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Czech Republic sperm chromatin structure test for bull and boar DNA integrity evaluation

Sk. Rubina, S. Sabiya Sulthana

ABSTRACT: The fertility potential of men examined for artificial insemination may be ascertained by analysis of sperm parameters, which are also crucial for forecasting the success of assisted reproductive procedures. Using the Sperm Chromatin Structure Assay (SCSA), we were able to quantify the degree of DNA damage in both boar and bull sperm. Acridine orange (AO) labeling makes DNA changes (strand breaks) in sperm nuclear chromatin more susceptible to in situ denaturation, which may be detected by flow cytometry. This is the basis of the test. The DNA Fragmentation Index (DFI) was used to quantify sperm chromatin damage. The spermatozoa were categorized into two groups: moderate (m-DFI) and high (h-DFI). Also measured was the proportion of immature cells, or HDS, or cells with high DNA stainability. We collected 68 boar samples from a single location and 37 bulls from two separate groups to assess sperm SCSA parameters. Out of all the bulls that were analyzed, six had a noticeably greater proportion of spermatozoa with detectable DFI (16.2%), while the other six had a noticeably larger percentage of immature cell types (HDS) (16.2%). There was a statistically significant difference between the first and second groups in terms of the mean percentages of spermatozoa with h-DFI and HDS ($P < 0.01$). Among the examined boars, five (or 7.4% of the total) had a noticeably greater percentage of spermatozoa with DFI and eighteen (or 26.5% of the total) had a noticeably higher percentage of sperm with HDS. There was a statistically significant difference between the other boars in the proportion of spermatozoa with DFI and HDS. Boars exhibited significantly greater proportions of spermatozoa containing h-DFI and HDS compared to bulls ($P < 0.0001$). The maximum percentages of spermatozoa with DFI and HDS were 20.8% and 3.5% for individual bulls, and 17.6% and 10.2% for boars, respectively. The percentages of spermatozoa with DFI and HDS did not show any significant associations. This delicate process seems to be useful as an extra tool for determining the quality of farm animals' semen prior to their use in breeding.

Keywords: Fertility, sperm, DNA integrity, flow cytometry, bull, boar

Introduction:

There are several agents, which can impair sper- matogenesis. In this complicated process stem sper-matogonia become mature spermatozoa through a series of events, such as mitosis, meiosis and cell differentiation. These steps can be easily affected anywhere along the process by certain environmen-tal and/or toxic agents. This may lead to decreased semen quality.

Sperm parameters are associated with male fertil-ity or infertility. Morphology evaluation is widely used for predicting fertility potential in farm ani- mals (Johnson *et al*., 1998; Chacon, 2001) and in hu-mans (Kruger *et al*., 1986; Vawda *et al*., 1996). Presently, conventional methods for evaluating male fertility are rather unreliable predictors of fertility because of small

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numbers of measured spermatozoa and the subjective evaluation of cells by the technician. This problem can be minimized by the flow cytometry measurement, which hasbeen used for sexing sperm (Johnson *et al*., 1987, 1989), sperm viability (Garner *et al*., 1986), acroso- me integrity (Graham *et al*., 1990) and sperm count (Evenson *et al.*, 1993b) and mitochondrial function determination (Evenson *et al*., 1985).

Male related embryonic death is linked to factors that result in reduced semen quality such as heat stress (Setchell *et al*., 1988; Sailer *et al*., 1997) and season (Colas, 1983). X-irradiation can affect testicu-lar cells and leads to damage of sperm DNA (Sailer*et al*., 1995b). The damaged sperm have the ability to fertilize the oocytes, but the embryonic develo- pment is very much related to the degree of DNA damage (Ahmadi and Soon-Chye, 1999). Mutagen exposure can lead to the decreased sperm produc- tion and altered sperm morphology (Wyrobek and Bruce, 1975). Some chemicals and toxicants may damage the protamines that protect and package DNA in sperm (Evenson *et al*., 1993a). Toxic agents can also cause DNA strand breaks, either directly or after damage of sperm production mechanisms (Van Loon *et al*., 1993). Associations were found between high air pollution and increased DNA fragmentation (Perreault *et al*., 2000; Selevan *et al*., 2000). Smoking in humans has an adverse effecton sperm quality (Pacifici *et al*., 1993); it especially causes endogenous DNA strand breaks (Potts *et al*.,1999). Age is another factor which plays a role in decreased sperm chromatin stability (Gogol *et al*., 2002). Sperm anomalies such as loosely packaged chromatin and damaged DNA are associated with poor quality semen samples (Sailer *et al*., 1995a). One of the main causes of sperm DNA damage is the exposure to reactive oxygen species (ROS) that are highly reactive and damaging to nucleic acids (Ochsendorf, 1999).

Increased percentages of spermatozoa with abnormal chromatin were found in bulls with lower fertility (Bochenek *et al*., 2001). It has also been observed that a part of motile spermatozoa of infertile men showed fragmented DNA (Lopes*et al*., 1998).

The SCSA method, first described by Evenson *et al.* (1980), characterizes sperm nuclear chromatinin spermatozoa, i.e. susceptibility to nuclear DNA denaturation *in situ*. That feature results from DNA damage that is easily detectable by flow cytometry using acridine orange (AO) staining. The methodis based on the fact that DNA without single and double strand breaks is not susceptible to denaturiz-ing conditions characterised by pH = 1.2. Disturbedchromatin integrity is characterized by the presenceof single and double strand breaks in DNA mol- ecules that lead to formation of denatured single stranded segments (ssDNA). These are quantified

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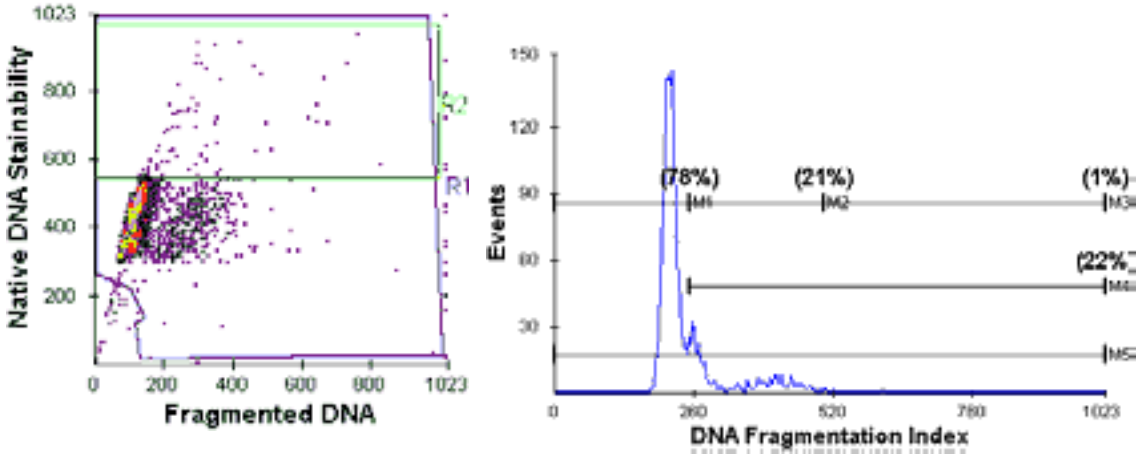

A **B**

Figure 1. Examples of SCSA two-parameter cytogram (A) and histogram (B) of individual bull sperm cells. Each cell is represented by dots for a total of 5000 cells per sample. Each cell's position is based on the amount of green (native DNA stainability) and red fluorescence (fragmented DNA) emitted from that cell. (A) Only cells falling in Region 1 (R1) are included in the analysis. Cellular debris (lower left hand corner) is excluded by the analysis. Region 2 (R2) contains the cell population with high green fluorescence, i.e. immature forms. Cells with decreased green and increased red fluorescence, i.e. cells with denatured DNA, fall down and to the right of the main population – the population with higher density situated on Y axis between 330 and 550 channels. (B) Markers for calculating SCSA parameters are shown here: Marker M1 represents cells of the main population with non-detectable DFI, marker M2 shows population of cells with m-DFI, marker M3 demonstrates cells with h-DFI. M4 combines all cells with DFI,
i.e. cells with altered integrity of chromatin. M5 is a total of M1, M2, M3 and M4 – or the total population of cells measured

by red fluorescence intensity, a characteristic of AOwhen associated with ssDNA after excitation with monochromatic 488 nm blue laser light. Under the same conditions, acridine orange associated with a double stranded molecule (dsDNA) emits green fluorescence. DNA damage of each sperm is expressed by the ratio of red to red plus green fluo-rescence. After sample analysis, several cell popula-tions appear in the resulting cytogram (Figure 1A):The main population characterized by percentage of mature spermatozoa with non-detectable DFI (the population with higher density situated between 330 and 550 channels on Y axis), mature sperm population with various levels of denaturation (DFI) – the large population with lower density on the right hand side and a population of immature forms of spermatozoa (HDS), in particular sper-matids which show about fivefold lower levels of chromatin condensation compared to mature forms(Evenson and Jost, 2000). Figure 1B then shows the main population (markerM1) and DFI population (marker M4). Cells with de-tectable DFI were divided into moderate DFI (m-DFI
– medium sperm damage – marker M2) and h-DFI (high sperm chromatin damage – marker M3). Fromstudies on human semen samples, there is strong evidence that DFI thresholds of 0–15%, 16–29% and

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≥30% relate to high, moderate and very low fertilitypotential, respectively (Evenson and Jost, 2000).
The objective of this study was to use the Sperm Chromatin Structure Assay to determine the level and variability of damage of sperm DNA integrity in bulls tested for artificial insemination and breed-ing boars.

MATERIAL AND METHODS

Semen

Sperm from two groups of bulls (group 1, *n* = 27; group 2, *n* = 10), Czech Simmental breed, 2 years old, were collected via artificial vagina at two in- semination stations in different localities. Fertilizing ability of respective bulls was expressed as the pregnancy rates in heifers on Day 90 after the first insemination. Sixty-eight breeding boar semen sam-ples obtained from one insemination station were analysed. Information on the fertility of the boars was not available. All semen samples were frozen in liquid nitrogen at –196°C. Only once frozen and thawed samples were tested.Sperm chromatin structure assay (SCSA)

Increased susceptibility of altered DNA (strand breaks) in sperm nuclear chromatin to *in situ* denaturation was measured by flow cytometry after staining with acridine orange (AO). AO as- sociated with single (denaturated) and double (na- tive) stranded DNA emitted red and green light, respectively. Chromatin damage of each sperm was quantified by red/(red + green) fluorescence. Each semen sample contained percentage of ma- ture cells with non-detectable (main population of spermatozoa in semen) and detectable (percentage of mature spermatozoa with increased chromatin damage) DFI. Spermatozoa with detectable DFI were divided into two subsets (spermatozoa with moderate and high DFI, according to the level of sperm chromatin damage). The next evaluated pa- rameter was the percentage of immature cells (HDS;cells with High DNA Stainability).
Samples were rapidly thawed in a 37°C water bath,placed on ice and diluted with TNE buffer (0.015 NNaCl, 0.01 M Tris, and 0.001 M EDTA, pH = 6.8) to
bring sperm concentration to 1.5×10^6 per ml. 200 µlof diluted samples were treated with 400 µl acid- detergent solution (0.08 N HCl, 0.1% Triton-X 100, pH = 1.2) for exactly 30 s to induce DNA denatura- tion. Then, 1.2 ml AO staining solution (6 µg/ml chromatographically purified AO in phosphate citrate buffer) was added to intercalate to single stranded or double stranded DNA. Samples were placed on the flow cytometer (FACSCalibur™ flow cytometer, Becton Dickinson, Mountain View, CA, USA, operated by the CELLQuest™ software).
We used one donor reference sample for each species to ensure comparable instrument settings throughout the measurements. Semen samples were exposed to 488 nm monochromatic laser lightand red (ssDNA) and green (dsDNA) fluorescence values collected and stored on 5 000 spermatozoa per sample after 2.5 minutes. In every sample, duplicate measurements were performed in suc- cession for statistical considerations; the second sample was taken from the same thawed aliquot, diluted appropriately, processed for the SCSA and

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measured.

Evaluation and statistical analysis

In each sample, green and red fluorescence of5 000 stained sperm cells were measured and the

Table 1. The mean values (±SD) measured by SCSA in sperm of bulls and their fertilizing ability

Group					
	(min-max) **				
	94.7 ± 4.5 (79.2–98.5)	5.3 ± 4.5 (1.5–20.8)	4.9 ± 4.4 (0.9–20.2)	0.4 ± 0.2[a] (0.2–0.7)	1.2 ± 0.7[a] (0.4–3.4)
	93.4 ± 4.2 (83.9–97.5)	6.6 ± 4.2 (2.5–16.1)	6.1 ± 4.1 (2.3–15.6)	0.5 ± 0.2[b] (0.3–0.8)	1.9 ±0.7[b] (1.1–3.5)

data were saved in the list mode and transferredto an offline computer for final statistical analysis using SCSA-Soft software (SCSA® DIAGNOSTICS, INC, Multiplex Research & Technology Center Brookings, USA).

Then, all data (DFI, m-DFI, h-DFI and HDS) wereanalysed by SPSS package computerised software, version 11.1 for Windows (SPSS, Inc. Chicago, IL, USA), using the non-parametric exact test and the k-means cluster test analysis.

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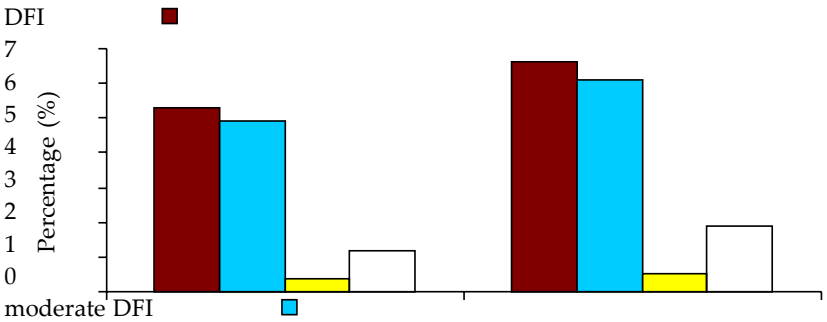
RESULTS AND DISCUSSION

Variability of percentages of spermatozoa with detectable DFI and HDS was assessed in bulls. Table 1 shows the mean values of SCSA param- eters and minimal and maximal values detectedfor individual bull spermatozoa. The highest per- centages of spermatozoa with DFI and HDS were 20.8% and 3.5%, respectively. The mean percentages of spermatozoa with h-DFI and HDS of bulls from the second group were statistically higher ($P < 0.01$)than those from the first group. The data of each group of bulls were compared in Figure 2.

On the basis of individual values, bulls were di- vided into two groups with significant differences between them determined by the k-means cluster test analysis ($P < 0.0001$). The first cluster was represented by 31 bulls with the mean percentage of spermatozoa with DFI 4% and the second one comprised six bulls with the mean percentage of spermatozoa with DFI 14.2%. These six bulls had significantly higher percentages of spermatozoa with DFI compared to the others. Two of them ex- ceeded the 15% threshold of DFI (16.1% and 20.8%).Other six bull spermatozoa had significantly higher percentages of spermatozoa with HDS. Using non-parametric exact test, significantly higher percentages of spermatozoa with h-DFI and HDS cells ($P < 0.0001$) were detected for boars than bulls. We did not find differences in mean percentages of spermatozoa with DFI between bulls and boars (Table 2). The highest percentageof spermatozoa with DFI detected in one boar was 17.6% and thus exceeded the 15% threshold, an-



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HDS (%)
(min-max)

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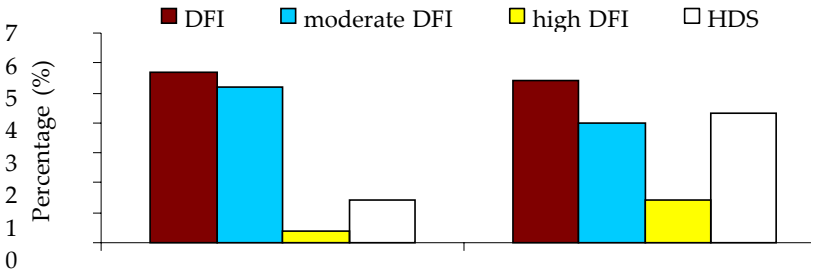
Bulls	37	94.4 ± 4.4 (79.2–98.5)	5.7 ± 4.4 (1.5–20.8)	5.2 ± 4.3 (0.9–20.2)	0.4 ± 0.2[a] (0.2–0.8)	1.4 ± 0.8[a] (0.4–3.5)
Boars	68	94.6 ± 2.7 (82.4–98.38)	5.4 ± 2.7 (1.6–17.6)	4.0 ± 2.4 (0.8–15.1)	1.4 ± 0.9[b] (0.3–4.2)	4.3 ± 1.8[b] (1.0–10.2)

[a, b]Values with different subscripts in the same column were highly significantly different ($P < 0.0001$)

other boar was found to be close to the thresholdof 15%. The highest percentage of spermatozoa with HDS was 10.2%. Boars were also divided into two clusters according to the levels of mean percentages of spermatozoa with DFI. Five boars had significantly higher percentages of sperma- tozoa with DFI than the other 63 boars. The mean percentages of sperm with DFI in the first and the second cluster were 4.9% and 12.4%, respec- tively. The k-means cluster test analysis showed that eighteen boars had significantly lower HDS compared to the other fifty boars. One boar had significantly higher percentage of spermatozoa with both DFI and HDS.

The comparison of the mean percentage of SCSA parameters of spermatozoa in groups of bull and boar semen is demonstrated in Figure 3. No cor- relation was found between DFI and HDS in all data measured and it is consistent with findingsof Evenson and Jost (2000). Cells with m-DFI and h-DFI likely have distinct biochemical properties that differentially influence male fertility poten- tial (Evenson *et al*., 2002). Therefore we examined whether an ejaculate with a high percentage of spermatozoa with DFI cells would also show a highpercentage of spermatozoa with h-DFI.



Significant correlations between percentages of spermatozoa with h-DFI and DFI were found withinall boars and bulls evaluated ($r = 0.284$, $P < 0.01$).

Fertilizing ability of one bull from group 1, with sperm with the highest percentage of spermatozoa with DFI 20.8% was approximately by 10% lower than the mean fertilizing ability of all bulls fromthe same group (49.8% vs. 60.6%). Also fertility of bulls from group 1 was significantly higher than in bulls from group 2 (60.6% vs. 43.3%) as shownin Table 1. Despite significantly decreased fertility of bulls from group 2 in comparison to group 1, differences between mean percentages of sperm with DFI of respective groups were not signifi- cant. Percentage of spermatozoa with h-DFI was significantly higher in group 2 compared to group

1. Percentages of spermatozoa with DFI in our studywere relatively low, but in spite of that there was evidence, that these data

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corresponded with the factthat fertilizing ability decreased with increased per-centage of spermatozoa with DFI (Ballachey *et al*., 1987; Evenson *et al*., 1999; Bochenek *et al*., 2001).

A threshold of >30% DFI was statistically de-rived for significant lack of fertility potential inhumans (Evenson *et al*., 2002). Larson-Cook *et al*. (2003) observed significant decrease in fertility if

1 2

Bulls Boars

Figure 3. The mean percentages of spermatozoa with respective SCSAparameters in bull and boar semen

the percentage of spermatozoa with DFI exceeded 27%. Fecundability declines as a function of thepercentage of sperm with abnormal chromatin and becomes low when aberrant cells reach >40% (Spano *et al*., 2000).

The objective of this study was to measure spermchromatin integrity in farm animals (bulls and boars) by the SCSA method, which is defined as susceptibility to denaturation *in situ*. SCSA is a pre-dictive tool of the time necessary to conception ora failure to conceive. Some animal studies measure semen samples to obtain diagnostic and prognos- tic values (Ballachey *et al*., 1987; Evenson and Jost, 1994). It was documented that sperm DNA integrityis a more objective marker of sperm function as opposed to the standard sperm parameters (Zini *et al*., 2001; Evenson *et al*., 2002). Recent studies have shown negative correlation between DNA damage and *in vitro* fertilization in humans (Sun *et al*., 1997;Lopes *et al*., 1998). Some authors published nega- tive correlations between DNA fragmentation indexand other conventional semen parameters, such as motility, morphology and concentration (Sun *et al*.,1997; Giwercman *et al*., 2003; Sills *et al*., 2004).

Optimal sperm DNA packaging seems to be nec-essary for full expression of male fertility poten-tial (Spano *et al*., 2000). Freezing-thawing samples several times deteriorate sperm quality (Evenson and Jost, 2000). Generally, overall sperm quality deteriorates after cryopreservation (Spano *et al*., 1999). On the other hand, frozen sperm samples, thawed one time and then immediately refrozen, do not have significantly altered SCSA data relativeto fresh samples (Evenson and Jost, 2000). Sperm DNA quality from some subfertile stallions may decline at a greater rate than spermatozoa fromfertile stallions, when exposed to similar storage conditions (Love *et al*., 2002).

Normal sperm chromatin has approximately fivefold decrease in binding capacity for DNA dyesand fluorochromes relative to the same DNA con- tent in round spermatids. Percentage of immature spermatozoa appeared to have a threshold 17% for pregnancy success in humans, but the confidence level is not significant (Larson *et al*., 2000). Evenson*et al*. (2003) observed significantly lower fertilizationrates in the IVF process in men with HDS higher than 15%. Some studies have shown that patients attending infertility clinics often had an increased DNA stainability (Evenson and Melamed, 1983; Engh *et al*., 1992). Our results do not show high levels of immature forms of spermatozoa withinall animals evaluated. In most cases, the defects of DNA stainability and DNA denaturation are mu- tually excluded and any single cell has not both defects (Evenson and Jost, 2000).

SCSA method is widely used all over the world and our data confirm the fact, that this

analysis maybe of a good diagnostic value for predicting fertilitypotential of farm animals, such as bulls and boars.

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