SUMMARY

Keywords: bull sperm, sperm DNA fragmentation, flow cytometry, CASA

The PhD thesis entitled "Comparative research regarding the biological evaluation of bull semen from beef and dairy breeds " was developed in the Doctoral School of the University of Agricultural Sciences and Veterinary Medicine "Ion Ionescu de la Brad" University, as part of the Improve and develop human resources program for research and innovation through the doctoral school, HRD / CPP107 / DMI1.5 / S / 77222, Sectoral Operational Programme Human Resources Development 2007 -2013.

The thesis contains 228 pages and according to the currently regulations consists of two main parts. As a source of information and documentation, a number of 254 bibliographic titles on the subject of the thesis, from the literature in the country and abroad were used. The information was subsequently used to interpret the data obtained in the second the second part. A total number of 63 figures and 27 tables supported the data presented in the second part.

The originality of the research resides in the use of the latest technology ("state of art") to assess the biological value of semen in an objective, accurate and rapid manner. Additionally, the research aimed at testing a biomimetic selection method (single layer centrifugation) to observe the extent to which this technology may be implemented in laboratory practice.

The first part, entitled "ACTUAL STATE OF KNOWLEDGE", is divided into three chapters (42 pages), which presents data from the literature regarding the morphology and physiology of the reproductive system in bull, semen collection methods and the evaluation of biological value of bull semen by conventional and special methods.

The second part, entitled "Personal Contributions", contains a total of 119 pages and is divided into 9 chapters, which focus on the objectives and the importance of the research, the materials and methods used for research, results of the performed analyzes and related discussions, as well as general conclusions and practical recommendations.

The thesis contains original data published in scientific papers presented at the symposiums and congresses organized by the Faculty of Veterinary Medicine, congresses abroad and scientific journals indexed in Thomson-Reuters database (ISI).

The first chapter of the second part, entitled "The purpose and objectives of the thesis" presents the research objectives as follows:

- Establishing the importance of using modern techniques for bull semen evaluation in the assessment of the biological value of frozen bull semen, from beef and dairy breeds;
- Determination of DNA fragmentation by three different methods (Toluidine blue stain; Halomax kit Bos-Taurus, SCSA- flow cytometry) and the significance of this parameter in the evaluation of semen;
- Assessing the influence of environmental factors on the parameters from the cryobiological spermiogram in bull;
- Assessment of sperm mobility by computer asissted analysis (CASA) and sperm concentration by Nucleocounter equipment SP-100;
- Determining correlations between different parameters of frozen bull semen and fertility in cows, expressed as a non-return rate at 60 days;
- Determination of possible correlations between usual sperm parameters (morphology, mobility, and viability) and sperm chromatin integrity assessed by Toluidine Blue Stain;
- Evaluation of the biological value of sperm bulls from dairy and beef breeds by determining the functional sperm parameters (membrane integrity, acrosome integrity, assessing oxidative stress) using continuous flow cytometry;
- Implementation of biomimetic technologies of the last generation (simple layer centrifugation) to improve the quality of bull semen;

The objective of CHAPTER V. ASSESSING THE INFLUENCE OF ENVIRONMENTAL FACTORS ON THE BIOLOGICAL VALUE OF FROZEN-THAWED BULL SEMEN USING FLOW CYTOMETRY was to analyze the spermiogram parameters of donor bulls used for AI programs by flow cytometry and evaluate the influence of environmental factors on the biological value of bull sperm. Flow cytometry is a breakthrough technology, that enables the assessment of semen in an objective and nevertheless least, fast manner.

To achieve the purpose of the research, cryopreserved semen from 10 bulls Swedish Red and White breed housed at a semtest in Skara, collected in three distinct seasons, was used in the study. For the assessment of biological value of semen, a series of functional sperm parameters were analyzed using continuous flow cytometry and various combinations of fluorochroms. The following functional spermatic parameters were analyzed: plasma membrane integrity, acrosome membrane integrity, sperm chromatin structure, sperm mitochondrial membrane potential as well as oxidative stress.

The values of the daily weather parameters such as temperature (° C), humidity (%), pressure (hPa) and the daily amount of visible light (minutes per day), recorded in December 2010 - January 2012, were obtained from the Swedish Meteorological and Hydrological Institute (SMHI).

Membrane integrity was assessed by flow cytometry using propidium iodide staining and SYBR 14 (Thomas CA, 1997), while acrosome membrane changes were measured using fluorescein isothiocyanate FITC-PNA, and calcium ionophore A23187. In order to distinguish viable cells from non-viable ones, propidium iodide (PI) was added to this combination, according to the protocol described by Watson and Cariese, 2002.

A combination of several fluorochromes was used to determine the free radicals from thawed semen. The superoxide oxygen (O2) was detected using Hidroethidine (HE; Molecular Probes, Inc.), while for the detection of hydrogen peroxide (H2O2), 2 ', 7'- diclorodihidro-fluorescein diacetate (DCFDA, Molecular Probes, Inc.) was used. The Differentiation of the living and dead sperm cells was performed by adding Hoechst 33258 (HO) in the sample.

In order to assess the mitochondrial membrane integrity, semen was diluted with Buffer B to give a concentration of approximately 2.5 x 106 SPZ / mL, and an amount of 300 µl was mixed with 1.2 µl JC-1 (solution Stock 3 mM) and incubated for 40 minutes at 38 ° C.

Sperm chromatin integrity was evaluated using the sperm chromatin structure assay (SCSA) developed by Evenson et al. (1980). This test uses a metachromatic staining based on acridine orange (AO), to assess the susceptibility of sperm deoxyribonucleic acid (DNA-sperm) to acid-induced distortion. DNA fragmentation Index (DFI%) is defined as the ratio of denatured DNA, simple stranded (red fluorescence) and the amount of DNA integrity, double stranded (green fluorescent) and denatured DNA, simply stranded.

Our research showed that the proportion (%) (mean ± RMSE) of live sperm, dead, or dying varied between seasons, a significant difference ($p < 0.05$) being observed between the spring and summer season in terms of the proportion of live sperm cells. The lowest percentage of live sperm (41.54%) was recorded in summer season while in spring the highest percentage (47.62%) was registered. Moreover, the degree of DNA fragmentation sperm assessed using the sperm chromatin dispersion test (SCSA) was lowest in spring (4.57%), value that differed statistically ($p < 0.04$) compared with the summer season, when there was the highest percentage of sperm with fragmented DNA (6.4%).

The percentage of live sperm, positive H2O2 recorded in winter (0.08%) was lower compared to the one observed in the spring season (0.33%) and summer (0.13%), this result being explained by a decrease in the metabolic activity during winter season.

Our research showed that in Spring season, the percentage of live sperm with reacted acrosome was higher (0.36%) than in winter (0.30%), this result is a consequence of the involvement of H_2O_2 in the acrosome reaction.

The results suggest that an extensive study, conducted over a longer period of time, including seminal plasma enzymes testing is justified, since the evaluation of oxidative stress tends to be a prognostic tool in assisted reproductive technology.

The objective of CHAPTER VI. SEASONAL DYNAMIC OF MOBILITY, MORPHOLOGY AND CONCENTRATION OF FROZEN-THAWED SEMEN FROM RED-WHITE SWEDISH BULLS USED FOR A.I refers to the analysis of mobility, concentration and sperm morphology of frozen semen from Red White Swedish dairy bulls, to identify possible seasonal variations of these parameters. Sperm mobility was assessed by computer-assisted analysis (CASA) using SpermVision ™ system (MiniTűb, Tiefenbach, Germany), and the concentration of sperm was determined using Nucleocounter equipment SP-100.

To assess sperm morphology wet smears were prepared as described by Barth and Oko AD RJ (1989) using the "wedge" (Anon, 2010), and stained according to the protocol of Williams stain.

In our study, the highest values for hyperactivity were observed in winter (16.38%), associated with elevated curvilinear trajectory velocity (VCL) (138.10%), amplitude of head (ALH) (5.5%) and a decrease in the index of linearity (LIN) (0.34%), while in spring ALH parameter value was lower (4.95%) and the mean values ± RMSE parameter LIN, STR were

higher compared to other seasons, this pattern being characteristic for progressive, non-hyperactivate sperm cells.The highest values of sperm concentration were recorded in spring season (64.78%), this value being statistically significant different ($p < 0.05$) compared to summer (56.76%).

Following the evaluation of sperm morphology by Williams-Lagerlöof staining, no statistically significant differences were observed between seasons on the proportion of sperm cells with normal morphology, but the lowest percentage of sperm with normal morphology was recorded in the summer (84%). The percentage of sperm with secondary anomalies was similar in the three seasons, while the percentage of primary anomalies, differed significant ($p = 0.03$) between the spring season ($5 \pm 0.45\%$) and summer ($6.5 \pm 0.45\%$).

CHAPTER VII. CORRELATIONS REGARDING MOTILITY, MORPHOLOGY, VIABILITY AND CROMATIN INTEGRITY OF FROZEN-THAWED BULL SEMEN investigated possible correlations between the percentage of sperm with damaged sperm chromatin assessed using toluidine blue stain, and some conventional sperm parameters such as mobility, viability and morphology of sperm.

Cryopreserved semen straws from six bulls from dairy and beef breed (Holstein, Charolais) were used in this study. The percentage of viable sperm, was determined by observing the clear field, at x 1000magnitude. Morphological examination was conducted using eosin-nigrosină based method on the same analysis method used for assessing viability (Rodríguez-Martínez H., 2000). Sperm chromatin integrity was assessed using toluidine blue stain, according to the protocol described by Agarwal et al, 2004.

Cryopreserved semen from bulls in the study showed a low level of sperm with abnormal chromatin (4.658 ± 1.89), this parameter being negatively correlated with viability, the percentage of live sperm (mean ± SD) being 51.68 ± 9.44. The percentage of sperm with normal morphology varied between 83.33% and 91.8%, with a mean of 87.24 ± 4.89 while the average mobility was 40.18 ± 10.33 recorded.

The main purpose of CHAPTER VIII. ASSESSMENT OF FUNCTIONAL PARAMETERS IN FROZEN-THAWED SEMEN FROM DAIRY AND BEEF BULLS was to analyze the biological value of bull semen from two European units for semen processing, Viking Genetics, Skara (Sweden) and the Centre Cattle Breeders Association (Estonia) to establish

weather differences in in the biological value of semen might explain the variations in fertility observed in the field.

Advanced technologies such as flow cytometry, CASA system and Nucleocounter SP-100 were used for the comparative assessment of bull semen, the following parameters being determined: sperm concentration, sperm mobility, sperm morphology, membrane integrity, acrosome integrity, oxidative stress, mitochondrial potential and the degree of DNA fragmentation.

The mean concentration ± standard deviation (DV) was 92.5 ± 22 (million / ml) for beef bulls, valued that differed significantly ($p < 0.001$) compared to the one recorded for dairy breed bulls, respectively 54.75 ± 19 million / ml. Morphological examination revealed a statistically significant difference ($p < 0.05$) on the proportion of sperm with normal morphology. In the case of dairy breeds of bulls, the average (± SD) proportion of sperm with normal morphology was 87 ± 6.2%, while in the case of beef bulls, 73 ± 5.34% spermatozoa with normal morphology were observed.

The average total motility was 64.92 ± 12.85 % in beef bulls and 59.41 ± 14% in dairy breed bulls, while the average progressive motility in the two groups was 58.56 ± 12.31%, 55.59 ± 14.06% respectively. The average percentage of viable sperm was 45.8± 7.6% for dairy breed bulls, while in the case of beef bulls, a lower percentage of live sperm (39.45 ± 11%) was recorded. After assessing the acrosome integrity, it has been shown that the average percentage of sperm cells with reacted acrosome registered for beef bulls was 0.47 ± 0.74%, the amount being statistically different from that result obtained in the case of dairy breeds bulls (0.28 ± 0.18%) ($p < 0.05$). The percentage of sperm that had damage to the sperm DNA was significantly ($p < 0.01$) higher for beef bulls (6 ± 3.3%) compared to dairy one (3.77 ± 1.14%).

CHAPTER IX. SINGLE LAYER ON EFFECT OF SPIN CYCLE thawed bull sperm characteristics evaluated bull sperm functional parameters before and after separation by centrifugation simple layer during a test incubation for 6 hours. Centrifugation in simple colloidal layer involves centrifuging sperm using a substrate that colloidal Otherwise, seminal plasma is removed and is selected subpopulation of sperm with specific characteristics such as mobility, viability, chromatin integrity and normal morphology.

Qualitative assessment of the semen was performed at initial time (T0) and during a 6hours survival test (T6) before and after the preparation by colloid centrifugation. Additionally, sperm

parameters such as acrosome integrity, mitochondrial potential and sperm DNA fragmentation were evaluated.

After 6 hours of incubation, the progressive motility decreased dramatically in the control samples, while in the samples selected by centrifugation, the decrease was more discrete, the progressive motility varying between 47.8% and 65.8. The mean percentage (± SD) of progressive motile spermatozoa was approximately 18.8% in the control samples at T6, a higher percentage ($p < 0.001$) of progressive motile spermatozoa being observed in the SLC selected samples (47.8%). The percentage of sperm with damaged DNA increased during incubation test, ranging between 8.5 and 12.6, with a mean of 10.2 in samples that were not selected by SLC. Colloid centrifugation selected motile sperm with plasma membrane integrity, high respiratory activity and reduced degree of DNA fragmentation compared with control samples. Sperm characteristics were maintained during the test incubation, thus prolonging the longevity of sperm. Significant differences between control samples and those selected by SLC were more evident after 6 hours of incubation.

CAPITOLUL X. CORRELATIONS REGARDING THE BIOLOGICAL VALUE OF BULL SEMEN AND FERTILITY IN COWS focused on the correlations between functional parameters of frozen-thawed bull spermatozoa from beef and dairy breeds, assessed by flow cytometry and fertility in cows, expressed as the coefficient of non-return to 60 days.

A total of 9733 females were artificial inseminated, the average non return rate being 61.22% for beef bulls and 67.37% for dairy breed bulls. In the case of beef breed bulls, the average (mean±SD) total mobility was 64.93 ± 5.61% and the average percentage of progressive sperm wa 58.82± 4.32%. Positive correlations were observed between the non-return rate (NRR), and the percentage of progressive sperm ($r = 0.214$, $p < 0.05$), the average curvilinear velocity (VCL) to ($r = 0.247$, $p < 0.05$), as well as the oscillation index (WOB), ($r = 0.588$, $p < 0.05$).

The dairy bulls presented an average total mobility of 52.12 ± 3.87%, lower compared to the value recorded for beef bulls. Positive correlations were observed between non-return rate (NRR) and the percentage of hyperactive sperm ($r = 0.275$; $p < 0.05$) and motility ($r = 0.260$; $p < 0.05$) and progressive motility ($r = 0.458$; $p < 0.05$). In our study, the percentage of sperm with high mitochondrial activity was 12.01 ± 2.33 a% for beef breeds bulls, while the dairy bulls presented a significantly higher amount of sperm cells with high mitochondrial activity (47.25 ±

2.65%). In both cases, the DNA fragmentation index was negatively correlated with the non-return rate, r = -0.415 ($p < 0.05$) in beef bulls and r = -0.890, $p < 0.05$ in dairy bulls.

The objectives of the research presented in CHAPTER XI. CHAPTER X. CORRELATION REGARDING CERTAIN MORPHOLOGICAL CHARACTERISTICS OF CRYOPRESERVED BULL SPERMATOZOA AND FERTILITY IN COWS, were aimed at assessing the variation in the length of the sperm mid-piece from dairy and beef breed bulls and possible correlations with other parameters such as functional sperm motility and sperm mitochondrial potential. Additionally, the influence that this variation may have on the fertility bulls expressed by non-return rate at 60 days was also investigated.

For the study we used cryopreserved semen from four different breeds: Swedish Red White (5 bulls), which represented the dairy breed category, Charolais bulls (3 bulls), Limousin (6 bulls), Simmental (2 bulls) that represented the beef breed category. Semen smears were stained by Williams protocol and before taking measurements, the digital image was increased to a level that allows a clear distinction of the sperm mid piece from the the main piece.

The average (Mean ± SD) mid piece length was 13.66 ± 0.37 µm for beef bulls, and 13.33 ± 0.29 was µm for dairy bulls. In terms of non-return rate (NRR), the beef bulls recorded an overall non NRR of 65.49%, inferior to that seen for dairy breed bulls (67.37%). There was a positive correlation between the non-return rate and length sperm mid piece of dairy breed bulls (r = 0.691) and beef breed bulls (r = 0.560), indicating that as the length of the sperm mid piece is higher, the value of the non-return rate may increase.

Inter-relationships between mitochondrial activity, motility and sperm mid piece lenght were confirmed by a positive correlation between the sperm mid piece length and membrane potential ($\Delta\Psi m$), for beef bulls (r = 0.269), respectively dairy bulls (r = 0.472).

A correlation coefficient was observed between the percentage of live sperm with reacted acrosome and the sperm mid piece length in both dairy (R=0.578) and beef bulls (r=0.434). The dairy bulls presented a positive correlation, statistically powerful, between the average length of sperm mid-piece and BCF (r = 0.833), STR (r = 0.701), DAP (r = 0.815), DCL (R = 0947), DSL (r = 0.894), VAP (0822) and VCL (r = 0.935) and a moderate one between the sperm mid piece length and ALH (p = 0.575), VCL (p = 0.660), LIN (r = 0.525) and VSL (r = 0.452).

CHAPTER XII. GENERAL CONCLUSIONS AND RECCOMENTATIONS broadly outlines the most important conclusions of this PhD thesis, and offers a series of practical recommendations for the Biotechnology of Evaluation of bulls semen.