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FUNCTIONAL CHARACTERISTICS OF BOVINE SPERMATOZOA IN RELATION TO THE BODY CONDITION SCORE OF BULLS

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ABSTRACT

This study was aimed at examining possible impact of the body condition of breeding bulls on the motility and viability of the sperm following freezing-thawing. The breeding bulls (n = 16) of Holstein and Czech Fleckvieh breeds were classified to the body condition score (BCS) grade according to a five-point scale with an accuracy of 0.25 points. The sperm samples of the bulls estimated as BCS grades 2, 3 or 4 were frozen in a programmable freezing device and stored in a liquid nitrogen for approximately one year. Following thawing the motility parameters (total motility – TM; progressive movement – PM) were analysed using the CASA Sperm Vision™ system at the intervals of 0, 0.5 or 2 h post-thaw. Viability parameters of sperm - plasma membrane integrity (*peanut* agglutinin, PNA-FITC), apoptotic rate (Yo-Pro-1) and dead/necrotic cell (propidium iodide /DAPI) rates were analysed at the same day using fluorescent microscope. Only minor non-significant difference was observed between BCS2 and BCS3 bull sperm in all studied parameters. However, significant differences in the sperm parameters were noted between bulls with BCS2 and BCS4. In particular, the BCS4 bulls showed significantly lower ($p < 0.5$) TM and PM than the BCS2 bulls. Moreover, significantly higher proportion ($p < 0.5$) of sperm with damaged plasma membrane and dead/necrotic sperm was revealed in the BCS4 bulls compared to the bulls of BCS2. These observations indicate that higher score of the body condition may negatively affect the quality of bull ejaculates.

Key words: bull; BCS; sperm; motility; viability

INTRODUCTION

Fertility of the bull is a major factor contributing to overall reproductive performance of cattle. Predicting the fertility of bulls is one of key area of agricultural research. Semen quality varies greatly from bull to bull. Semen from certain bulls may be of acceptable quality at collection but does not survive cryopreservation. The freezing and thawing process can adversely affect the nucleus, plasma membrane, acrosomal and mitochondrial membranes of spermatozoa (Chatterjee *et al.*, 2001; Aires *et al*., 2003; Amirat *et al*., 2004). This can adversely affect processes required for successful *in vivo* fertilization of the oocyte (Bailey *et al*., 2000).

Previous studies showed that many factors affect semen quality and the bull fertility including scrotal circumference (abnormal scrotal thermoregulation; Coulter *et al.*, 1997), sperm

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morphology, motility, physical normality of the bull and his mating ability, libido, age and the body condition (Barth *et al.*, 1995). The evaluation of body condition score (BCS) is an assessment of the proportion of muscle and of body fat, which the male has, and it is determined on a scale of 1 to 5, where "1" – being very thin and "5" – being overweight. BCS, or degree of fatness, seems to be the most reliable indicator of well-being of an animal, and, when coupled with changes in body weight, provide a useful indicator to assess reproductive potential (Dunn and Moss, 1992). If the animal is too thin or overweight, breeding strength and quality of the ejaculate may be affected (Neary and Yager, 2002). The quality of bull semen may be positively affected by quality and level of livestock feed. Production of quality ejaculates requires a diet which ensures adequate nutrition levels and the sufficient amount of essential vitamins and mineral substances. Bulls in poor body condition have lower fertilizing ability (Neary and Yager, 2002).

There are only few reports about effect of BCS on sperm characteristics and fertilizing ability of beef bulls (Addas, 2011; Barth and Waldner, 2002; Dunn and Moss, 1992). However, there are no sufficient data about effect of BCS in bulls on the sperm viability in regards to fluorescent markers of the cell death, membrane status and CASA-measured motility. The aim of this study was to evaluate effect of body condition score of breeding bulls on several functional parameters of spermatozoa.

MATERIAL AND METHODS

Semen collecting and processing

The observations were made in a bull housing facility and laboratory at a single AI center, where 8 Holstein (H) and 8 Czech Fleckvieh (F) bulls were selected for monitoring. The selected bulls were from 1 to 6 years old and the frequency of semen collection for all was once weekly. One sample of ejaculate was obtained from each bull using an artificial vagina. The BCS of selected bulls was evaluated by a five point scale with an accuracy of 0.25 points at the time of ejaculate collecting according to methodology of body condition scoring especially for H and F breeds, respectively. The optimum BCS on five point scale differed between evaluated breeds in relation to different requirements in accordance with production type - milk in H and dual purpose in F. The volume of semen samples (VOL) was measured using an electronic scale (Scout Pro, OHAUS®), sperm concentration (DEN) using a spectrophotometer (GENESYS 10vis, Thermo Scientific®), and percentage of motile spermatozoa (ACT) subjectively by phase contrast microscopy (LP 3000, Arsenal®) immediately after collecting. In addition, we evaluated the percentage of live sperm by staining before diluting and freezing in accordance with standard methodology as following: a drop of semen was mixed with eosin on a preheated microscope slide, spread, then examined under a phase contrast microscope at 1000x magnification and with oil immersion. We classified a minimum of 100 spermatozoa as either dead (with red heads) or live (with white heads) and expressed this as a percentage rate.

Only fresh semen with required quality (minimum progressive motility 70 % and sperm concentration 0.7×10^6 mm^{-3}) was used for the subsequent processing of samples for observation according to common standards used for producing AI doses. The samples of semen were diluted with AndroMed® (Minitüb, Tiefenbach, Germany), a commercially produced extender containing soybean lecithin extract. Polyvinyl chloride (PVC) straws (0.25 cm^3; IMV) were filled, cooled to 4 °C and equilibrated for 90 min. Subsequently, they were frozen in a programmable freezing device (IMV-Digitcool, L'Aigle, France) then plunged into liquid nitrogen for storage.

Sperm motility analysis

The straws were thawed in a water bath at 37 °C for 1 min. Sperm total motility and progressive movement were measured using a CASA system (Sperm Vision, Minitub Slovakia Ltd) at 0, 0.5 and 2 h after removal from storage. Between these time points the samples were incubated in a saline with 1 % of foetal calf serum (saline-FCS) at 37 °C in the incubator. Spermatozoa were transferred by a pipette into a Leja counting chamber with the depth of 10 µm. The chamber was placed under a Zeiss Axioscope A.1 phase-contrast microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) with heating plate (37 °C) at 200x magnification. The camera transferred the image into a computer,

where sperm motility was determined by the Sperm Vision software. In each sample at least 6 view fields were counted. The values of a total motility (M) and progressive movement (PM), measured at several time points (0, 0.5 and 2 h) during the day, were summarized and the average values per each day were presented in the tables.

Fluorescent assays

For plasma membrane integrity the sperm samples were stained with fluorescence-labelled lectin - *Arachis hypogaea* peanut agglutinin (PNA-FITC; Molecular Probes, Lucerne, Switzerland) in combination with propidium iodide (for the detection of dead/necrotic cells). The sperm samples were unfixed, allowing PNA-FITC labelling only in spermatozoa with disrupted or otherwise damaged plasma membrane, whilst sperm cells with intact membranes remained unstained. Following washing in saline-FCS, the sperm suspension was incubated in staining solution containing 20 µmol.l^{-1} of PNA-FITC and 5 µg.ml^{-1} PI in saline-FCS for 20 min at room temperature. After the incubation, sperm samples were washed in saline and, following centrifugation, 4 µl of the sperm suspension was placed onto a microscope slide, gently mixed with 4 µl of Vectashield mounting medium with DAPI (H-1200, Vector Laboratories Inc., Burlingame, CA, USA), a blue-fluorescent DNA stain which marks nucleoplasma of all sperm cells in samples. The obtained drops were flattened with a coverslip and immediately observed under a Leica inverted fluorescent microscope with respective bandwidth filters for green, red and blue fluorescence.

For detection of apoptosis the sperm were stained with a Yo-Pro-1 specific green fluorochrome (Molecular Probes, Lucerne, Switzerland) in combination with PI for identification of dead sperm cells. Following washing in saline-FCS the sperm suspension was incubated in a staining solution: saline-FCS with 5 µmol.l^{-1} Yo-Pro-1 and 5 µg.ml^{-1} of PI. After 20 min staining at room temperature, the sperm samples were washed in a saline-FCS solution and 4 µl of sperm suspension were placed onto a microscope slide, mixed with 4 µl of Vectashield with DAPI and the drop was flattened with a coverslip. The preparations were immediately evaluated under a Leica fluorescent microscope (Leica Microsystems, Wetzlar, Germany) with special filters for green, red and blue fluorescence. The green fluorescing sperm cells were regarded as apoptotic cells. The cells colored pink (stained by propidium iodide) were considered as dead or necrotic cells.

Statistics

The results of a whole or progressive motility were statistically processed after summarization of the values obtained from several measurements. For sperm motility analysis at least 7 view fields per each group were evaluated (at least 750 sperm cells per one experiment). Average values were calculated from three measurements during the day. The sperm images were made by a camera equipped with the Leica fluorescent microscope from and number of sperm cells was counted from a PC monitor. The experiments were performed in five repeats. In each experiment, about 8 - 10 microscopic view fields per each group were photographed. Totally, more than 1500 spermatozoa per each group were counted. Comparisons of arithmetic means between BCS groups were performed by a repeated measure ANOVA and t-test. The statistical analysis was performed with original data using the Statistix analytical software (Version 8.0; Anonymous, 2001). The data in table are represented as the mean ± standard error of the mean.

RESULTS

Sperm motility characteristics

The tested bull (n = 16) were arranged into three groups according to their body condition score (BCS) as belonging to BCS2 (n = 6), BCS3 (n = 6) and BCS4 (n = 4). All examined bulls showed average sperm motility (M) after thawing 42.5 % and progressive movement (PM) was 39.6 % (Table 1.). The higher average motility and progressive movement ($p < 0.05$) were observed in the group of the bulls with BCS2 when compared with the BCS4 bulls. The lowest sperm total M and PM was determined in the BCS4 bulls. In the group of BCS3 bulls an average total M and PM were about 42 % and 39 %, respectively.

Table 1. Sperm motility parameters in relation to BCS of bulls (mean ± S.E.M.)

BCS of bulls	No. bulls	Motility	Progressive movement
BCS2	6	56.26 ± 2.21[a]	52.93 ± 2.37[a]
BCS3	6	42.21 ± 2.06	39.31 ± 2.02
BCS4	4	28.94 ± 1.98[b]	26.61 ± 1.86[b]
Average	16	42.47 ± 2.08	39.62 ± 2.10

a versus b – significant difference between BCS groups at $p < 0.05$

Sperm viability characteristics

Similar trends were observed at the evaluation of sperm viability characteristics according to BCS (Table 2.). The ratio of sperm with disintegrated plasma membrane (PNA-positive) was significantly lower ($p < 0.05$) in the BCS2 bulls than in the BCS4 bulls. Similarly, the occurrence of dead/necrotic spermatozoa was lowest in the BCS2 bulls. The highest ratio of apoptotic sperm (Yo-Pro-1-positive) was revealed in the BCS3 bulls. In this group of bulls the ratio of dead/necrotic, apoptotic and the sperm with disintegrated plasma membrane was higher compared with the BCS2 bulls. On the other hand, the highest proportion of dead/necrotic and plasma membrane-disintegrated sperm was observed in the BCS4 bulls.

Table 2. Sperm viability parameters in relation to BCS of bulls (mean ± S.E.M.)

BCS of bulls	No. bulls	PNA	Yo-Pro-1	PI
BCS2	6	24.03 ± 2.30[a]	15.62 ± 1.83	12.63 ± 1.49[a]
BCS3	6	27.07 ± 2.08[a]	18.85 ± 1.66	15.22 ± 1.62[a]
BCS4	4	35.06 ± 2.72[b]	15.37 ± 1.56	20.25 ± 1.85[b]

a versus b – significant difference between BCS groups at $p< 0.05$

Correlations among sperm characteristics

Highly significant ($p < 0.0001$) positive correlation was observed between the total motility and progressive movement in a whole population of bulls, irrespective to BCS. Moderate negative correlation was observed between the motility and sperm viability parameters: the PNA-, PI- and Yo-Pro-1-positive spermatozoa (Table 3.). When BCS was taken into consideration, the highly significant ($p < 0.0001$) positive correlation was noted between the total motility and progressive movement in the BCS2 and the BCS3 bulls. The high negative correlation between total motility and the apoptotic sperm ratio was determined in the BCS2 bulls ($r = -0.7458$). In the BCS3 group, the high negative correlation was observed between total motility and dead/necrotic sperm ($r = -0.7019$). In the BCS4 bulls a correlation was not determined due to low number of animals available in this group).

Table 3. Correlations between the sperm motility and other perm parameters according to BCS of bulls

BCS of bulls	M/PM r	M/PNA r	M/Yo-Pro-1 r	M/PI r
BCS2	0.996*	-0.358	-0.746	-0.213
BCS3	0.997*	-0.626	-0.530	-0.702
BCS4	N/D	ND	ND	ND
Average	0.998*	-0.634	491	-0.584

r - Pearson´s correlation coefficient (t-test); no correlation – 0.111 ± 0.333; moderate correlation – 0.334 ± 0.666; high correlation – 0.667 ± 0.999; * p < 0.0001; N/D – not determined; M- motility; PM – progressive movement

DISCUSSION

In this study we investigated the impact of BCS of breeding bulls on motility and viability characteristics of spermatozoa after freezing-thawing. Sperm motility is the most important indicator of the ejaculate quality. Motility of 30 % or higher is considered to be of acceptable quality (Person *et al.*, 2007). Good post-thawing motility parameters in association with low proportion of dead, apoptotic spermatozoa, or spermatozoa with damaged plasma membrane contribute to an improvement of fertilizing ability and higher percentage of embryo cleavage rate (Watson, 2000).

The results of our study demonstrate possible negative effect of higher body condition score of bulls on sperm total motility and progressive movement. Bulls with BCS4 showed considerably lower motility and progressive movement than the bulls with BCS2 or BCS3. Similarly, Coulter *et al.* (1997) reported that bulls, fed with a high-energy diet (having high BCS), showed decreased proportion of motile sperm and very poor progressive movement than bulls fed a moderate-energy diet.

Besides good motility characteristics, low content of apoptotic and dead/necrotic sperm in the ejaculate is also very important for proper fertilizing ability. Presence of high percentage of apoptotic spermatozoa in the semen dose could be one of the reasons for poor fertility of breeding bulls (Anzar *et al.*, 2002) and, similarly, spermatozoa with damaged or inactive membranes will have limited viability and fertilizing potential (Correa and Zavos, 1994). In our study, high negative correlation was found between the total motility and apoptotic sperm occurrence in BCS2 bulls. Similarly, negative correlations between motility and apoptotic sperm rate was determined by Zhang *et al*. (2008) on human spermatozoa. No large difference in the proportion of apoptotic sperm was recorded among tested groups in our experiments. This apoptosis occurrence was the highest in the BCS3 bulls.

For successful fertilization, high proportion of sperm with the intact membrane is an essential requirement for proper sperm cell function (Makarevich *et al.*, 2011). Significantly higher ratio of sperm with damaged plasma membrane was revealed in BCS4 bulls compared to BCS2 group. Only moderate negative correlations were confirmed between PNA-positive sperm and motility rate in the BCS2 and BCS3 bulls. Alm *et al.* (2001) found low but significant correlation between the proportion of viable cells and fertility, suggesting that the plasma membrane integrity evaluation can serve as a quality control method for frozen-thawed spermatozoa in breeding bulls.

The present results imply that overfeeding could adversely affect ejaculate, because overfed bulls have lower semen quality than underfed bulls. The BCS4 bulls showed higher occurrence of dead/necrotic spermatozoa than bulls from BCS2 or BCS3 groups. Similarly, other studies on beef bulls (Barth and Waldner, 2002) demonstrated that significantly fewer bulls with BCS2 or BCS4 grade or greater had satisfactory semen quality than bulls with a BCS3 grade (where satisfactory semen quality comprises at least 70 % of morphologically

normal sperm with the progressive movement of at minimum 60 %). Bulls fed a high-energy diet (having high BCS) showed greater proportion of sperm with various morphological defects than bulls fed a moderate-energy diet (Swanepoel *et al.*, 2008).

Body condition or degree of fatness seems to be the most reliable indicator of well-being of an animal, and, when coupled with changes in body weight, provides a useful key to assess reproductive potential (Dunn and Moss, 1992). Our results support an idea that high body condition of breeding bulls may have a negative effect on reproductive characteristics of the ejaculate.

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