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Boar Fertility and Sperm Chromatin Structure Status: A Retrospective Report

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ABSTRACT: Little information exists about boar sperm chromatin quality and fertility within a commercial setting. The objective of this report is to provide information about boar sperm chromatin integrity and its relationship to fertility. The sperm chromatin structure assay (SCSA) was used retrospectively to characterize sperm from 18 sexually mature boars having fertility information. Boar fertility was defined by farrow rate (FR) and average total number of pigs born (ANB) per litter of gilts and sows mated to individual boars. Fertility data was compiled for 1867 matings across the 18 boars. The SCSA uses flow cytometry to evaluate the structural integrity of sperm nuclear DNA. The SCSA parameters measured in this retrospective analysis were the percentage DNA fragmentation index (%DFI) and standard deviation of the DNA fragmentation index (SD DFI). The %DFI and SD DFI showed the following significant negative correlations with FR and ANB; %DFI vs FR, $r = -0.55$, $P < .01$; SD DFI vs FR, $r = -0.67$, $P < .002$; %DFI vs ANB, $r = -0.54$, $P < .01$; and SD DFI vs ANB, $r = -0.54$, $P < .02$. Although more information is required to better understand the relationship between DFI and boar fertility, this report suggests that the SCSA assay may be an important assay for identification of boars having potential for lowered fertility.

Key words: Semen analysis, SCSA, sperm DNA fragmentation.
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Artificial insemination (AI) has been a successful reproductive management tool for making improvements in livestock production efficiency. A significant impact on genetic progress is accomplished through the use of high–genetic-merit sires for inseminating a group of females. The use of sperm at low concentrations per AI dose can enable greater efficiency with significant genetic impact. As the trend of AI continues to become more efficient (Martinez et al, 2001) it then becomes more important to identify the subfertile or infertile male and ideally to do so prior to entering the breeding herd. Therefore, the identification, and culling, of males producing subfertile ejaculates allows livestock producers an opportunity to enhance overall reproductive efficiency.

The primary method for evaluating sperm quality has been to use classical semen parameters of viability, motility, concentration, and morphology and correlate the information to individual male fertility (Salisbury et al, 1961; Pace et al, 1981; Saacke et al, 2000). When sperm quality parameters are poor for a particular male, that individual's fertility can often be restored or "compensated" by adjusting the quantity of sperm in the dose. In contrast, individuals producing ejaculates with uncompensable sperm traits (fertility is not attainable by adjusting the quantity of sperm) are masked relative to their competency to effect fertilization and sustain embryo development (Saacke et al, 1994). Therefore, more information is needed at the cellular and molecular levels of sperm from fertile and subfertile males. This information will help to characterize and identify factors associated with the uncompensable traits (Evenson, 1999). Continued development of a battery of laboratory assays to identify subfertile males would benefit AI as used within various livestock industries.

To that end, the sperm chromatin structure assay (SCSA) produces information about sperm DNA status and its impact on male fertility (Evenson, 1999). Extensive data from livestock animals and humans provide evidence that sperm nuclear DNA fragmentation/degeneration negatively impacts paternal pregnancy outcomes (Evenson et al, 2002). The SCSA protocol, more fully described under "Materials and Methods," exposes raw semen to a 30-second treatment with a pH 1.2 buffer that denatures sperm DNA at the sites of single-stranded and double-stranded DNA breaks. Sperm DNA is normally highly condensed and stable; however, the SCSA detects sperm having chromatin vulnerable to heat or acid denaturation. Therefore, according to the SCSA, the proportion of these sperm is expressed as the DNA fragmentation index (DFI). A

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computer calculation determines the percentage of sperm in the semen sample with fragmented DNA (%DFI). In livestock species, Ballachey et al (1988) found SCSA data highly correlated with heterospermic performance in bulls (-0.94, $P < .01$). In heterospermic boar trials, the SCSA correctly predicted 3 high-fertility and 3 low-fertility boars (Evenson et al, 1994). Seasonal pregnancy rate and %DFI were negatively correlated in stallions (Kenney et al, 1995). In that study, pregnancy rate was 86% in fertile stallions, whereas %DFI was an average of 16%. For subfertile stallions, the pregnancy rate was 38%, with an average %DFI of 28%. Results from a recently published meta-analysis indicate that human couples with no known infertility problems were 7.0 times (confidence interval [CI] 3.17, 17.7) more likely to achieve a natural pregnancy/delivery if the DFI was <30% ($n = 362$, $P = .0001$) (Evenson and Wixon, 2006).

Although a previous SCSA evaluation was conducted in a heterospermic experiment involving only 6 boars and 18 inseminations (Evenson et al, 1994), the relationship between sperm chromatin quality and fertility within a large-scale, commercial setting remains unknown in pigs. The present retrospective study provides information on 18 boars having various levels of fertility in conjunction with individual SCSA data. The objectives were to: 1) provide an information base to begin establishing a DFI threshold that impacts fertility, and 2) determine whether correlations exist between SCSA-defined parameters (%DFI and standard deviation of the DFI [SD DFI]) and boar fertility (farrow rate [FR] and average number of pigs born per litter).

Materials and Methods

Boars, Semen Collection, and Semen Processing

Six genetic backgrounds or hybrid lines were represented (Landrace, Large White, Pietrain, Duroc, Hampshire, and Yorkshire) among the 18 boars. The hybrid lines were developed in response to selection for phenotypic parameters that impact economic traits of interest. To maintain genetic purity, the inseminations were made only within hybrid lines. The boars (average age = 21.0 months) were maintained in individual pens at a swine production unit located in western Kansas, and semen was collected during the fall months from each boar once per week for use in an ongoing AI program. Females were checked for estrus at AM and PM using a parade boar. Females in estrus in the PM were bred the following AM and PM, whereas females in estrus in the AM were bred that PM and the AM of the following day (if in standing estrus). The insemination dose was 3 billion total viable sperm (determined microscopically using motility as an estimate) in an 80-mL vial with BTS (Pursel and Johnson, 1975) as the semen extender. Information on the individual female parity status for the 1867 matings was not available. Ejaculated semen was collected using the gloved-hand technique. Only the sperm-rich fraction was collected into a prewarmed (36°C) 500-mL plastic collection vessel fitted with gauze to filter out gel material from the semen. The container was then transferred to the semen-processing laboratory within 5 minutes of semen collection. The samples were evaluated microscopically for motility and morphology by placing a 7.0-μL aliquot on a prewarmed (36°C) microscope slide and mounted with a coverslip for viewing at 400× magnification using an Olympus upright BX51 phase-contrast microscope (Olympus America Inc, City, State). Sperm concentration was determined spectrophotometrically (Model 690; Turner, City, State) using a 100-μL aliquot of neat semen added to 7.9 mL of 2.9% sodium citrate in a glass cuvette. One ejaculate per boar was used as the sample source for the SCSA. Only those semen samples possessing greater than 25 million sperm per mL, greater than 80% motility, and less than 20% abnormalities were used in the study. Each of two 0.5-mL aliquots of the semen sample from each of the 18 boars was placed in prelabeled (boar identification and date of semen collection) 2.0-mL cryotubes and flash frozen in liquid nitrogen within 20 minutes of semen collection. The cryotube vials were maintained in liquid nitrogen until the day of shipment. The frozen samples were shipped overnight in a Styrofoam box containing 4.5 kg of dry ice and transferred to a −110°C freezer upon arrival at SCSA Diagnostics, Brookings, South Dakota.

All animal procedures followed guidelines published in the National Pork Board Swine Care Handbook (2003). All boars were observed twice daily for injury or disease, and the herd veterinarian was consulted for diagnosis and treatment as needed.

Definition of Boar Fertility

Boar fertility was defined in terms of: 1) FR, which is the number of bred females (gilts or sows) that farrowed divided by the number of females bred; and 2) the average total number of pigs born per litter (ANB) for farrowed females.

SCSA Procedures

The SCSA protocol used for the present study has been previously described (Evenson and Jost, 2003). Boar samples were thawed in a 37°C water bath and then placed on crushed liquid ice. The SCSA measures the amount of sperm DNA fragmentation in each of 5000 sperm per sample at a flow rate of ~250 sperm per second and then relates that measurement to a statistical risk of subfertility or infertility. Sperm DNA fragmentation is measured by staining acid-treated sperm with acridine orange, a fluorescent dye, and then passing the stained sperm single file through a glass channel with the laser beam of the flow cytometer focused on the stream of sperm. Each sperm emits a fluorescent light when hit by the laser beam: sperm without detectable levels of DNA fragmentation will emit a green fluorescence and those with moderate to high sperm DNA fragmentation will emit various levels of red fluorescence. The amounts of green and red fluorescence of 5000 individual sperm are quantified by a multichannel

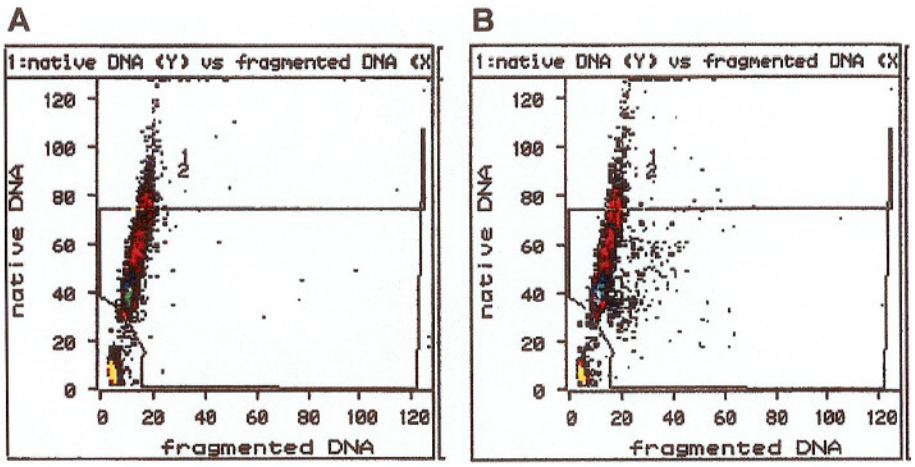

Figure. Green (native DNA) versus red (fragmented DNA) fluorescence cytograms corresponding to excellent 2.56% DNA fragmentation index **(A)** and poor 7.20% DNA fragmentation index **(B)** sperm DNA integrity in boar samples. The position of each dot is due to the amount of red fluorescence (X axis) and green fluorescence (Y axis) on a scale of 0 to 1024 channels (units). The line in the lower left hand corner is a computer gate to exclude debris signals from the sperm measurements.

analyzer with 1024 channels of intensity by flow cytometry that collects green light (515–530 nm) and red light (>630 nm). For each sample, 5000 individual sperm are measured to determine the %DFI and SD DFI (Figure). Numerous aliquots of a reference boar sample were used to ensure the stability of the sample run. The reference sample DFI was ~2%–3%. The SD DFI, which measures extent of sperm DNA fragmentation, was also reported. The reference SD DFI was ~32–35.

A second, independent aliquot of each sample was measured for quality control. The coefficient of variation between the first and second independent measurements was very low, in the range of 1%–3%. A Cytofluorograf II flow cytometer (Ortho Diagnostics Inc, Westwood, Massachusetts) was used for sample analysis that was interfaced with a Cicero data-handling unit (Cytomation, Fort Collins, Colorado). The SCSA calculations were made with SCSAsoft software (SCSA Diagnostics, Brookings, South Dakota). Flow cytometry data were used to create a DFI histogram profile (0–1024 channels) of the entire sperm population. Computer gates of the DFI histogram (red/red + green fluorescence) were used to determine the %DFI.

Statistical Analyses

An evaluation was made of the linear relationship between the SCSA parameters and boar fertility using Pearson's correlation (Table 1). A receiver operating characteristic curve was calculated to determine whether events and nonevents (ie, high %DFI versus low %DFI) could be discriminated. The software program used was SAS (SAS Institute Inc, Cary, North Carolina).

Results

All ejaculates from the boars were satisfactory upon evaluation and were used for the SCSA. Eighteen sexually mature boars (average age = 21.0 months), representing 6 different genetic lines, were used in the study. Fertility information (% FR and ANB) were documented for each of the 18 boars prior to the SCSA analysis. Individual boar fertility and SCSA data are shown in Table 2. A total of 1867 matings were made across the 18 boars (average of 103 matings per boar) with the number of matings ranging from 61 to 235 per boar.

A total of 1161 farrowings occurred for an overall FR of 62.2% ± 27.8 SD (an average of 64.5 farrowings per boar). The FR range was 38.9%–82.7% with a 67.9% median. The ANB for each boar ranged from 8.9 to 11.0. The range of %DFI (as determined via SCSA and represented in the Figure) among the 18 boars was 1.2%–28.3%. Two boars (Nos. 732 and 738) were >6% DFI and 16 boars were <6% DFI. Using 6% as the receiver operating characteristic threshold, evaluation of the 18 boars found the odds ratio for %DFI to be 1.5x (P = .0003, CI 1.21, 1.94), and sensitivity was 83%. The receiver operating characteristic established threshold

Table 1. *Pearson correlation coefficients (r) of SCSA variables and fertility for 18 boars bred to 1867 females*

SCSA Variable	Farrow Rate	Average No. Total Pigs Born
DNA fragmentation index	−0.55[a]	−0.54[a]
SD of DNA fragmentation index	−0.67[b]	−0.54[c]

Abbreviation: SCSA, sperm chromatin structure assay.
[a] $P < .01$.
[b] $P < .002$.
[c] $P < .02$.

Table 2. *Boar fertility and sperm chromatin structure variables for 18 boars*

Boar No.	No. Matings	No. Farrowed	Farrow Rate, %	ANB	%DFI	SD DFI
732	95	37	38.9	8.9	12.0	70.8
738	126	57	45.2	9.0	28.3	76.6
327	233	119	51.0	9.3	3.0	40.3
879	81	44	54.3	9.9	1.9	26.4
196	79	43	56.1	10.4	1.6	27.2
32	235	132	62.8	10.0	5.4	44.2
443	70	44	64.7	11.0	2.3	37.1
577	71	46	67.4	10.4	1.2	30.7
878	86	58	68.4	10.2	1.2	24.2
755	130	89	70.4	9.6	2.6	36.9
96	61	43	71.2	9.5	2.1	27.1
708	108	77	72.0	9.8	1.9	33.8
751	136	98	72.4	9.6	6.3	37.4
293	69	50	74.6	10.8	1.7	37.0
310	63	47	76.4	10.2	1.5	26.0
102	68	52	76.4	10.8	4.9	43.5
290	69	53	76.8	10.9	2.2	30.4
438	87	72	82.7	9.7	2.5	26.3
Mean	103.7	64.5	65.6	10.0	4.6	37.5
Total	1867	1161				

Abbreviations: ANB, average total number of pigs born; %DFI, DNA fragmentation index; SD DFI, standard deviation of DFI.

was 40 for SD DFI; odds ratio was 2.5x ($P = .001$, CI 1.87, 3.32), and sensitivity was 92%. The %DFI had a significant negative relationship to FR and ANB, $r = -0.55$, $P < .01$, and $r = -0.54$, $P < .01$, respectively. The SD DFI also had negative relationships to FR and ANB, $r = -0.67$, $P < .002$), and $r = -0.54$, $P < .02$, respectively.

Discussion

An important element for enhancing swine AI success is accurate semen evaluation. Generally, semen evaluation provides information on sperm motility, morphology, and concentration for a given collection. Nevertheless, ejaculates having satisfactory profiles for the above variables do not always equate to satisfactory fertility (Evenson, 1999). Therefore it is important to continue developing assays that enable the identification of low-fertility or infertile boars.

To that end, the SCSA is a powerful and statistically rigorous diagnostic technique that has the potential to identify subfertile boars and therefore improve semen production efficiency by a boar-culling process. Opportunities are present within today's swine production practices to leverage these sperm biomarkers, such as SCSA, prior to the time boars enter the AI stud. For example, young boars are physically isolated for several weeks prior to entering the AI stud and during this time are trained for semen collection. Semen samples could be collected during this time and assessed for various biomarkers. The identification and subsequent culling of boars possessing poor sperm DNA integrity could be advantageous to production efficiency.

The results of this study showed %DFI having a significant negative relationship to FR and ANB ($r = -0.55$, $P < .01$, and $r = -0.54$, $P < .01$, respectively). Interestingly, 2 boars (Nos. 732 and 738) had DFI greater than 6% (12% and 28.3%, respectively) and corresponding %FR of 38.9 and 45.2, respectively. The data suggests that DNA fragmentation negatively impacts factors that influence fertilization and/or embryonic development. However, more detailed studies are required to better understand where within the reproductive processes the negative impact may be occurring.

The SD DFI also had negative relationships to FR and ANB and has potential to be a more sensitive measure for FR versus %DFI ($r = -0.67$, $P < .002$, and $r = -0.54$, $P < .02$, respectively). Similarly in bulls, Ballachey et al (1987) found SD DFI to be more sensitive than %DFI vs nonreturn rates (-0.65, $P < .01$, and $-.053$, $P < .05$, respectively). The present data suggest that boar sperm possessing fragmented DNA can effect embryonic development (albeit a lower embryonic development), corroborating earlier studies in mice showing that fertilization occurs whether the sperm has damaged DNA or not (Ahmadi and Ng, 1999).

Relative to the prevalence of boar semen possessing fragmented sperm DNA as reported in other studies, Martinez (2005) found 86% of 173 AI boars to have less than 5% DFI (and 14% to have greater than 5% DFI) as determined by a modified fluorescent microscope SCSA

(Waberski et al, 2002). Similarly, Rybar et al (2004) evaluated 68 breeding boars from 1 location using the flow cytometric method as described in the present study and found 92.6% of the boars (63 of 68) having nondetectable %DFI. However, information on the fertility of the 68 boars in the Rybar study was not available. Boe-Hansen et al (2008) reported on a study of ejaculates from 145 boars used in 3276 experimental inseminations in Danish breeding herds. The total number of piglets born (litter size) for Hampshire, Landrace, and Danish Large White boars was, respectively, 0.5, 0.7, and 0.9 piglets smaller per litter when the SCSA-defined %DFI values were above 2.1% as opposed to below this value. It is indeed surprising to have a statistical threshold at such a low %DFI. However, several aforementioned studies on boars confirm a threshold in the 2%–6% range. In a related note, a single semen collection from 6 highly inbred
9 miniature pig boars (NIH miniature pig) was evaluated using SCSA. All of these boars had acceptable semen profiles as determined microscopically. Four of these boars produce litters, whereas 2 have produced none. The mean %DFI for the 2 infertile boars was 18.8 ± 9.9 SD, whereas the fertile boars had a 10.9 ± 8.7 SD %DFI (Steve Terlouw, personal communication).

The bulk of sperm DNA fragmentation studies suggest that sperm with damaged DNA will fertilize eggs (Ahmadi and Ng, 1999). It has been shown in the present study that the ANB is correlated with SCSA data suggesting that the pig eggs may be fertilized but the embryos with damaged paternal genomes die in utero. Thus, both the Danish study (Boe-Hansen et al, 2008) and the present result suggest that sperm with measurable defective DNA may be capable of fertilizing oocytes, but the embryos are not developmentally competent because of compromised DNA integrity.

The present study suggests that a >6% DFI places certain commercial boars into a statistical group that produces a reduced FR and ANB. However, more information is needed to validate this threshold value, because the number of boars in the present study is limited. The significant decrease in fertility for values above a certain DFI threshold is consistent with human data (Evenson and Wixon, 2006). For example, couples
10 using intrauterine insemination were 7.3 times (CI 2.88, 18.3) more likely to achieve a pregnancy/delivery if their DFI was <30% (n = 518, P = .0001). As to why the statistical thresholds for SCSA parameters %DFI and SD DFI are lower for the boar relative to the human or the stallion (Kenney et al, 1995), that is not understood at this time. However, we can speculate that the low
C1 %DFI threshold in boars (Rybar et al, 2004; Martinez 2005) may be an indirect, selective response of semen evaluations performed on AI boars. For example, the boars represented in the present study were required to meet certain seminal threshold levels for concentration, motility, and morphology prior to entering the AI facility. Therefore an indirect selection was placed on the boars prior to use in AI, and this is nonexisting in human couples or stallions (to a lesser extent).

One additional thought as to what might influence or be responsible for the low %DFI threshold in boars relative to other species: sows and gilts have 2 fertility indices, FR and ANB, whereas humans, horses, and cattle have 1 (birthing rate). Moreover, sows and gilts require a minimum number of fetuses to maintain pregnancy. Perhaps swine will only tolerate a relatively low %DFI for those reasons. Nevertheless, additional studies confirming the present observations are required.

In conclusion, it is well known that both semen quality and quantity are considered in the equation for optimal male fertility potential (Salisbury et al, 1961; Pace et al, 1981; Saacke et al, 2000). The present study suggests that boar sperm possessing a %DFI beyond 6% may lead to reduced FR and/or ANB. It is interesting that ANB is correlated with SCSA data suggesting that fertilization occurs but the embryo possessing a damaged paternal genome dies in utero. Finally, if the present observations are confirmed with additional fertility studies, the potential exists for an additional assay to be considered for improving swine production efficiency.

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