachey BE, Miller HL, Jost LK, Evenson DP, 1986b. Flow cytometry evaluation of testicular and sperm cells obtained from bulls implanted with zeranol. J Anim Sci 63:995–1004

Bedford JM, Bent MJ, Calvin H, 1973. Variations in the structural character and stability of the nuclear chromatin in morphologically normal human spermatozoa. J Reprod Fertil 33:19–29

Bishop MWH, 1964. Paternal contribution to embryonic death. J Reprod Fertil 7:383–96

Darzynkiewicz A, Traganos F, Sharpless T, Melamed MR, 1975. Thermal denaturation of DNA *in situ* as studied by acridine orange staining and automated cytofluorometry. Exp Cell Res 90:411–28

Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR, 1976. Lymphocyte stimulation: a rapid multiparameter analysis. Proc Natl Acad Sci USA 73:2881–84

Elliott FI, 1978. Significance of semen quality. In: Salisbury GW, VanDemark NL, Lodge JR (eds.), Physiology of Reproduction and Artificial Insemination of Cattle. San Francisco: W.H. Freeman and Co., pp. 429–41

Evenson DP, 1986. Flow cytometry of acridine orange stained sperm is a rapid and practical method for monitoring occupational exposure to genotoxicants. In Sorsa M, Norpa H (eds.), Monitoring of Occupational Genotoxicants. New York: Alan R. Liss Inc., pp. 121–32

Evenson DP, Arlin Z, Welt S, Claps ML, Melamed MR, 1984a. Male

reproductive capacity may recover following drug treatment with the L-10 protocol for acute lymphocytic leukemia (ALL). Cancer 53:30–36

Evenson DP, Baer RK, Jost LK, Gesch RW, 1986. Toxicity of thiotepa on mouse spermatogenesis as determined by dual parameter flow cytometry. Toxicol Appl Pharmacol 82:151–63

Evenson DP, Darzynkiewicz Z, Melamed MR, 1980a. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 240:1131–33

Evenson DP, Darzynkiewicz Z, Melamed MR, 1980b. Comparison of human and mouse sperm chromatin structure by flow cytometry. Chromosoma (Berl) 78:225–38

Evenson DP, Darzynkiewicz Z, Melamed MR, 1982. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J Histochem Cytochem 30:279–80

Evenson DP, Higgins PH, Grueneberg D, Ballachey BE, 1985. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry 6:238–53

Evenson DP, Klein FA, Whitmore WF, Melamed MR, 1984b. Flow cytometric evaluation of sperm from patients with testicular carcinoma. J Urol 132:1220–25

Evenson DP, Melamed MR, 1983. Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J Histochem Cytochem 31:248–53

Everett RW, Bean B, 1986. Semen fertility–an evaluation system for artificial insemination sires, technicians, herds, and systematic fixed effects. J Dairy Sci 69:1630–41

Garner DL, Gledhill BL, Pinkel D, Lake S, Stephenson D, Van Dilla MA, Johnson LA, 1983. Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry. Biol Reprod 28:312–21

Garner DL, Pinkel D, Johnson LA, Pace MM, 1986. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol Reprod 34:127–38

Gledhill BL, 1970. Enigma of spermatozoal deoxyribonucleic acid and male infertility: a review. Am J Vet Res 31:539–45

Gledhill BL, Lake S, Dean PN, 1979. Flow cytometry and sorting of sperm and other male germ cells. In: Melamed MR, Mullaney PF, Mendelsohn ML (eds.), Flow Cytometry and Sorting. New York: John Wiley and Sons, pp. 471–84

Kempthorne O, 1957. An Introduction to Genetic Statistics. New York: John Wiley and Sons, Inc.

Linford E, Glover FA, Bishop C, Stewart DL, 1976. The relationship between semen evaluation methods and fertility in the bull. J Reprod Fertil 47:283–91

Matyus L, Szabo G Jr, Resli I, Gaspar R Jr, Damjanovich S, 1984. Flow cytometric analysis of viability of bull sperm cells. Acta Biochim Biophys Acad Sci Hung 19:209–14

Monesi V, 1971. Chromosome activities during meiosis and spermiogenesis. J Reprod Fertil 13:1–14(Suppl.)

Pinkel D, Garner DL, Gledhill BL, Lake S, Stephenson D, Johnson LA, 1985. Flow cytometric determination of the proportion of X- and Y-chromosome-bearing sperm in samples of purportedly separated bull sperm. J Anim Sci 60:1303–07

Saacke RG, 1983. Semen quality in relation to semen preservation. J Dairy Sci 66:2635–44

Saacke RG, 1984. Semen quality, importance of and influencing factors. Proceedings of the Tenth Technical Conference on Artificial Insemination and Reproduction. National Association of Animal Breeders, Milwaukee, WI, April, 1984, pp. 30–36

Salisbury GW, Hart RG, Lodge JR, 1977. The spermatozoan genome and fertility. Am J Obstet Gynecol 128:342–50

SAS, 1984. SAS User's Guide. Cary, NC: SAS Institute Inc.