Reprinted from JOURNAL OF ANDROLOGY, March-April 1988
Vol. 9, No. 2

The Sperm Chromatin Structure Assay
Relationship with Alternate Tests of Semen Quality and Heterospermic Performance of Bulls

BRENDA E. BALLACHEY,* DONALD P. EVENSON,* AND RICHARD G. SAACKE†

Data obtained by the sperm chromatin structure assay (SCSA) on spermatozoa from nine bulls were correlated with fertility, measured by heterospermic performance (-0.94, $P < 0.01$) and by alternate tests of sperm quality, including motility, acrosome integrity, Sephadex filtration and morphology of spermatozoa (all significant at $P < 0.05$ to $P < 0.01$). The SCSA uses flow cytometry to determine the susceptibility of nuclear DNA to low pH-induced denaturation *in situ* as measured by the ratio of acridine orange binding to double- or single-stranded DNA. The error associated with multiple SCSA measurements was relatively low. The primary finding is that the assay of chromatin structure stability performed on killed spermatozoa was as highly correlated with the heterospermic performance of semen as the best of the classical tests for semen quality. The SCSA may therefore be a highly useful technique for evaluation of sperm quality.

Key words: Semen quality, fertility, chromatin structure, bulls, heterospermic insemination, flow cytometry, acridine orange, spermatozoa.

J Androl 1988; 9:109–115.

From the Department of Chemistry, South Dakota State University, Brookings, South Dakota and the Department of Dairy Science, Virginia Polytechnic Institute and State University Blacksburg, Virginia†*

The sperm chromatin structure assay (SCSA; Evenson et al, 1980, 1985; Evenson, 1986a; Ballachey et al, 1987) is a technique that uses acridine orange staining and flow cytometry to evaluate the structural stability of sperm nuclear chromatin. Using extended, frozen bovine semen samples, Ballachey et al (1987) obtained significant correlations (-0.41 to -0.68, $P < 0.05$ to $P < 0.01$) between SCSA values, measured on one or a few collections per bull, and sire fertility ratings, based on non-return rates over a period of several years.

Numerous other tests for laboratory evaluation of semen quality have been developed (Elliott, 1978; Graham, 1978; Saacke, 1982, 1984). The measures most widely used in the artificial insemination industry currently include percentages of motile and morphologically normal spermatozoa, although a consistent relationship between the results of these methods and male fertility has not been clearly demonstrated. Determination of the relationship between these

Supported in part by USDA Grant No. 86-CRCR-1-2170, NIH Grant No. R01ES03035 and EPA Grant No. R812363. Publication No. 2236 of the South Dakota Agricultural Experiment Station.

Reprint requests: D. P. Evenson, Ph.D., Department of Chemistry, ASC 136, South Dakota State University, Brookings, South Dakota 57007.

Submitted for publication March 17, 1987; revised version received June 18, 1987; accepted for publication July 7, 1987.

tests of sperm quality and fertility has often been complicated by inaccurate estimation of male fertility, since many factors other than semen quality influence pregnancy rates (Oltenacu and Foote, 1976; Pace, 1980). Furthermore, laboratory tests are often subject to a higher degree of measurement error than desirable, particularly when replicate measurements are made by different technicians and/or in different laboratories. Problems associated with laboratory assays were reviewed by Graham et al (1980).

Heterospermic insemination refers to insemination of the female(s) with a mixture of spermatozoa from two or more males. In heterospermic studies it has generally been observed that competition of the spermatozoa exists among various males, since one male will sire a greater proportion of offspring than expected from the ratio of sperm cells in the mixture (Edwards, 1955; Beatty, 1960; Beatty et al, 1969; Saacke et al, 1980). Based on the proportions of offspring resulting from heterospermic matings containing known numbers of spermatozoa from competing males, estimates of relative male fertility potential can be derived. In several species, the relative fertility has been shown to have the same relationship under homospermic and heterospermic conditions (Napier, 1961; Beatty et al, 1969; Martin and Dzuik, 1977). However, the efficiency of fertility measurement can be greatly improved using heterospermic insemination.

Saacke et al (1980) conducted an experiment to evaluate the relationships between heterospermic performance of bulls and semen quality, measured in a series of laboratory tests, using spermatozoa from the same extended, frozen ejaculates throughout their study. They concluded that the fertilizing ability of the semen samples, measured by heterospermic insemination, was highly related to certain laboratory tests of sperm cell quality.

As frozen semen samples from that experiment were still available, the present study was initiated to compare results obtained by the SCSA with other quality tests, and to determine the relationship between the SCSA and sire fertility, based on heterospermic performance.

Materials and Methods

As previously described (Saacke et al, 1980), semen samples were obtained from nine beef bulls, extended and frozen in 0.5-cc polyvinylchloride straws by routine procedures. A sufficient number of straws were processed to ensure an adequate supply of semen for insemination of females by heterospermic matings, ie, matings in which equal numbers of spermatozoa from two bulls were mixed prior to insemination. The parentage of calves resulting from these matings was determined and, based on the number of calves sired, a competitive index was derived for each bull. Additional samples were subsequently analyzed by a series of laboratory semen quality tests, as outlined by Saacke et al (1980). These tests were : 1) motility estimate (a subjective measurement); 2) motility, measured by the photomotility technique (Elliott et al, 1973); 3) percentage intact acrosomes, based on the presence of the apical ridge (Saacke and Marshall, 1968); 4) percentage filterable cells, using Sephadex filtration (Graham et al, 1978); 5) percentage morphologically normal spermatozoa; 6) head-to-head serum-induced agglutination of spermatozoa (Senger and Saacke, 1976); 7) computerized measurement of sperm motility, and 8) computerized measurement of sperm velocity. Results from the first five of these tests have been included in this paper for comparison with the SCSA. Two aliquots of six to 10 pooled straws were measured, and values for motility estimate, photomotility, acrosome integrity and filterable cells were obtained immediately after the samples were thawed.

For the present study, samples were analyzed by the sperm chromatin structure assay (SCSA). Straws were removed from liquid nitrogen, placed on crushed ice for 5 to 7 minutes, and then the semen was extruded into a test tube. Thawing by this method compared with thawing in a 35 C waterbath does not affect the results obtained in the SCSA (unpublished data). For each measurement, a 5-μl aliquot of the sample was diluted with 195 μl of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl and 1 mM disodium EDTA, pH 7.4) to give a concentration of approximately 1 to 2×10^6 spermatozoa per ml. The diluted sample (200 μl) was mixed with 400 μl of 0.1% (v/v) Triton X-100, 0.08 N HCl and 0.15 M NaCl (pH 1.2). This low pH, nonionic detergent solution improves dye uptake, and potentially induces partial denaturation of the DNA in chromatin. After 30 seconds, 1.2 ml of acridine orange (chromatographically purified, Polysciences, Warrington, PA) 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 μg acridine orange per ml) was added. Samples were placed in the flow cytometer, and 2 minutes later, red ($F >_{600}$ nm) and green -(F_{530} nm) fluorescence measurements were taken at a rate of about 150 cells per second for a total of 5000 cells per sample. For each bull, three aliquots from a single straw were stained and measured.

The differential fluorescence (green and red) results from the metachromatic properties of acridine orange. When intercalated into double-stranded DNA, acridine orange fluoresces green (Lerman, 1963), whereas when associated with single-stranded DNA, it fluoresces red (Bradley and Wolf, 1959; Darzynkiewicz, 1979). Levels of RNA in the sperm nucleus are negligible (Monesi, 1971) and do not contribute to the measured fluorescence. Other studies (Evenson et al, 1985; 1986; Ballachey et al, 1987) have shown no significant differences in ratios of green to red fluorescence between whole spermatozoa and isolated nuclei measured by the SCSA. Elevated levels of red fluorescence are not reduced by RNAse treatment (Evenson et al, 1985). Thus, levels of green and red fluo-

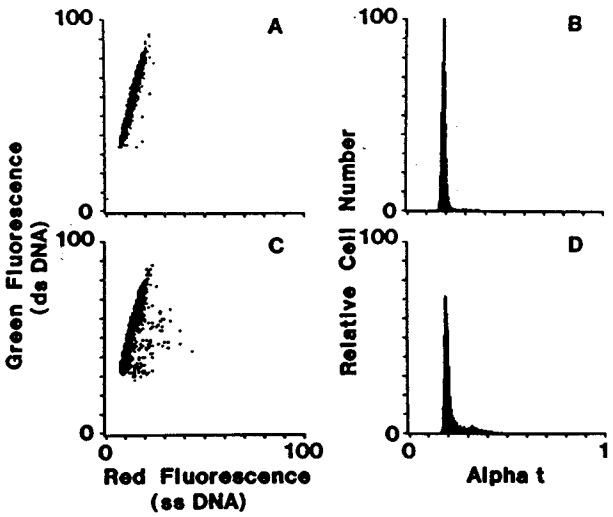

Fig. 1. Examples of data generated by the sperm chromatin structure assay. Fig. 1A and C are cytograms of green [y axis; double-stranded DNA] vs. red [x axis; single-stranded DNA] fluorescence; Fig. 1B and D are the corresponding α_t frequency histograms. The $\overline{X}$ and SD of the α_t distribution describe the overall extent of denaturation, ie, shift from green fluorescence (double-stranded DNA) to red fluorescence (single-stranded DNA). Most of the cells fall within a cluster; those cells outside that cluster are termed "cells outside the main population of α_t" (COMPα_t) and if sufficient in number are visible as a "shoulder" on the right side of the main peak in the α_t distribution.

TABLE 1. Means, Standard Deviations and Percentages of Error in Multiple Samples for Alpha-t Variables from the Sperm Chromatin Structure Assay (SCSA) and Other Semen Tests

Test	Mean ± Standard Deviation*	Error†
SCSA - α t values‡		
$\overline{X}\,\alpha$ t	205.1 ± 10.8	17
SD α t	49.0 ± 8.6	9
COMP α t	9.0 ± 4.3	3
Motility estimate, %	38.3 ± 14.4	34
Photomotility, %	23.3 ± 9.6	18
Acrosome integrity, %	74.3 ± 10.3	13
Sephadex filtration, %	24.8 ± 12.8	10
Normal morphology, %	75.8 ± 13.3	3

*N = 9.

†The error, which was computed using the expected mean squares from an analysis of variance, indicates the percentage of variance associated with replicate measurements, on separate aliquots of each sample (N = 3 for the SCSA variables; N = 2 for the other variables). The remainder of the variance was associated with differences between samples.

‡$\overline{X}\,\alpha$t, SD α t and COMP α t = Mean of α t, standard deviation of α t,a nd cells outside the main population of α t, respectively, based on the α t distributions generated by the SCSA.

rescence correspond to the relative content of double- and single-stranded DNA, respectively.

The flow cytometer used was a Cytofluorograf II (Ortho Diagnostic Systems, Westwood, MA), equipped with a Lexel 100-mW argon ion laser operated at 35-mW output and 488-nm wavelength. The Cytofluorograf was interfaced to an Ortho Diagnostics 2150 data handling system. Using computer protocols, alpha-t, defined as the ratio of red to total (red + green) fluorescence [α_t = red/(red + green); Darzynkiewicz et al, 1975], was computed for each cell and the distribution of α_t values was determined. The theoretical distribution of α_t values is from 0 to 1. However, because measurements are actually made over 1000 channels (levels) of fluorescence, the α_t values presented here are expressed on a scale of 0 to 1000.

The mean ($\overline{X}\alpha_t$) and standard deviation (SDα_t) of the α_t distribution were used to describe the extent of sperm DNA denaturation of a sample. An additional descriptive measurement was the proportion of cells falling outside the main population of α_t (COMPα_t). The COMPα_t values were obtained by visually inspecting the α_t distribution on the computer screen, and enumerating the cells on the upper side of the "main" peak, which has an α_t value of approximately 200. (Refer to Fig. 1 for clarification.)

Results

Examples of the data obtained with the SCSA and presented in Fig. 1A and 1C are cytograms or scattergrams of green (y axis) vs. red (x axis) fluorescence. Alpha-t was computed for each cell, and the corresponding distributions of α_t values are shown in Fig. 1B and 1D. The first sample (Fig. 1A, 1B) was from the bull with the highest rank for heterospermic performance (competitive index = 24.5), and the second (Fig. 1C, 1D) from the bull with the second lowest rank (competitive index = −19.1). Note the broader α_t distribution for the second sample, resulting in increased α_t values: SD α_t was 38.4 vs. 56.4, and COMPα_t was 5% vs. 19%, for the first and second samples, respectively.

Means of the α_t values from the SCSA, and of five other semen quality tests (motility estimate, photomotility, acrosome integrity, filterable cells and normal morphology) are presented in Table 1. Also listed are the errors for each of the tests, that is, the percentages of the total variance that were associated with repeat measurements made on a single sample. The errors associated with measurement of SDα_t, COMPα_t and normal morphology were relatively low, at 9, 3 and 3%, respectively. For the other tests, errors ranged from 10% (filterable cells) to 34% (motility estimate).

Correlations among the various measures of semen quality and competitive indices of bulls are listed in Table 2. Significant correlations, ranging from −0.68 to −0.93 ($P < 0.05$ to $P < 0.01$), were obtained for

TABLE 2. Correlations among Alpha-t Variables from the Sperm Chromatin Structure Assay (SCSA), Other Semen Quality Tests, and Competitive Index*

	SD αt	COMP αt	MOTest	MOTphot	ACR	FILT	NORM	CI‡
$\bar{X}$ αt†	0.80	0.63	−0.56	−0.78	−0.60	−0.68	−0.25	−0.81
SD αt†		0.75	−0.76	−0.93	−0.84	−0.92	−0.62	−0.94
COMP αt†			−0.68	−0.68	−0.87	−0.87	−0.60	−0.74
Motility estimate, % (MOTest)				0.83	0.89	0.66	0.84	0.89
Photomotility, % (MOTphot)					0.76	0.76	0.64	0.93
Acrosome integrity, % (ACR)						0.87	0.81	0.90
Sephadex filtration, % (FILT)							0.63	0.82
Normal morphology, % (NORM)								0.72

*Correlation coefficients greater than 0.79 are significant at $P < 0.01$; greater than 0.65 are significant at $P < 0.05$; greater than 0.57 are marginally significant at $P < 0.10$. N = nine bulls.

†$\bar{X}$ αt, SD αt and COMP αt = Mean of αt, standard deviation of αt, and cells outside the main population of αt, respectively, based on the αt distributions generated by the SCSA.

‡CI = Competitive index, based on heterospermic performance.

either SDα_t or COMPα_t with motility estimate, photomotility, acrosome integrity and filterable cells. Higher values of α_t indicate a greater extent of DNA denaturation and lower sample quality, but for the other semen quality tests, higher values correspond to increased quality, and thus these correlations were negative in sign. Correlations of SDα_t or COMPα_t with normal morphology were not as large, and did not quite reach significance ($P < 0.10$). Positive correlations (0.63 to 0.89) were obtained among motility estimate, photomotility, acrosome integrity, filterable cells and normal morphology.

All measures of semen quality correlated ($P < 0.05$ to $P < 0.01$) with the competitive index. Bulls with the best heterospermic performance had lower α_t values. Correlations of $\bar{X}\alpha_t$, SDα_t and COMPα_t with competitive index were −0.81, −0.94 ($P < 0.01$) and −0.74 ($P < 0.05$), respectively. The multiple correlation (R^2) of the competitive index with motility estimate and SDα_t was 0.96 ($P < 0.01$), and the partial correlation between SDα_t and competitive index, holding motility estimate constant, was −0.90. The relationships between SDα_t and COMPα_t with the competitive index are graphed in Fig. 2 to illustrate the distribution of α_t values over the range of fertility observed in this study.

Discussion

Based on the variation observed in semen quality traits, this group of bulls was representative of beef bulls used in commercial artificial insemination. Previous use of the SCSA on frozen bull semen has been limited largely to samples from Holstein artificial insemination sires, and the α_t values for these beef bulls generally correspond to those obtained with the Holsteins (Ballachey et al, 1987). However, the range of cells outside the main population of α_t (COMPα_t) was somewhat lower in the present study, at 5 to 19%, whereas in the previous work it was 3 to 40%. Low variation in a semen quality trait might be expected to make the detection of a relationship between that trait and sire fertility more difficult, but

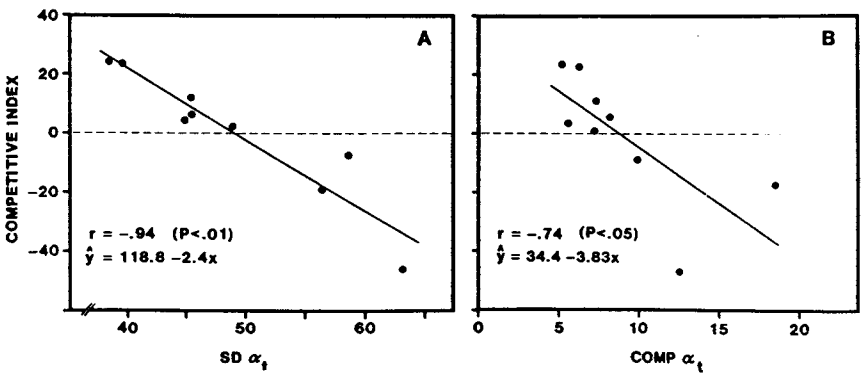

Fig. 2. Relationship of Competitive Index with A) Standard deviation of α_t (SDα_t), and B) Cells outside the main population of α_t (COMPα_t).

the range of COMPα_t in the present study was evidently sufficient to enable detection of quality differences associated with heterospermic performance.

The relatively low error rate associated with SDα_t and COMPα_t (9% and 3%, respectively) are encouraging if the SCSA is to be useful in routine quality analysis. Prior estimates of within-sample variance have also been low, particularly for COMPα_t (Ballachey et al, 1987). One factor contributing to a small rate of error is the large number (N = 5000) of cells counted for each measurement.

At present, we know of no other laboratories utilizing the SCSA on spermatozoa, and the degree of error that would be obtained if repeat measurements were made in different laboratories has not been ascertained. However, it is anticipated that COMPα_t would remain highly repeatable since, relative to SDα_t and $\bar{X}\alpha_t$, it is far less subject to daily variation in instrument settings (Ballachey et al, 1987). In this laboratory, flourescent beads are currently used to standardize the fluorescence gains each day, providing increased accuracy in comparison of samples measured at different times; these beads could also be used for standardization of measurements made in other laboratories.

Correlations among various measures of semen quality were relatively high. Because, for example, a 95% confidence interval on r = 0.80 (with N = 9) extends from 0.29 to 0.96, it is not very meaningful to discuss differences among the r values. It is apparent that variation in sperm cell quality is detected by all of the tests, and to some extent they are providing similar information. Particularly noteworthy was the fact that SCSA, a very different kind of test, provided information equivalent to the best classical tests of semen quality regarding prediction of heterospermic performance. SCSA is an evaluation of chromatin stability performed on killed spermatozoa, while the other tests in this study (with the exception of morphology) were measures of viability based upon the motility or membrane characteristics of live specimens. Other tests included in the original study by Saacke et al (1980) were: percentage head-to-head agglutination and computerized measurement of both motility and velocity. Generally, correlations of α_t values with these tests were also significant (data not presented) and agreed with the results presented in Table 2.

Correlations of SDα_t and COMPα_t with normal morphology did not quite reach significance ($P < 0.10$), as also noted in an earlier study (Ballachey et al, 1987). Immature spermatozoa containing a cytoplasmic droplet were by far the most commonly occurring abnormal form. Greater variation in the specific types of abnormalities present might have resulted in altered correlation coefficients, particularly if abnormalities of the sperm head had been more prevalent.

The absolute value of correlations of the various semen quality tests with the competitive index ranged from 0.72 to 0.94. However, these differences are not sufficient to permit ranking of these tests for prediction of fertility. Furthermore, although heterospermic performance appears to provide a highly useful means of ranking bulls for fertility, the relative index values could vary to some extent if an increased number of progeny were measured.

The value of the SCSA relative to or in combination with other methods of quality evaluation raises an important question. To clarify this relationship, the multiple regression of motility estimate and SDα_t on the competitive index was computed. A partial correlation of −0.90 was obtained between the competitive index and SDα_t after adjusting for motility estimate, indicating that when SDα_t was added, the residual error in the model relative to using only motility estimate for prediction of the competitive index was reduced by 81%. Although the R^2 value (0.96) for the full model is extremely high, the R^2 values for either motility estimate (0.79) or SDα_t (0.89) alone with competitive index were also very high. Because of the large simple correlations obtained in this data set and the small number of samples (N = 9), it was felt that extensive interpretation of the results with respect to ranking the individual tests of semen quality or further multiple regression analyses would not be meaningful. The simple correlations obtained confirm previous results (Ballachey et al, 1987), and do indicate a potentially useful role for the SCSA in semen quality analyses. Additional studies on larger data sets would help to determine which tests or combinations of tests will be of greatest use in quality and fertility evaluation.

In the previous study (Ballachey et al, 1987) on Holsteins involving two separate data sets, the magnitude of the correlations between SDα_t and fertility (measured either as a "fertility rating" or by nonreturn rate) were greater than those obtained between COMPα_t and fertility. For mature Holstein sires (N = 49), the correlation of fertility rating with SDα_t was −0.58 ($P < 0.01$), whereas with COMPα_t, it was −0.40 ($P < 0.01$). In that data set, one or a few semen collections were evaluated per bull, whereas the fertility rating was based on lifetime mating records. In

the present study, however, there was a 1:1 correspondence between the samples evaluated in the laboratory and those used to determine heterospermic performance. In addition, the variance in fertility detected among bulls was increased by using the competitive fertilization (heterospermic) approach. Both of these factors likely contributed to improved correlations between α_t values and fertility.

The relatively higher correlation between $SD\alpha_t$ and the competitive index compared with that between $COMP\alpha_t$ and competitive index (−0.94 vs. −0.74) agrees with the relative values obtained previously on Holstein sires. However, reasons for the apparent superiority of $SD\alpha_t$ in predicting fertility are not thoroughly understood. $COMP\alpha_t$ describes only the proportion of spermatozoa falling outside the main or "normal" populations, that is, those cells with greater susceptibility to DNA denaturation *in situ*, and it provides no information as to the relative extent of denaturation in these cells. In contrast, $SD\alpha_t$ is influenced by several factors, including the number of cells in $COMP\alpha_t$, the mean of the $COMP\alpha_t$ cells, and also the uniformity of the "normal" population itself. As indicated by the high correlation (0.75) between $SD\alpha_t$ and $COMP\alpha_t$, the two values are closely but not perfectly related. Furthermore, although $COMP\alpha_t$ was highly correlated with photomotility, for example, the actual percentage of spermatozoa in $COMP\alpha_t$ was not the same as the actual percentage of nonmotile cells.

The factors possibly contributing to chromatin structural differences and consequent variation in susceptibility of DNA to denaturation *in situ* have not been identified, but have been discussed by Evenson et al (1985) and Ballachey et al (1987). It is known that, in mice, highly repeatable, dose-response differences in α_t values can be induced by toxic chemical treatment (Evenson et al, 1985, 1986, 1987). Studies of human spermatozoa have shown that fertility problems, chemotherapy and disease are associated with increased α_t values (Evenson, 1986b). Higher α_t values have been observed in spermatozoa from bulls implanted with zeranol, a growth promotor (Ballachey et al, 1986), as well as in spermatozoa collected from bulls during periods of illness (unpublished data). Further work is necessary to determine the relationship of chromatin stability to the post-ejaculatory history of spermatozoa as well as abnormal spermatogenesis in order to determine the biologic association of SCSA with other forms of semen evaluation, particularly measures of sperm viability.

In conclusion, the SCSA utilizes flow cytometry for multiparameter measurement of sperm cells, and provides a unique measurement of sperm chromatin structure and quality. Rapid, objective measurements can be made on a large number of cells per sample, resulting in relatively low error associated with the SCSA measurements. The present results indicate that the SCSA is equivalent to alternate tests of semen quality in its association with heterospermic performance, which is a predictor of sire fertility, and it may be useful, either alone or in combination with other tests, for analysis of semen quality. Of particular interest in this study was the observation that the SCSA, a test of chromatin stability employing killed spermatozoa, gave equivalent predictions of heterospermic performance to the classical tests of sperm viability performed on live cells. Because the SCSA evaluates a characteristic of sperm cells that cannot presently be determined by any other method, it could be of particular significance in identifying defective samples that appear normal with other criteria.

Acknowledgments

The authors thank Select Sires, Inc., Plain City, Ohio for their cooperation in providing semen samples for this study, and R.K. Baer, P. Bennett, and L. Jost for excellent technical and secretarial support.

References

Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol Reprod 1987; 36:915–925.

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

Beatty RA. Fertility of mixed semen from different rabbits. J Reprod Fertil 1960; 1:52–60.

Beatty RA, Bennett GH, Hall JG, Hancock JL, Stewart DL. An experiment with heterospermic insemination in cattle. J Reprod Fertil 1969; 19:491–502.

Bradley DF, Wolf MK. Aggregation of dyes bound to polyanions. Proc Natl Acad Sci USA 1959; 45:944–952.

Darzynkiewicz Z. Acridine orange as a molecular probe in studies of nucleic acids *in situ*. In: Melamed MR, Mullaney PF, Mendelsohn ML, eds. Flow cytometry and sorting. New York: John Wiley & Sons, 1979; 285–316.

Darzynkiewicz Z, Traganos F, Sharpless T, Melamed MR. Thermal denaturation of DNA *in situ* as studied by acridine orange staining and automated cytofluorometry. Exp Cell Res 1975; 90:411–428.

Edwards RG. Selective fertilization following the use of sperm mixtures in the mouse. Nature 1955; 175:215–216.

Elliott FI. Significance of semen quality. In: Salisbury GW, Van Demark NL, Lodge JR, eds. Physiology of reproduction and artificial insemination of cattle; 2nd edition. San Francisco: WH Freeman & Co, 1978; 429–441.

Elliott FI, Shermann JK, Elliott EJ, Sullivan JJ. A photographic method of measuring percentage of progressively motile sperm cells using dark-field microscopy. VIII Intl Symp Zootech, Milan, Italy 1973; 160–166.

Evenson DP. Flow cytometry of acridine orange stained sperm is a rapid and practical method for monitoring occupational exposure to genotoxicants. In: Sorsa M, Norppa H, eds. Monitoring of occupational genotoxicants. New York: Alan R. Liss Inc, 1986a; 121–132.

Evenson DP. Male germ cell analysis by flow cytometry: effects of cancer, chemotherapy and other factors on testicular function and sperm chromatin structure. In: Andreeff MA, ed. Clinical cytometry. NY Acad Sci 1986b; 350–367.

Evenson DP, Baer RK, Jost LK, Gesch RW. Toxicity of thiotepa on mouse spermatogenesis as determined by dual parameter flow cytometry. Toxicol Appl Pharmacol 1986; 82:151–163.

Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 1980; 240:1131–1133.

Evenson DP, Higgins PH, Grueneberg D, Ballachey BE. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry 1985; 6:238–253.

Evanson DP, Janca FC, Jost LK. Effects of the fungicide methylbenzimidazol-2-yl carbamate (MBC) on mouse germ cells as determined by flow cytometry. J Toxicol Environ Health 1987; 20:387–399.

Graham EF. Fundamentals of the preservation of spermatozoa. In: The integrity of frozen spermatozoa. Conf Proc, Natl Acad Sci, Washington, DC 1978; 4–44.

Graham EF, Schmehl MKL, Evensen BK. An overview of column separation of spermatozoa. Proc 7th Tech Conf Artif Insem Reprod, Natl Assoc Anim Breeders, 1978; 69–73.

Graham EF, Schmehl MKL, Nelson DS. Problems with laboratory assays. Proc 8th Tech Conf Artif Insem Reprod, Natl Assoc Anim Breeders, 1980; 59–66.

Lerman LS. The structure of the DNA-acridine complex. Proc Natl Acad Sci USA 1963; 49:94–102.

Martin PA, Dzuik PJ. Assessment of relative fertility of males (cockerels and boars) by competitive mating. J Reprod Fertil 1977; 49:323–329.

Monesi V. Chromosome activities during meiosis and spermiogenesis. J. Reprod Fertil 1971; 13[Suppl]: 1–14.

Napier RAN. Fertility in the male rabbit. III: estimation of spermatozoan quality by mixed insemination, and the inheritance of spermatozoan characters. J Reprod Fertil 1961; 2:273–289.

Oltenacu EAB, Foote RH. Monitoring fertility of artificial insemination programs. Proc 6th Tech Conf Artif Insem Reprod, Natl Assoc Anim Breeders, 1976; 61–68.

Pace MM. Fundamentals of assay of spermatozoa. 9th Intl Cong Anim Reprod Artif Insem (Madrid) 1980; 1:133–146.

Saacke RG. Semen quality in relation to semen preservation. J Dairy Sci 1982; 66:2635–2644.

Saacke RG. Semen quality, importance of and influencing factors, Proc 10th Tech Conf Artif Insem Reprod, Natl Assoc Anim Breeders, 1984; 30–36.

Saacke RG, Marshall CE. Observations on the acrosomal cap of fixed and unfixed bovine spermatozoa. J Reprod Fertil 1968; 16:511–514.

Saacke RG, Marshall CE, Vinson WE, O'Conner ML, Chandler JE, Mullins KJ, Amann RP, Wallace RA, Vincel WN, Kellgren HC. Semen quality and heterospermic insemination in cattle. 9th Intl Cong Anim Reprod Artif Insem (Madrid) 1980; 5:75–78.

Senger PL, Saacke RG. Serum-induced head-to-head agglutination of bovine spermatozoa. J Reprod Fertil 1976; 47:215–219.