Bull and boar sperm DNA integrity evaluated by sperm chromatin structure assay in the Czech Republic

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ABSTRACT: Analysis of sperm parameters is very important for predicting the outcome of assisted reproductive techniques and is necessary for determination of fertility potential of males tested for artificial insemination. In our study we have determined the level of bull and boar sperm DNA damage by Sperm Chromatin Structure Assay (SCSA). This test is based on increased susceptibility of altered DNA (strand breaks) in sperm nuclear chromatin to *in situ* denaturation measured by flow cytometry after staining with acridine orange (AO). Sperm chromatin damage was quantified by percentages of spermatozoa with detectable DNA Fragmentation Index – DFI divided into moderate (m-DFI) and high (h-DFI) DFI. Percentage of immature cells (HDS; cells with High DNA Stainability) was also evaluated. We measured sperm SCSA parameters in a total of 37 bulls in two groups from different localities and 68 boar samples from one locality. Significantly higher percentage of spermatozoa with detectable DFI was detected in six bulls (16.2%) and a significantly higher percentage of immature cell forms (HDS) was found in other six bulls (16.2%) among all tested bulls. The mean percentages of spermatozoa with h-DFI and HDS of bulls from the second group were statistically higher than those from the first group ($P < 0.01$). Five boars (7.4%) of all tested boars had significantly higher percentage of spermatozoa with DFI and 18 boars (26.5%) had significantly higher percentage of sperm with HDS compared to the other boars. Both percentages of spermatozoa with DFI and HDS were significantly higher in one boar compared to the others. Boars had significantly higher percentages of spermatozoa with h-DFI and HDS ($P < 0.0001$) in comparison to bulls. For individual bulls, the highest percentages of spermatozoa with DFI and HDS were 20.8% and 3.5%, respectively while for boars these were 17.6% and 10.2%, respectively. No significant correlations were found between percentages of spermatozoa with DFI and HDS. This sensitive procedure seems to be convenient as additional method for semen quality detection in farm animals before their exploitation in breeding.

Keywords: sperm; DNA integrity; flow cytometry; bull; boar; fertility

There are several agents, which can impair spermatogenesis. In this complicated process stem spermatogonia 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 environmental and/or toxic agents. This may lead to decreased semen quality.

Sperm parameters are associated with male fertility or infertility. Morphology evaluation is widely used for predicting fertility potential in farm animals (Johnson *et al*., 1998; Chacon, 2001) and in humans (Kruger *et al*., 1986; Vawda *et al*., 1996).

Presently, conventional methods for evaluating male fertility are rather unreliable predictors of fertility because of small numbers of measured spermatozoa and the subjective evaluation of cells by the technician. This problem can be minimized by the flow cytometry measurement, which has been used for sexing sperm (Johnson *et al*., 1987, 1989), sperm viability (Garner *et al*., 1986), acrosome 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 testicular 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 development is very much related to the degree of DNA

damage (Ahmadi and Soon-Chye, 1999). Mutagen exposure can lead to the decreased sperm production 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 effect on 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 chromatin in 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 method is based on the fact that DNA without single and double strand breaks is not susceptible to denaturizing conditions characterised by pH = 1.2. Disturbed chromatin integrity is characterized by the presence of single and double strand breaks in DNA molecules that lead to formation of denatured single stranded segments (ssDNA). These are quantified

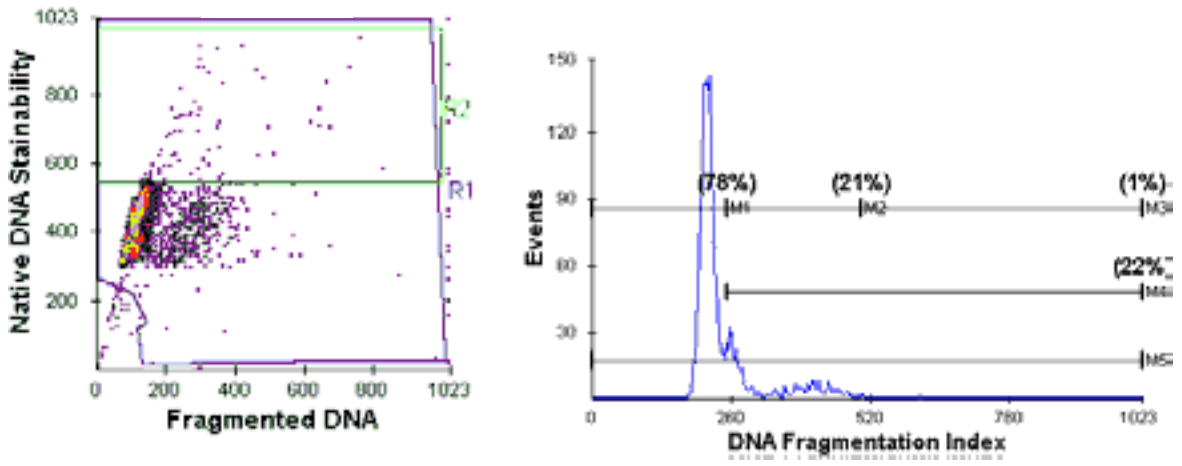

Figure 1. Examples of SCSA two-parameter cytogram (A) and histogram (B) of individual bull sperm cells. Each cell is represented by dots for a total of 5000 cells per sample. Each cell's position is based on the amount of green (native DNA stainability) and red fluorescence (fragmented DNA) emitted from 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 AO when 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 fluorescence. After sample analysis, several cell populations 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 spermatids which show about fivefold lower levels of chromatin condensation compared to mature forms (Evenson and Jost, 2000).

Figure 1B then shows the main population (marker M1) and DFI population (marker M4). Cells with detectable DFI were divided into moderate DFI (m-DFI – medium sperm damage – marker M2) and h-DFI (high sperm chromatin damage – marker M3). From studies on human semen samples, there is strong evidence that DFI thresholds of 0–15%, 16–29% and ≥30% relate to high, moderate and very low fertility potential, 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 breeding 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 insemination 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 samples 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 associated with single (denaturated) and double (native) 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 mature 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 parameter 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 N NaCl, 0.01 M Tris, and 0.001 M EDTA, pH = 6.8) to bring sperm concentration to 1.5×10^6 per ml. 200 µl of 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 denaturation. 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 light and 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 succession for statistical considerations; the second sample was taken from the same thawed aliquot, diluted appropriately, processed for the SCSA and measured.

Evaluation and statistical analysis

In each sample, green and red fluorescence of 5 000 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	Number of bulls (*n*)	Pegnancy rate* (%)	Non-detectable DFI (%) (min-max) **	DFI (%) (min-max)	m-DFI (%) (min-max)	h-DFI (%) (min-max)	HDS (%) (min-max)
1	27	60.6 [a]	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)
2	10	43.3 [b]	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)

[a, b]values with different subscripts in the same column were significantly different ($P < 0.01$)

*in heifers on Day 90 after the first insemination

**minimal and maximal values of respective bull spermatozoa

data were saved in the list mode and transferred to 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) were analysed 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.

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 parameters and minimal and maximal values detected for individual bull spermatozoa. The highest percentages 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 divided 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 exceeded 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 percentage of spermatozoa with DFI detected in one boar was 17.6% and thus exceeded the 15% threshold, an-

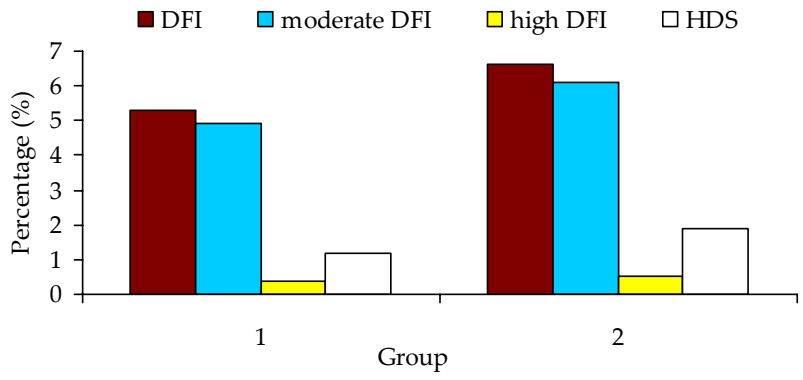

Figure 2. The mean percentages of bull spermatozoa with respective SCSA parameters

Table 2. The mean values (±SD) measured by SCSA in bull and boar sperm

Species	Number of males (*n*)	Non-detectable DFI (%) (min-max)*	DFI (%) (min-max)	m-DFI (%) (min-max)	h-DFI (%) (min-max)	HDS (%) (min-max)
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 ($P < 0.0001$)
*minimal and maximal values of respective bull and boar spermatozoa

other boar was found to be close to the threshold of 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 spermatozoa 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%, respectively. 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 correlation was found between DFI and HDS in all data measured and it is consistent with findings of Evenson and Jost (2000). Cells with m-DFI and h-DFI likely have distinct biochemical properties that differentially influence male fertility potential (Evenson *et al.*, 2002). Therefore we examined whether an ejaculate with a high percentage of spermatozoa with DFI cells would also show a high percentage of spermatozoa with h-DFI.

Significant correlations between percentages of spermatozoa with h-DFI and DFI were found within all 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 from the 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 shown in 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 significant. Percentage of spermatozoa with h-DFI was significantly higher in group 2 compared to group 1. Percentages of spermatozoa with DFI in our study were relatively low, but in spite of that there was evidence, that these data corresponded with the fact that fertilizing ability decreased with increased percentage of spermatozoa with DFI (Ballachey *et al.*, 1987; Evenson *et al.*, 1999; Bochenek *et al.*, 2001).

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

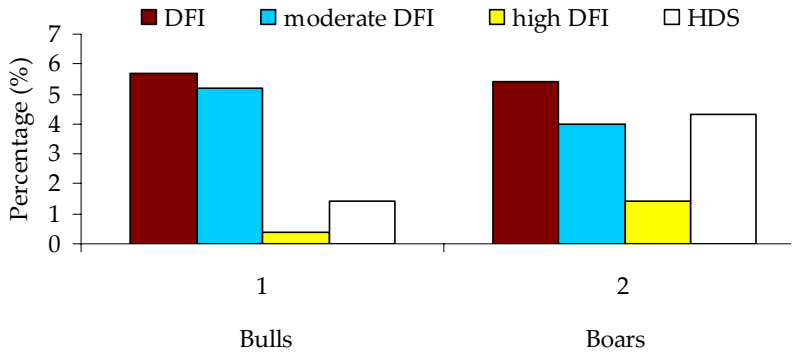

Figure 3. The mean percentages of spermatozoa with respective SCSA parameters in bull and boar semen

the percentage of spermatozoa with DFI exceeded 27%. Fecundability declines as a function of the percentage 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 sperm chromatin integrity in farm animals (bulls and boars) by the SCSA method, which is defined as susceptibility to denaturation *in situ*. SCSA is a predictive tool of the time necessary to conception or a failure to conceive. Some animal studies measure semen samples to obtain diagnostic and prognostic values (Ballachey *et al*., 1987; Evenson and Jost, 1994). It was documented that sperm DNA integrity is 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 negative correlations between DNA fragmentation index and 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 necessary for full expression of male fertility potential (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 relative to fresh samples (Evenson and Jost, 2000). Sperm DNA quality from some subfertile stallions may decline at a greater rate than spermatozoa from fertile stallions, when exposed to similar storage conditions (Love *et al*., 2002).

Normal sperm chromatin has approximately fivefold decrease in binding capacity for DNA dyes and fluorochromes relative to the same DNA content 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 fertilization rates 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 within all animals evaluated. In most cases, the defects of DNA stainability and DNA denaturation are mutually 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 may be of a good diagnostic value for predicting fertility potential of farm animals, such as bulls and boars.

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