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FLOW CYTOMETRIC EVALUATION OF BOAR SEMEN BY THE SPERM CHROMATIN STRUCTURE ASSAY AS RELATED TO CRYOPRESERVATION AND FERTILITY

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ABSTRACT

Boar semen from a heterospermic mating trial and semen cryopreserved by various methods were evaluated by the flow cytometric sperm chromatin structure assay (SCSA), which measures the susceptibility of sperm nuclear DNA to acid-induced denaturation in situ. Spermatozoa were treated with a pH 1.4 buffer and then stained with the metachromatic dye acridine orange. Acridine orange intercalated into double-stranded DNA (native) fluoresces green while single-stranded DNA (denatured) fluoresces red when excited with 488 nm light. The ratio of red to total fluorescence provides an index of normality/abnormality. The SCSA data on neat boar semen or semen in either Kiev-Merck or Pursel-Johnson extender and frozen directly on dry ice blocks or plunged into LN_2 did not differ within individual boars. Therefore, chromatin structure, as measured by the SCSA, was not influenced differently by these 2 methods of semen cryopreservation. When semen from 6 boars was mixed in equal sperm numbers in six 3-way combinations and inseminated into at least 3 Duroc gilts per combination, 4 of the 6 combinations yielded 2 litters, while the remaining 2 combinations yielded 3 litters. The SCSA correctly predicted both the high and low fertility boars based on a ratio of offspring as deviated from the theoretical percentage. Thus, the SCSA was found to be a valuable adjunct method for evaluating boar semen quality.

Key words: sperm chromatin, flow cytometry, acridine orange, boar semen cryopreservation, fertility

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INTRODUCTION

Neither the data nor the analysis of records on boar fertility has been as extensively developed as that for the bovine, especially for the dairy industry. The reasons for this include the lack of good cryopreservation of semen, the need for large numbers of spermatozoa for successful insemination, and the fluctuation in selection goals in contrast to that of the single, long-term major goal of enhanced milk production for dairy cattle (4). The swine industry is currently directing efforts to better the cryopreservation techniques in order to improve swine production.

Methods for accurately evaluating boar fertility would have a positive effect on pork production. Presently, conventional assays are rather unreliable predictors of fertility, largely due to the small number of spermatozoa measured and the subjective evaluation of cells by the technician. For example, classical light microscope assays provide for assessment of fertility including percentages of sperm motility and morphologically normal spermatozoa, and these estimates are subjective. Automated instrumentation interfaced with computer capabilities is needed to provide multiple measurements on a large number of cells. In addition to sperm motion analyzers which are rapidly becoming part of the semen quality assessment, flow cytometry (FCM;29) is emerging as a powerful tool for providing rapid, multiparameter, and objective measurements of sperm characteristics that likely contribute to fertility potential. Large numbers of sperm cells can be measured and analyzed without the bias of selection.

To date, FCM measurements of sperm parameters have included DNA content for sexing sperm (21,22), sperm viability (17), sperm count (16), acrosome integrity (20), mitochondrial function (14,20), and sperm chromatin structure integrity, which is defined as the susceptibility of DNA to acid- or heat-induced denaturation in situ (1,8,9).

Normal sperm development leads to a chromatin structure in which the DNA in situ is fully resistant to denaturation under the conditions described here (6). The DNA of spermatozoa with an abnormal chromatin structure is susceptible to denaturation in situ; the percentage of cells with abnormal chromatin and the extent of the abnormality can be detected by the sperm chromatin structure assay (SCSA). Over the past decade, the SCSA has been used to study the relationship between integrity of sperm chromatin structure and fertility potential (1,2,8) as well as the changes in sperm chromatin structure induced by environmental exposure to chemicals (7,9,11,12), disease (6) and other factors. The SCSA measure is equal to or more sensitive than some other reproductive toxicology measures (6).

A competitive mating or heterospermic insemination study has been suggested as the most accurate and definitive environment in which to evaluate fertility differences between males (3,27). Heterospermic insemination facilitates the use of significantly fewer females, yet affords greater accuracy due to reduction of the female variance component of fertility (18). The relationship between bull fertility potential and semen quality measures was carefully tested with a heterospermic insemination experiment (28). An excellent correlation of -0.94 ($P<0.01$) was found between susceptibility to DNA

denaturation and bull fertility potential, and it is likely that the chromatin structure has an important bearing on fertility and on sustaining pregnancy.

The above studies indicate the potential value of the SCSA for the livestock industry. The successful development of the SCSA protocol for analysis of bull spermatozoa as related to fertility can be used and/or adapted for evaluation of swine spermatozoa. Our purpose was to demonstrate the value of the SCSA in evaluating 1) fresh and frozen boar spermatozoa, 2) boar semen extended by several different methods, and 3) the semen used in a boar heterospermic trial.

METHODS AND MATERIALS

Fresh Versus Frozen Boar Semen

Fresh semen was collected from 3-yr-old boars (n=3) at the International Boar Semen Center, Eldora, Iowa. Half the semen from each boar was left as neat semen and half was extended in Kiev-Merck liquid semen extender[a] and shipped to South Dakota State University, by overnight delivery, in a styrofoam box containing several Freez Paks[b] precooled to 4°C. Extended and neat semen samples were measured by the SCSA as fresh samples the day after collection and later as frozen/thawed semen. Two methods of freezing were tested; 0.2-ml aliquots were either 1) frozen into pellets by dropping onto dry ice (≈15 min) and then stored in LN_2 or 2) frozen in a cryovial which was plunged directly into LN_2. Frozen pellets were thawed by dropping 1 pellet into 2 ml of TNE buffer (0.01 M Tris HCl, 0.15 M NaCl, 0.001 M disodium EDTA, pH 7.4) at 37°C and then placing on ice (4°C) for FCM measurement, while the semen frozen in cryovials was thawed for 30 sec in a 37°C water bath and diluted in 2 ml of TNE before being placed on ice.

Data were analyzed by least squares procedures using the general linear model (GLM) procedure of the Statistical Analysis System (SAS;30). The main effects were boar, extender and freezing method. The extender effect and the freezing method effect were tested using the interaction of boar with extender and boar with freezing method, as the error term, respectively. The residual was used to test all other effects. The results are presented as least squares means (±SEM).

Fresh semen samples from 3 boars were also measured as whole cells and as sonication-liberated nuclei to determine whether potential cytoplasmic droplets influenced the data. Semen from 1 of the boars was also measured as whole cells and as sonicated nuclei prepared at all 3 temperature levels (fresh, dry-ice pellet freezing, and LN_2 freezing). One-half milliliter of diluted boar semen (≈2 to 3 X 10^6 spermatozoa/ml) in a Corning #25702 cryogenic vial[c] was sonicated for 40 sec at 4°C, power setting 3, and

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[a]Swine Genetics International, Ltd., Cambridge, IA.
[b]Lifoam® Leisure Products, Baltimore, MD.
[c]Corning, NY.

70% of 1-sec pulses in a Branson 450 sonifier.[d] Under these conditions, somatic cells are totally disrupted and ≥ 95% of the sperm tails are dissociated from the heads.

Extender Effects on Boar Semen

Semen was collected from 5 boars (ages 18 mo to 4.5 yr) at the University of Illinois, and each ejaculate was divided so that half remained as neat semen and half was extended by the Beltsville method (26) modified to exclude egg yolk. Half of each of the neat and extended semen was frozen into 0.2-ml pellets on dry ice and placed into LN_2, while the remaining half was frozen immediately in LN_2. The extended semen was first concentrated by centrifugation to a sperm density of ≈ 6 billion in 10 ml of solution before freezing. The neat semen was not concentrated before freezing, and sperm density was recorded for 1 ml from each of the boars. The semen was thawed as in the previous experiment for FCM measurements. A preliminary study showed that the boar effect had the most variation in the analysis of variance, and so as to minimize possible instrument error when looking for treatment differences, sampling order was by boar through each of the 4 treatments.

Data were analyzed by least squares procedures using the GLM procedure of SAS. The main effects were boar and treatment. The treatment effect was tested using the interaction of boar with treatment as the error term. The residual was used to test all other effects. The results are presented as least squares means (±SEM).

Boar Heterospermic Trial

According to Wentworth and Lush (32), the inheritance patterns of coat color in swine provide a reliable phenotypic marker by which sires of different breeds can be compared in the same female. A reference sire of one breed serves as the standard by which sires of other breeds are compared. With 3 color alleles, white is dominant over black and red, and black is dominant over red. Use of red females (homozygous for red coat color) allows for competitive mating of 3 sires simultaneously, 1 red, 1 black and 1 white, providing that all are homozygous for coat color. If all the boars had the same fertility potential, it would be expected that each boar would sire 1/3 of each litter's piglets, i.e., the theoretical estimate. Comparison of the ratios of offspring by sire from several litters allows for the ranking of sires on the basis of fertility.

Heterospermic mating trials were conducted at the University of Illinois Teaching Farm where they farrowed. Six boars (18 mo to 4.5 yr old) with known heterogeneous fertility based on single-sire inseminations under field conditions, were mated to 9-mo-old Duroc crossbred gilts in various combinations (Table 1). The boars were selected for specific matings on the basis of previous fertility records and phenotype to facilitate the comparison of the 3 sires for each litter. The sire of each piglet was established by coat color at farrowing. Semen was collected once the gilts were in estrus and the sperm

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[d]VWR Scientific, Danbury, CT.

concentration was determined using a hemacytometer counting chamber. Each sire contributed 2 X 10^9 sperm cells to a pooled inseminate containing 6 X 10^9 sperm cells for each mating. The gilts were inseminated once at approximately 30 h after the first signs of estrus. From the ejaculate of each sire, a sample was cryopreserved using the Beltsville method modified to exclude egg yolk, as described by Pursel and Johnson (26), for later measurement by the SCSA.

Table 1. Heterospermic combinations

Combination	No. of litters	Boars[a]		
A	2	1	2	3
B	3	1	2	4
C	3	1	2	6
D	2	1	3	5
E	2	1	4	5
F	2	1	5	6

[a]1 = Hampshire; 2 and 5 = Duroc; 3 = Chester White; 4 and 6 = Yorkshire.

The SCSA results divided the boars into 2 groups consisting of 3 boars of higher fertility and 3 of lower fertility. The GLM procedure of SAS was used to analyze the data. The main effects were fertility grouping, boar and collection. The fertility group effect was tested using the boar with fertility group as the error term. The residual was used to test all other effects. The results are presented as least squares means (±SEM).

Sperm Chromatin Structure Assay (SCSA)

The SCSA is an acridine orange (AO) staining technique, initially described by Darzynkiewicz et al. (5) for staining somatic cells, that was adapted by Evenson and colleagues (1,8,9,15) to study sperm chromatin structure. This procedure measures the susceptibility of DNA in sperm chromatin to acid-induced denaturation (1,8,9). Acid-treated sperm cells are stained with AO, a metachromatic fluorescent dye. When exposed to 488 nm light, AO intercalated into double-stranded (normal) DNA fluoresces green (24) while that associated with denatured, single-stranded DNA, fluoresces red (23).

Fresh or frozen/thawed semen was diluted in TNE buffer to a final concentration of 1 to 2 million cells/ml. Then 0.2 ml of semen was admixed with 0.40 ml of a detergent/acid solution consisting of 0.1% Triton X-100 in 0.08 N HCl and 0.15 M NaCl (pH 1.4). After 30 sec, 1.2 ml of staining solution containing 6 µg/ml electrophoretically

purified AO[e] in staining buffer were added to the sample. The AO staining buffer is prepared by mixing 370 ml of 0.1 M citric acid monohydrate, 630 ml of 0.2 M Na_2HPO_4 (dibasic), and adding .372 g disodium EDTA and 8.77 g NaCl (pH 6.0), as previously described (10). All steps were maintained at 4°C and FCM measurements were begun 3 min after staining.

Flow Cytometric (FCM) Measurements

Samples were measured in an Ortho Cytofluorograf II equipped with a 100 mW argon-ion laser operated at 35-mW output and 488-nm wavelength, and interfaced to a Data General 2150 computer.[f] The flow cytometer detected the green (native DNA) and red (denatured DNA) fluorescence of each cell as it passed through the focal point in the quartz flow cell at rates of about 250 cells/sec. The flow cytometer is capable of distinguishing up to 1000 channels (fluorescent intensities) of both red and green fluorescence on each cell. Data on 5000 sperm cells per sample were collected and analyzed.

Before each SCSA experiment, a reference boar sperm sample was aliquoted into several hundred 0.5 ml snap cap tubes and frozen (-80°C). These reference samples were individually thawed and run periodically throughout the experiment to check for instrument reliability and stability, keeping the mean red and green fluorescence within a 10 channel range.

Figure 1 shows examples of raw FCM data of spermatozoa from a boar with normal fertility (panels A and B) and one with lower fertility (panels C and D). Each dot in the cytograms (panels A and C) corresponds to 3 or more single cells and its position is dependent upon the amount of native (green) and denatured (red) DNA. The degree of normality/abnormality is expressed by the term alpha t (α_t), which is the ratio of red/(red+green) fluorescence for each cell in the sample population. Normal spermatozoa produce a narrow α_t distribution, while the distribution from spermatozoa with denatured DNA is broader. Red + green fluorescence is total fluorescence. By definition, and as shown in the frequency histograms (panels B and D) α_t values range from 0 to 1.0 (5); however, for convenience of working with whole numbers, they are expressed here as ranging from 0 to 1000 and correspond to the 1000 channels of fluorescence from the cytometer.

The most useful α_t variables for expressing abnormalities are 1) COMPα_t, the percentage of cells outside the main (normal) population of α_t, i.e. those cells with abnormal chromatin structure, determined by placing a computer region from the right hand edge of the main population to the end of the α_t histogram, 2) SDα_t, the standard deviation of the distribution, a measure of the extent of abnormality and 3) Xα_t, the mean

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[e]Polysciences, Warrington, PA.
[f]Becton Dickinson Immunocytometry Systems, Braintree, MA.

channel of the distribution. Higher α_t values indicate increased levels of abnormality. In Figure 1, the lower fertility boar has a higher percentage of cells in the COMPα_t box (panel C) and a higher SDα_t (panel D) than the fertile boar (panels A and B). The methodology chapter (10) contains a detailed description of the materials and procedures of the SCSA.

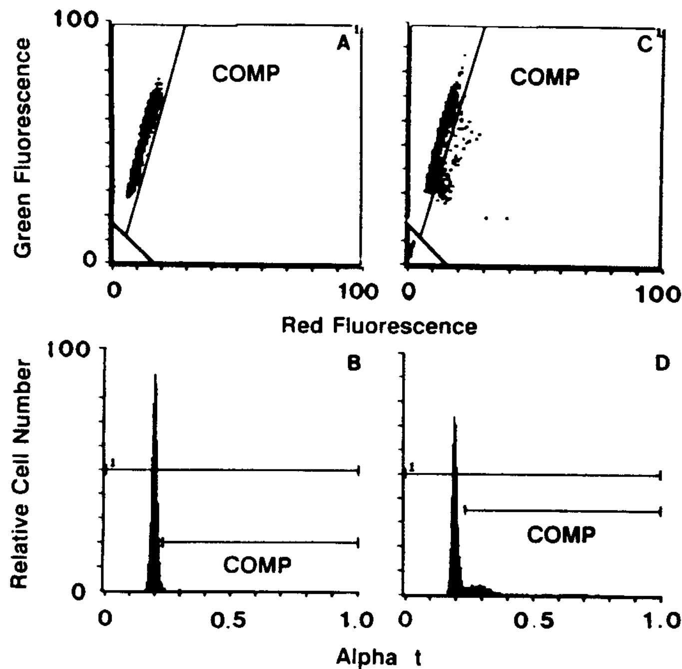

Figure 1. Green versus red fluorescence cytograms and corresponding alpha t (α_t) frequency histograms of sperm chromatin structure assay data on spermatozoa from a fertile boar (panels A and B) and a less fertile boar (panels C and D). The COMP areas are the cells outside the main population of the α_t distribution.

Although normal spermatozoa have the same amount of DNA stainability, there is a wide distribution in DNA fluorescence of mammalian spermatozoa due to an optical artifact (19) caused by their asymmetrical shape and high refractive index. This effect results in an elongated green DNA fluorescence distribution (Figure 1) which causes problems when measuring DNA content but not when measuring α_t values, since they are derived from a ratio of fluorescence values.

RESULTS

Assessment of Freezing and Extender Effects

Figure 2 shows results of the experiment on fresh versus frozen and extended semen. Analysis of variance revealed that the 3 boars were significantly different for all SCSA variables. The freezing method (fresh, frozen on dry ice, or frozen in LN_2) was significantly different for COMPα_t ($P<0.05$) only. The COMPα_t values for fresh semen, semen frozen on dry ice and semen frozen in LN_2 were 5.4±0.1, 4.8±0.1, and 4.5±0.1%, respectively. Although a statistical difference between fresh semen and semen frozen in LN_2 was evident, this difference was not considered to be biologically meaningful. There were significant boar by extender interactions for Xα_t ($P<0.01$) and SDα_t ($P<0.01$), which suggests that the semen of each boar responded differently to the extender.

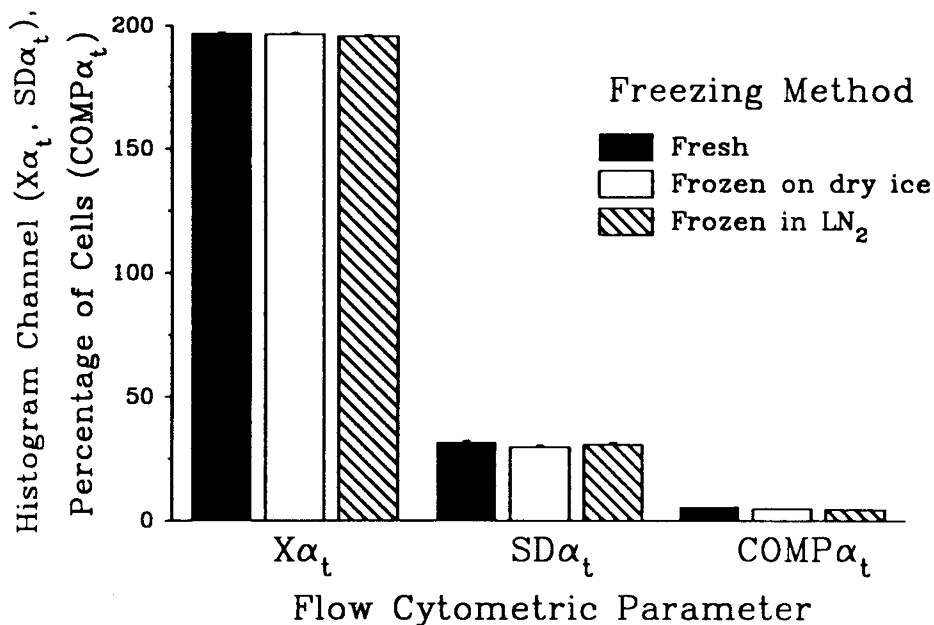

Figure 2. Least squares means of sperm chromatin structure assay variables for fresh versus frozen semen. Xα_t = the mean channel, SDα_t = the standard deviation and COMPα_t = the percentage of cells outside the main population of the α_t distribution.

No differences were found in SCSA results when fresh sperm cells from the 3 boars were measured as sonicated or whole spermatozoa or when semen from one of

the boars, processed at all temperature levels (fresh, dry ice and LN_2), was measured as sonicated or whole cells.

In the experiment with 5 boars, analysis of variance again showed the boar effect to be a significant source of variation when comparing different freezing methods on neat and extended boar semen. No differences were found between neat and extended semen for the SCSA variables when pooled across boars and freezing method (Figure 3), nor for freezing method when pooled across boars and extender treatment.

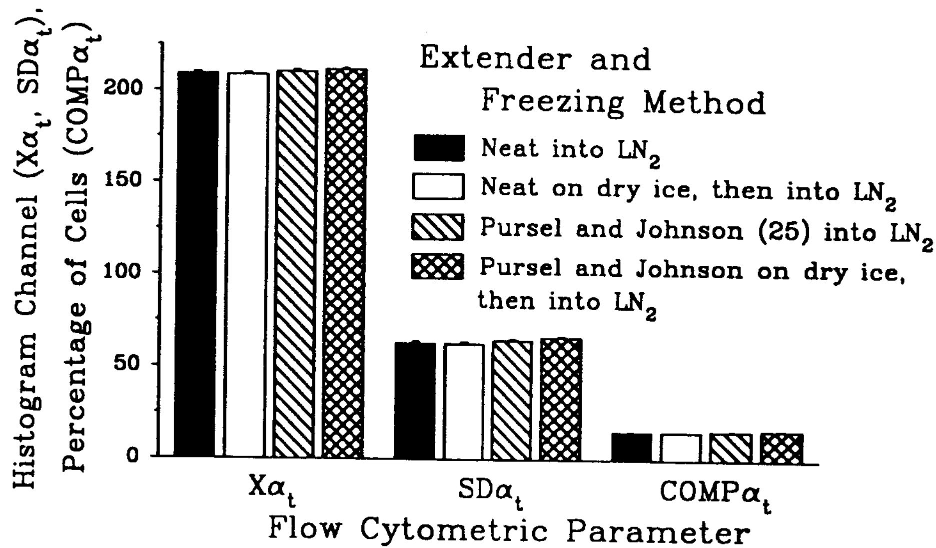

Figure 3. Least squares means of sperm chromatin structure assay variables for neat versus extended semen under different freezing conditions. $X\alpha_t$ = the mean channel, $SD\alpha_t$ = the standard deviation and $COMP\alpha_t$ = the percentage of cells outside the main population of the α_t distribution.

Potential of the SCSA as a Fertility Predictor

Results of the heterospermic trial are shown in Table 2. When the actual number of piglets sired was compared with the expected number (1/3), a percentage of this theoretical estimate was calculated. Two groups of boars were established based on the expected number of progeny. Three of the boars sired a higher than expected percentage of offspring and 3 sired a lower than expected percentage. When these results were compared to the FCM data shown in Figure 4, the value of the SCSA for measuring fertility potential can be seen.

Table 2. Heterospermic trial data for each boar

Boar	No. of litters	Total no. of piglets in litters	Expected no. of offspring	Actual no. of piglets sired	Percentage of the theoretical estimate
1	14	164	54.7	14	26%
2	8	102	34.0	63	185%
3	4	42	14.0	7	50%
4	5	62	20.7	10	48%
5	6	59	19.7	27	137%
6	5	57	19.0	40	210%

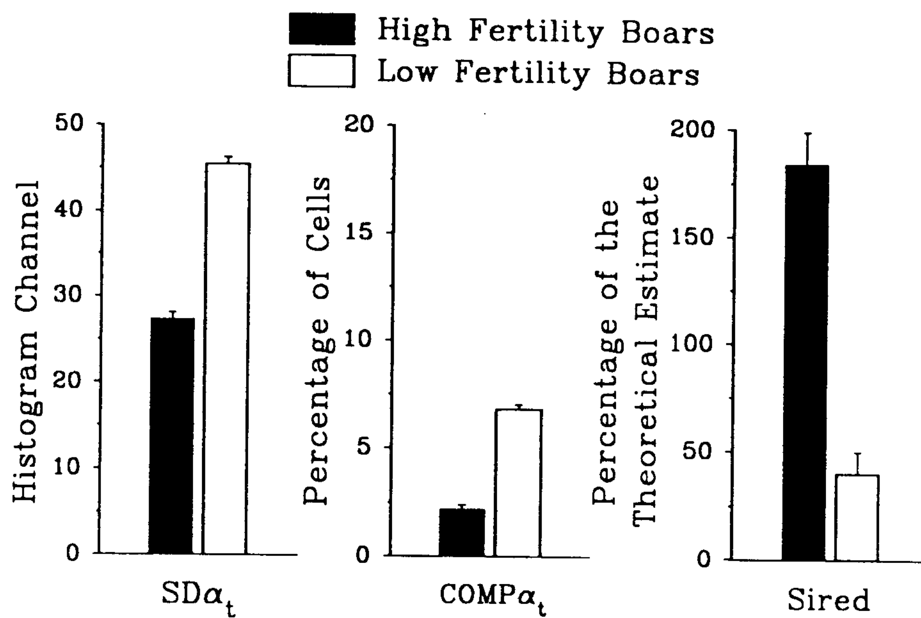

Figure 4. Relationship between sperm chromatin structure assay data and heterospermic fertility potential expressed as a percentage of the theoretical estimate (each boar sires 1/3 of the piglets). $SD\alpha_t$ = the standard deviation and $COMP\alpha_t$ = the percentage of cells outside the main population of the α_t distribution.

Boars siring more piglets than expected had significantly lower $X\alpha_t$ ($P<0.05$), $COMP\alpha_t$ ($P<0.05$) and $SD\alpha_t$ ($P<0.01$) than the less fertile boars, while as a group, the low fertility boars exhibited greater variation in the SCSA variables. Figure 5 compares the least squares means for $SD\alpha_t$, $COMP\alpha_t$ and the number of piglets sired as a percentage of theoretical estimate for the high and low fertility boars. These data show that SCSA results were consistent with the results of the heterospermic fertility ranking.

Each boar in a heterospermic combination was assigned a within combination ranking of 1, 2 or 3 (highest to lowest number of piglets sired). Correlation coefficients were calculated between this ranking and the SCSA variables. There were 6 rankings in combinations A, D, E and F and 9 rankings in combinations B and C. These correlations from the various sire comparisons revealed the value of the SCSA as a predictor of subfertility. As shown in Table 3, in most combinations, as the fertility decreased (higher value), the SCSA variable also increased.

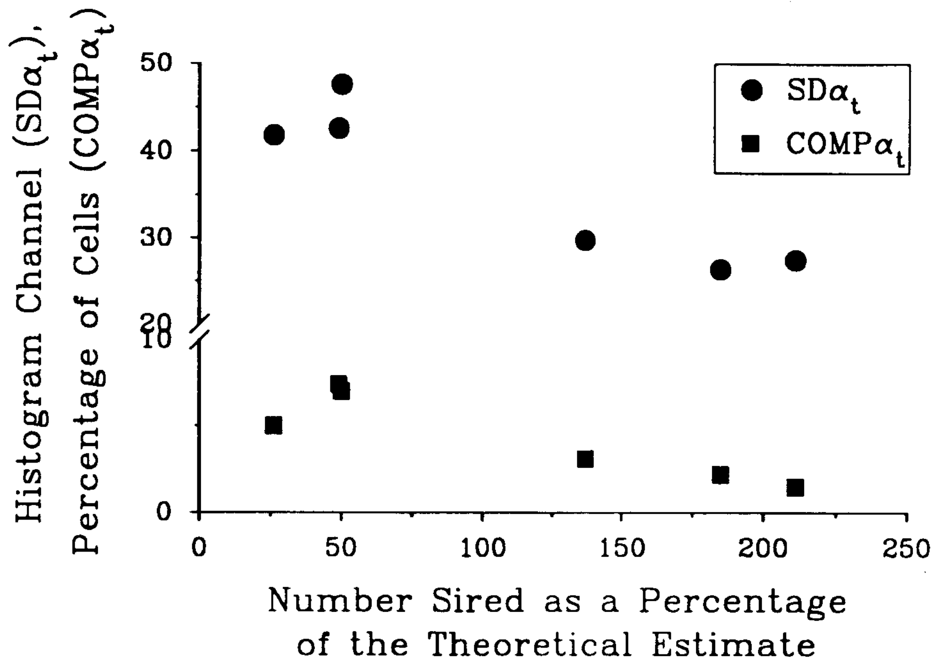

Figure 5. Least squares means for the sperm chromatin structure assay variables $SD\alpha_t$ and $COMP\alpha_t$, plotted with the number of piglets sired as a percentage of the theoretical estimate (each boar sires 1/3 of the piglets) for the 3 high fertility and the 3 low fertility boars. $SD\alpha_t$ = the standard deviation and $COMP\alpha_t$ = the percentage of cells outside the main population of the α_t distribution.

Table 3. Correlations of heterospermic within combination ranking with sperm chromatin structure assay variables

Variable[a]	Combination					
	A	B	C	D	E	F
$X\alpha_t$	0.27	0.66*	0.74*	0.49	0.70	0.92**
$SD\alpha_t$	0.27	0.92**	0.72*	0.75	0.88*	0.91*
$COMP\alpha_t$	0.19	0.77*	0.91**	0.71	0.91*	0.93**

*P<0.05
**P<0.01
[a] $X\alpha_t$ = the mean channel, $SD\alpha_t$ = the standard deviation, and $COMP\alpha_t$ = the percentage of cells outside the main population of the α_t distribution.

DISCUSSION

The $SD\alpha_t$ has consistently been the SCSA variable having the highest correlation with reproductive toxicology measures (7) and fertility. Increased values for α_t can be induced by exposure to drugs, chemicals and environmental stresses and after a recovery period can return to normal (6). The higher values may also be a result of genetic abnormalities.

Ballachey et al. (1) used the SCSA to measure 1 to 8 semen samples collected and frozen over the breeding lifetime of 49 bulls from the Eastern Artificial Insemination Cooperative. Because of the narrow range of the fertility ratings of the bulls, a good correlation between fertility rating and $SD\alpha_t$ might not be expected (25), but it was, in fact -0.58 (P<0.01). In a heterospermic trial (2), semen was collected from 9 bulls representing different phenotypes, and paired samples were mixed so that the mixture contained equal numbers of spermatozoa from each bull. The SCSA measurements of aliquots of the same semen samples had a correlation of -0.94 (P<0.01) between the heterospermic fertility index and $SD\alpha_t$.

This study showed that boar sperm chromatin structure was not altered, as determined by the SCSA, by freezing directly on dry ice or into LN_2, with or without different types of extenders. Thus, the loss of boar sperm fertility potential after freezing/thawing is likely due to factors other than damage to sperm chromatin structure. Sperm chromatin structure was, however, correlated with fertility potential in the boar as shown in the heterospermic experiment. Reduced fertility was correlated with increased α_t values; however, the percentage of abnormal cells ($COMP\alpha_t$) increased only from about 2 to 7% while the $SD\alpha_t$ increased from 27 to 47. This indicates that the low fertility boars had less homogeneous sperm chromatin than the high fertility boars. Although

these differences may appear small, our current interpretation is that these values represent a "tip of the iceberg" effect that reflects an increased level of damage to the whole population.

These data strengthen previous conclusions that sperm chromatin structure is correlated with bull fertility as shown by both field trials (1) and heterospermic studies (2). Current investigations strongly indicate that human fertility is correlated with sperm chromatin structure. A review article by Ward and Coffey (31) suggests that sperm chromatin structure is important for both fertility and viability of the embryo. Our view is that the unique packaging of the sperm chromatin is needed to prevent damage to the DNA as spermatozoa are transported from the testis to the site of fertilization; during this period the sperm cell is not capable of DNA repair. Thus, if DNA damage were extensive, then appropriate repair may not be possible after fertilization.

Other studies have shown that the direct freezing of semen samples or caudal spermatozoa at low temperatures (-80 to -120°C) and thawing once does not change sperm chromatin structure in humans (13) or in mice (7,12). Thus, the heterogeneity observed within a semen sample is likely due to dysfunctional spermatogenesis/ spermiogenesis. Samples obtained over time from individual bulls, stallions and humans (13) have shown remarkable repeatability over several months duration in the percentage of COMPα_t and SDα_t values, implying that particular stem cells possess a defect, leading to variable but specifically altered sperm chromatin that may be incompatible with fertility and sustained embryonic development.

Our present experiments show that the SCSA, which has been shown to be useful for bull and human fertility studies and animal toxicology studies, also appears to be useful for boar fertility studies. The SCSA correctly predicted the 3 high and 3 low fertility boars in a heterospermic fertility trial. The full potential of the SCSA for boar fertility prediction needs further exploration.

REFERENCES

1. Ballachey BE, Hohenboken WD, Evenson DP. Heterogeneity of sperm nuclear chromatin structure and its relationship to fertility of bulls. Biol Reprod 1987;36:915-925.
2. Ballachey BE, Saacke RG, Evenson DP. The sperm chromatin structure assay: Relationship with alternate tests of sperm quality and heterospermic performance of bulls. J Androl 1988;9:109-115.
3. Beatty RA, Bennett GH, Hall JG, Hancock J, Stewart DL. An experiment with heterospermic insemination in cattle. J Reprod Fertil 1969;19:491-502.
4. Crabo BG. Preservation of boar semen: a worldwide perspective. In: Johnson LA, Rath D (eds), Boar Semen Preservation II. Proceedings of the Second International Conference on Boar Semen Preservation. Paul Parey Scientific Publishers, Berlin, 1991;3-9.
5. Darzynkiewicz Z, Traganos F, Sharpless T, Melamed M. Thermal denaturation of DNA in situ as studied by acridine orange staining and automated cytofluorometry. Exp Cell Res 1975;90:411-428.
6. Evenson DP. Flow cytometry evaluation of male germ cells. In:Yen A (ed) Flow cytometry: Advanced Research and Clinical Applications, Vol I. CRC Press, Boca Raton, FL, 1989;217-246.

7. Evenson DP, Baer RK, Jost LK. Long-term effects of triethylenemelamine exposure on mouse testis cells and sperm chromatin structure assayed by flow cytometry. Environ Mol Mutagen 1989;14:79-89.
8. Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 1980;240:1131-1133.
9. Evenson DP, Higgins PH, Grueneberg D, Ballachey BE. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry 1985;6:238-253.
10. Evenson DP, Jost LK. Sperm chromatin structure assay (DNA denaturability). In: Darzynkiewicz Z, Crissman HA, Robinson OP (eds), Methods in Cell Biology: Flow Cytometry, Second Edition. Academic Press, Inc, New York, (in press).
11. Evenson DP, Jost LK. Hydroxyurea exposure alters mouse testicular kinetics and sperm chromatin structure. Cell Proliferation 1993;26:147-159.
12. Evenson DP, Jost LK, Baer RK. Effects of methyl methanesulfonate on mouse sperm chromatin structure and testicular cell kinetics. Environ Mol Mutagen 1993;21:144-153.
13. Evenson DP, Jost LK, Baer RK, Turner TW, Schrader SM. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. Reprod Tox 1991;5:115-125.
14. Evenson DP, Lee J, Darzynkiewicz Z, Melamed MR. Rhodamine 123 alters the mitochondrial ultrastructure of cultured L1210 cells. J Histochem Cytochem.1985;33:353-359.
15. Evenson DP, Melamed MR. Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J Histochem Cytochem 1983;31:248-253.
16. Evenson DP, Parks JE, Kaproth MT, Jost LK. Rapid determination of sperm cell concentration in bovine semen by flow cytometry. J Dairy Sci 1993;76:86-94.
17. Garner DL, Pinkel D, Johnson LA, Pace MM. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. Biol Reprod 1986;34:127-138.
18. Gianola D, Thompson LH. Theoretical consequences of heterospermic progeny testing in polytocous species. In:Insemination Artificielle et Amelioration Genetique: Bilav et Prespecteress Critiques, No 29. Les Colleques de l'INRA, Toulouse-Auzeville, France,1984.
19. Gledhill BL, Lake S, Steinmetz LL, Gray JW, Crawford JR, Dean PN, Van Dilla MA. Flow microfluorometric analysis of sperm DNA content: effect of cell shape on the fluorescence distribution. J Cell Physiol 1976;87:367-376.
20. Graham JK, Kunze E, Hammerstedt RH. Analysis of sperm viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biol Reprod 1990;43:55-64.
21. Johnson LA, Flook J, Hawk H. Sex Preselection in rabbits: Live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 1989;41:199-203.
22. Johnson LA, Flook J, Look M, Pinkel D. Flow sorting of X and Y chromosome-bearing spermatozoa into two populations. Gamete Res 1987;16:1-9.
23. Kapuscinski J, Darzynkiewicz Z, Melamed MR. Luminescence of the solid complexes of acridine orange with RNA. Cytometry 1982;2:201-211.
24. Lerman LS. The structure of the DNA-acridine orange complex. Proceedings of the National Academy of Science, USA, 1963;49:94-102.
25. Linford E, Glover FA, Bishop C, Stewart DL. The relationship between semen evaluation methods and fertility in the bull. J Reprod Fertil 1976;47:283-291.
26. Pursel VG, Johnson LA. Freezing boar spermatozoa: Fertilizing capacity with concentrated semen and a new thawing procedure. J Anim Sci 1975;40:99-102.
27. Saacke RG. Semen quality in relation to semen preservation. J Dairy Sci 1983;66:2635-2644.

28 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. In:Proceedings of the 9th International Congress on Animal Reproduction and Artificial Insemination. Madrid, 1980;5:75-78.
29 Shapiro HM. Practical Flow Cytometry, Second Edition. Alan R Liss, Inc, New York, 1988.
30 Statistical Analysis Systems: User's Guide; Statistics. SAS Institute, Cary, NC, 1985.
31 Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells. Biol Reprod 1991;44:569-574.
32. Wentworth EN, Lush JL. Inheritance in swine. J Agric Res 1923;23 557.