Relationships between Sperm Chromatin Structure, Motility, and Morphology of Ejaculated Sperm, and Seasonal Pregnancy Rate[1]

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ABSTRACT

The objectives of this study were to investigate further the sperm chromatin structure assay (SCSA) for use in stallion semen evaluation and to relate sperm chromatin structure to the routine measures of sperm quality as well as to fertility as measured by seasonal pregnancy rate (SPR). Routine clinical fertility and breeding record evaluations and the SCSA were conducted on 106 stallions in commercial breeding operations. Sperm and SCSA values were compared between fertile and subfertile breeding stallions. With the use of the metachromatic DNA probe acridine orange (AO), the SCSA provides a flow cytometric measurement of the relative amounts of double-stranded and single-stranded DNA in each of 5000 sperm cells of the sample population. Under 488-nm laser light, AO that is bound to double-stranded (native) DNA fluoresces green while that bound to single-stranded (denatured) DNA fluoresces red. The ratio alpha $_t$ (α_t) describes the amount of red to total (red + green) fluorescence emitted. Negative correlations were found between SPR and the SCSA variable COMPα_t (cells outside the main population of α_t, -0.40), percentage morphologically normal sperm (-0.41), and percentage progressively motile sperm (-0.35). The subfertile stallions had higher ($p < 0.05$) levels of COMPα_t (32%) than fertile stallions (16%). The SCSA appears to be useful for estimation of some forms of stallion subfertility.

INTRODUCTION

One way to try to assess the inherent fertility of a stallion is by the average number of cycles and services required to produce a live foal from a full book of mares. This will provide "apparent fertility" but is insufficient in itself for estimating degrees of intrinsic fertility because of other confounding factors, including 1) the intrinsic fertility of the brood mare band; 2) overall management, including such aspects as nutrition, housing, and the preventive medicine program; and 3) the breeding management of mares and stallions.

The approach currently used for clinical stallion fertility evaluation throughout North America is to compare the physical findings of the internal and external genital organs as well as the results of tests of semen quality of stallions of known fertility to those for stallions in question [Kenney et al., 1983].

In a continuing effort to increase the objectivity of the estimation of intrinsic stallion fertility by evaluation of various measures of sperm genetic material [Kenney et al., 1991], we report here our findings in evaluating sperm chromatin structure along with semen findings in the same ejaculates of stallions whose seasonal pregnancy rate was known.

MATERIALS AND METHODS

All 106 stallions evaluated were phenotypic males that had physically normal and functional genital organs and that were actively involved in breeding programs. Individuals with any form of the XY sex reversal syndrome [Kent et al., 1986; Power, 1986], pseudohermaphroditism [Hahn et al., 1980], or hermaphroditism [Dunn et al., 1974] were eliminated. Each stallion underwent a standard fertility evaluation [Kenney et al., 1983]. Those with normal semen had a minimum of 0.5×10^9 spermatozoa in the ejaculate of which $\geq 40\%$ were both morphologically normal and progressively motile.

Initially, stallions were divided into two

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groups, fertile and subfertile. The fertile group (group 1; n = 60) was composed of stallions standing at stud on local breeding farms with no history of subfertility. The subfertile group (n = 46) involved stallions that had been referred to the Georgia and Philip Hofmann Research Center for Animal Reproduction with a history of subfertility.

In order to refine the analysis of subfertility, we subdivided the subfertile group of 46 stallions into three groups (groups 2, 3, and 4), defined as follows. Group 2 (subfertile, n = 23) consisted of stallions with a history of subfertility shown attributable to the stallion after management of the animal, infectious disease, or subfertility of the mares had been ruled out. Group 3 (subfertile and chromosomally abnormal, n = 19) consisted of stallions originally placed in group 2 but subsequently shown to have an abnormal karyotype. Group 4 (functionally sterile, n = 4) was composed of stallions that rarely or never rendered mares detectably pregnant after numerous matings. Because complete data were not available for each individual in the study, subtotals for some of the variables (i.e., fertility class, pregnancy data, sperm measurements) were not complete, and therefore some totals vary from n = 106.

The fertility estimate for each stallion was based on its seasonal pregnancy rate (SPR), which is the number of mares pregnant to one stallion in the same season divided by the number of mares bred by that stallion in that season. Although cycles per pregnancy is a more precise measure of fertility, sufficient records were not available in all cases to enable use of this method.

Semen Collection and Storage

Semen samples were collected into a polyethylene bag (catalog B736[A]N; NASCO, Fort Atkinson, WI) by means of a Missouri artificial vagina (catalog C6248N; NASCO). After removal of the gel fraction by aspiration or polyester fiber filtration (catalog 41-0168; AGWAY Corp., Syracuse, NY), a well-mixed 4.5-ml aliquot was promptly placed in a screw-cap, polyethylene tube (catalog 5000-0020; Nalge Co., Rochester, NY) and placed in a freezer, on dry ice, or in liquid nitrogen.

To test the effect of room temperature incubation on sperm chromatin structure as measured by the sperm chromatin structure assay (SCSA), fresh semen from five stallions was aliquotted into tubes for freezing and allowed to remain at 22°C from 5 min up to 24 h before freezing. All SCSA data were the same up to 3 h of incubation.

Samples were stored frozen. Those that were collected on farms were stored in whatever freezer was available. Those collected at New Bolton Center were stored at −20°C or −70°C, depending upon which space was available. When it was necessary to transport samples, they were shipped on dry ice by commercial express carriers from North America or Europe.

Sperm Chromatin Structure Assay (SCSA)

Samples were analyzed by the SCSA, a flow cytometric procedure developed by Evenson and colleagues [Ballachey et al., 1987, 1988; Evenson et al., 1980; Evenson, 1989; Evenson & Jost, 1994]. Samples were thawed in a water bath at 37°C; a 5-µl aliquot of each semen sample was diluted with 195 µl TNE buffer (0.15 M NaCl, 0.01 M Tris, 1 mM EDTA, pH 7.4) to a concentration of $1-2 \times 10^6$ spermatozoa/ml. The diluted 200-µl sample was mixed with 400 µl of a solution containing 0.1% Triton X-100, 0.08 N HCl, and 0.15 M NaCl (pH 1.2). This low pH nonionic detergent solution provides for rapid dye uptake and potentially induces partial denaturation of the DNA in situ. After 30 sec, the cells were stained by adding 1.2 ml of a solution containing 6.0 µg of chromatographically purified acridine orange (AO; Polysciences, Warrington, PA) per ml of AO buffer (370 ml of 0.1 M citric acid and 630 ml of 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0) [Darzynkiewicz et al., 1976; Evenson et al., 1985]).

When intercalated into double-stranded DNA, AO fluoresces green [Lerman, 1963] upon laser excitation, whereas when associated with single-stranded DNA it fluoresces red [Bradley & Wolf, 1959; Darzynkiewicz, 1979]. Levels of RNA in the sperm nucleus are negligible [Monesi, 1965] and do not contribute to the measured fluorescence. Other studies [Ballachey et al., 1987; Evenson, 1986; Evenson et al., 1985] have shown no significant difference in ratios of green to red fluorescence between whole spermatozoa and isolated nuclei measured by SCSA. Elevated levels of red fluorescence are not reduced by RNAse treatment [Evenson et al., 1985]. Thus, levels of green and red fluorescence correspond to the relative content of double- and single-stranded DNA.

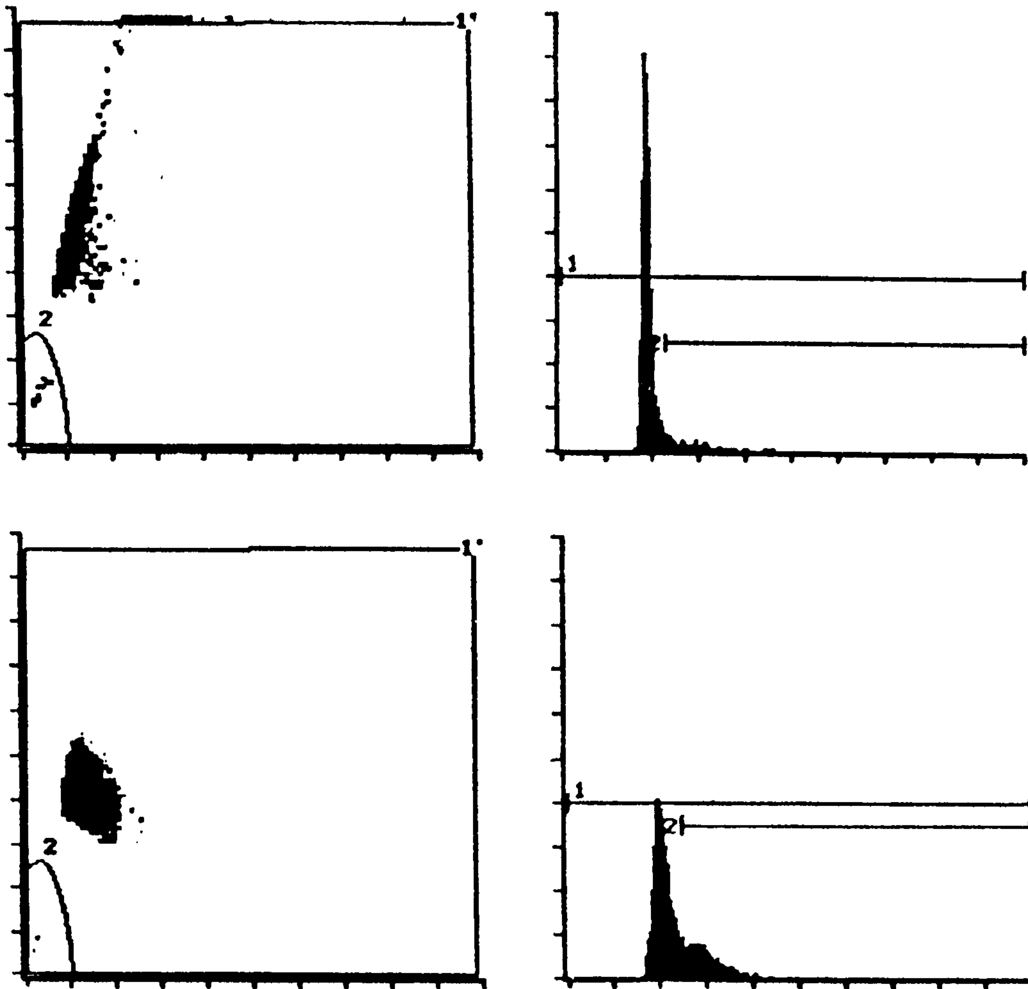

FIG. 1. Left) Typical SCSA two-parameter cytograms of stallion semen stained with acridine orange and measured by flow cytometry. Green fluorescence is on the Y-axis and red fluorescence is on the X-axis. Right) Corresponding α_t frequency histograms with number of cells on the Y-axis. The cells in region 2 are denatured and fall outside the main or normal population and are termed the cells outside the main α_t population, or COMPα_t cells. The upper panels are SCSA profiles from a good quality semen sample and the lower panels are from a poorer quality sample.

Flow Cytometric Measurement

A Cytofluorograf II flow cytometer (Ortho Diagnostic Systems, Westwood, MA) equipped with a Lexel 100-mW argon ion laser, operated at 35-mW output and 488-nm wavelength, was used. The Cytofluorograf was interfaced to an Ortho Diagnostics 2150 data handling system. The AO-stained samples were placed in the flow cytometer; 3 min later, red (> 630 nm) and green (515–530 nm) fluorescence measurements were taken at a rate of about 150 cells per second for a total of 5000 cells per sample.

Alpha$_t$, defined as the ration of red to total fluorescence (α_t = red/(red + green)) [Darzynkiewicz et al., 1975] was computed for each cell, and the α_t distribution values were determined. Measurements are made over 1000 channels of fluorescence and the values are presented here on a scale of 0 to 1000.

The SCSA variables used were the **C**ells **O**utside the **M**ain **P**opulation (COMPα_t = the percent of abnormal cells with denatured DNA), the Mean of α_t (Xα_t = mean channel of the entire distribution), and the Standard Deviation of α_t (SDα_t = an indicator of the extent or degree of denaturation).

TABLE 1. Sperm chromatin structure and sperm parameters of fertile and subfertile stallions (mean ± SD).

Stallions	SPR* (n)	$X\alpha_t$* (n)	$SD\alpha_t$* (n)	$COMP\alpha_t$* (n)	%Progressive motility* (n)	%Morphologically normal* (n)
Fertile	86 ± 8 (60)	220 ± 40 (48)	81 ± 31 (59)	16 ± 17 (54)	58 ± 17 (32)	56 ± 21 (29)
Subfertile	34 ± 23 (21)	262 ± 55 (16)	90 ± 32 (33)	32 ± 23 (33)	44 ± 22 (23)	42 ± 21 (26)

* Values within columns are different at $p < 0.05$

The SCSA protocol is highly repeatable for stallion sperm within an ejaculate ($X\alpha_t = 0.92$, $SD\alpha_t = 0.96$; $COMP\alpha_t = 0.99$).

Statistical Analysis

Seasonal pregnancy rate and SCSA variables ($X\alpha_t$, $SD\alpha_t$, and $COMP\alpha_t$) and sperm variables were analyzed by ANOVA using the SAS GLM procedure [SAS, 1985]. Fertility classification (two classes in Table 1 and four classes in Table 2) was used as the main effect. When the ANOVA indicated a significant effect of the fertility classification, group mean separation was carried out through use of the Waller-Duncan test at a K-ratio of 1:100 ($p < 0.05$).

A simple correlation analysis was performed to measure the association among sperm measures between SPR and the SCSA variables.

RESULTS

Figure 1 shows a typical example of SCSA data for stallion semen from a good quality sample and a poor quality sample. Note that the main population has a tightly clustered elliptical shape indicating a good quality sample with a very narrow α_t distribution. In contrast, the poor quality sample has a significant number of dots representing cells moved down and to the right from the main population, reflecting a loss of green fluorescence with a concomitant increase of red fluorescence, indicating an increased level of DNA denaturation.

Numerous samples from stallions of normal fertility were stored for 1–6 mo in a frost-free freezer; all of these samples had an increased susceptibility to DNA denaturation. Characteristic of repetitively frozen and partially thawed sperm, the samples had an increased level of green fluorescence resulting from an increased accessibility of the DNA to AO staining. Thereafter, storage in such freezers was avoided and samples were transported promptly on dry ice.

In Table 1, the seasonal pregnancy rate and sperm parameters were lower ($p < 0.05$) for subfertile stallions than for fertile ones. The SCSA variables were higher ($p < 0.05$) for the subfertile than for the fertile stallions.

In Table 2, the values for all parameters were consistent with the results presented in Table 1, and there were no differences among the subfertility classes. It should be noted that fertile stallions had a higher SPR (86%) than did subfertile (38%), genetically abnormal (37%), and very subfertile (0.5%) stallions. In addition, the $COMP\alpha_t$ of fertile stallions (16%) was lower than that for very subfertile (41%) and chromosomally abnormal subfertile (39%) stallions, but was not different from that for

TABLE 2. Sperm chromatin structure and sperm parameters of stallions in four fertility classes (mean ± SD).

Class	%SPR (n)	$X\alpha_t$ (n)	$SD\alpha_t$ (n)	$COMP\alpha_t$ (n)	%Progressive motility (n)	%Morphologically normal (n)
Fertile	86 ± 8^a (60)	220 ± 40^a (48)	81 ± 31 (54)	16 ± 17^a (54)	58 ± 17^a (32)	56 ± 21^a (24)
Subfertile	38 ± 24^b (14)	$254 \pm 55^{a,b}$ (11)	87 ± 32 (21)	$28 \pm 22^{a,b}$ (21)	46 ± 22^a (16)	45 ± 19^a (19)
Genetic	37 ± 15^b (5)	$272 \pm 23^{a,b}$ (2)	87 ± 22 (8)	39 ± 18^b (8)	47 ± 16^a (6)	40 ± 22^a (6)
Functionally sterile	0.5 ± 0.7^c (2)	289 ± 75^b (3)	109 ± 46 (4)	41 ± 36^b (4)	$0 \pm --^b$ (1)	$3 \pm --^b$ (1)

a,b,c Different superscripts within columns are different at $p < 0.05$.

TABLE 3. Correlation analysis between the SCSA values and SPR, percentage of morphologically normal sperm (%MN), percentage of progressively motile sperm (%PM), and percentage of morphologically normal-progressively motile sperm (#MNPM).

	COMPα_t	SDα_t	SPR
SPR	−0.399**	−0.154	-
	$p < 0.004$	$p = 0.282$	-
	n = 51	n = 51	-
%MN	−0.414	−0.358	0.340
	$p < 0.001$	$p < 0.003$	$p < 0.03$
	n = 59	n = 65	n = 40
%PM	−0.353	−0.374	0.26
	$p < 0.005$	$p < 0.002$	$p < 0.10$
	n = 60	n = 66	n = 42
#MNPM	0.189	0.372	0.29
	$p < 0.18$	$p < 0.004$	$p < 0.08$
	n = 53	n = 59	n = 37

subfertile stallions (28%). Furthermore, the SDα_t and sperm measures were not different among the four groups of stallions.

The Xα_t was lowest for fertile stallions (220.0), with a trend to higher levels in the other groups.

Table 3 contains correlation coefficients of the variables studied. The SPR was negatively correlated with COMPα_t ($r = -0.399$, $p < 0.004$) but was not correlated with the SDα_t. The SPR was correlated ($r = 0.34$, $p < 0.03$) with the percentage morphologically normal sperm, and there were significant negative correlations between the sperm measures and SCSA variables.

DISCUSSION

An etiological diagnosis of the cause of stallion subfertility is difficult to make in most cases in which causes of abnormal behavior or inability to copulate or ejaculate have been eliminated. After clinical fertility evaluations are made, the subfertility may be blamed on an insufficient number of total sperm or a high incidence of abnormal sperm morphological features, or on too low a percentage of progressive motility, or remain unknown. In the present study, which has extended our efforts to increase the objectivity of the estimation of intrinsic stallion fertility by evaluation of features of spermatogenetic material [Kenney et al., 1991], it has been possible to demonstrate that subfertility in stallions can be related to defects of the chromatin involving increased susceptibility to DNA denaturation in situ. This relationship has been previously shown to exist in both the bull and boar [Ballachey et al., 1987, 1988; Evenson et al., 1994].

The etiology of this type of defect may be induced by various toxic agents [Ballachey et al., 1986], or there could be a genetic predisposition as is perhaps occurring in our group of 19 subfertile stallions with chromosomal abnormalities. The definitive chromatin of mature sperm is formed during spermiogenesis when the round spermatid becomes elongated. In this critical process, the somatic cell histones are replaced in large measure by specific protamines that are stabilized in the chromatin by intra- and intermolecular disulfide bonds [Evenson et al., 1989].

The nature of the chromatin structure defect and its relationship to infertility are not well understood. However, it is reasonable to speculate that if sperm DNA is not properly protected by chromatin proteins, it would be much more susceptible to damage including single and double strand breaks. This hypothesis is supported by the studies of Evenson et al. [1995] that showed a significant correlation between stallion sperm demonstrating suscepti-

bility to DNA denaturation in situ and the presence of DNA strand breaks. If more strand breaks were present than could be repaired after fertilization, the risk would be high for early embryo death.

One of our stallions on first evaluation had a very high COMPα_t (48%), which decreased over a period of 2 mo to a normal level (10%). Since he came from a stable that was known to use various medications for racing stallions, it is suspected that this was a case of drug-induced increased denaturability of the sperm DNA.

It is evident in Table 1 not only that stallions presented to a clinic for problems of subfertility, and screened as mentioned here, will tend to have a subnormal pregnancy rate, but also that this rate may be indicated by an elevated COMPα_t (as well as possibly reduced percentage of progressive motility and morphologically normal sperm). The SCSA data can be of great assistance to examining clinicians, since the altered SCSA values are indicative of a serious molecular defect.

Table 2 shows that the subfertile group and the genetically subfertile group are similar in several parameters; this leads one to wonder whether cases in the subfertile group might have genetic defects not marked by a chromosomal anomaly.

Overall, it appears that as the percentage of COMPα_t approaches or exceeds 30%, it is associated with subnormal pregnancy rates. This finding has subsequently been supported by a more refined follow-up study [Love & Kenney, unpublished results].

We found that it is safe to hold semen samples at ambient temperature of about 22°C for about 3 h. However, it would be prudent to freeze them to −70°C as soon as possible to avoid experimental artifacts.

Data presented on stallion sperm lead to the suggestion that assessing sperm chromatin integrity as measured by the SCSA can provide useful information on the nature of a particular stallion's subfertility. It is clear that this lead should be followed in the future to uncover the nature of the spontaneous, congenital, or genetic factors that lead to abnormally increased denaturability of sperm DNA. In addition, it will be appropriate to study the mechanism by which abnormal chromatin structure can interfere with the fertility scheme of sperm migration through the female tract, capacitation, sperm binding to the zona pellucida, zona penetration, and post-fertilization events.

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