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REPRODUCTIVE BIOTECHNOLOGY IN ANIMAL HUSBANDRY – CURRENT STATUS AND FUTURE PROSPECTS[1]

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Abstract: To date there is still no optimal biotechnology which ensures maximum preservation of the functional parameters of the spermatozoa from buffaloes, boars and dogs. The aim of this research is to study the biological potential of seminal plasma proteins that are specific only to ejaculates with high cryotolerance and good quality parameters of the spermatozoa. The motility and velocity parameters of the spermatozoa were assessed by computer-assisted sperm analysis. Seminal plasma proteins were separated by size-exclusion liquid chromatography and characterized by polyacrylamide gel electrophoresis and mass spectrometry. Based on the results obtained, sperm diluents and methods for biological evaluation of the fertilization potential of the spermatozoa from buffalo bulls, boars and dogs were created and proposed for practical application.

Keywords: buffalo, boar, dog, seminal plasma proteins

Introduction

The preservation of genetic material from breeding animals is a priority for the livestock breeding in most developed countries in Europe and the world. The needs of the practice require the presence of gene banks in order to increase the number of nucleus herds, to conduct planned selection and to preserve gametes from valuable and highly productive animals, as well as endangered species. The successful functioning of a gene bank is always accompanied by an effective reproductive biotechnology for semen cryopreservation and artificial insemination (AI).

Reproductive biotechnologies are always associated with the quality control on reproductive traits of the breeding stock. This control includes both

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exterior parameters of the animal and functional assessment of the gametes**.** Many of the evaluation criteria are based on approved European parameters. Additional specific evaluation criteria are also used. They include precise control on the full reproductive cycle - from gametogenesis and in vitro fertilization to assessment of the fertilization potential of the gametes, as well as analyses on the implantation and pregnancy.

In Bulgaria there are long lasting traditions in the field of reproductive biology and in vitro technologies applicable to different types of farm animals. Biotechnologies for AI with preserved and cryopreserved semen are introduced in practice since mid-last century. Bulgarian scientists have developed one of the first efficient embryo biotechnology and from 1950 to 1990 new Bulgarian breeds were created: 5 in cattle- breeding, 8 in sheep, 1 in buffaloes, 1 in goats, 2 breeds and 3 hybrids in pig breeding. These results were achieved thanks to the rapid breed development and genetic improvement of our livestock and the massive implementation of the technology for AI. All this allowed the use of the elite breeding animals' reproductive potential in the most effective way.

Today, due to the new political and economic situation and after the transition from planned to market economy; there have been a lot of changes that affected in varying degrees the planned breeding and selection in livestock. These changes have affected the average annual production of meat and milk. The average annual milk production per capita declined from 275 kg for the period 1986-1989 to 165 kg for the period 2008-2011, meat production decreased for the respective periods from 90 kg to 30 kg.

Similar is the situation with the application of AI in animals. By 1991-1992, over 90% of the animals in Bulgaria were artificially inseminated. Today, these data are reflected in table 1.

Table 1. Artificial insemination in farm animals (Source: Ministry of Agriculture and Forestry of Bulgaria, direction "Agrostatistics"- survey "Number of livestock in Bulgaria up to 01.11.2002")

Animal species	Artificial insemination:	
	% of farms	% inseminated female animals
Cattles	37.5%	50.0%
Pigs	11.2%	27.8%
Sheep	1.7%	5.3%
Goats	0.8	0.2

In one of the best centers for AI in animals on the Balkan Peninsula, located in the town of Sliven, about 149 bulls were available in 1992-1993. Now the number of bulls is 18. Currently the national genetic reserve stores the following genetic material: 347 913 doses from 405 bulls of 33 breeds and 7300 doses from 23 rams of 10 autochthonous breeds.

This reduction of the milk and meat production is an illustration of the serious fall-off in Bulgarian livestock breeding in the area of planned breeding.
Complex approaches are required to deal with the situation, which can be summarized in the following directions:

- Sustainable development of livestock genetic resources;
- Increased number of animals undergoing selection control;
- Maintenance of an optimal number of animals in nucleus and reproductive herds;
- Expanded use of AI;
- Introduction of innovations in reproductive biotechnology for preservation and cryopreservation of gametes;
- Updated nutrition and breeding;
- Health control;
- Introduction of good manufacturing practices for sustainable production and animal welfare;
- Optimization of production, processing technology and marketing of food of animal origin.

Today, the biotechnology for cryopreservation of male gametes is not yet widely used in some species of farm and domestic animals such as boars, rams, buffaloes and dogs. The reasons for this stand in unresolved issues related to the lack of optimal biotechnology that ensures maximum preservation of the biological potential of the spermatozoa. A lot of additional research is required in these animal species. This is a prerequisite for search for new semen cryoprotectants and cryopreservation media in these species.

In recent years, more and more scientists focus their attention on the role of seminal plasma proteins (SPPs) and their association with the activation of signaling pathways responsible for the sperm functioning *(Furugen et al., 2012; De Lamirade et al., 1984; De Vries et al., 2003)*. It has been reported that some SPPs affect motility and survival rates of male gametes and can affect their fertilization capacity. To this end, there are still many unexplored concerns, especially when it comes to the role of SPPs on the spermatozoa of the boar, ram, buffalo bull and dog *(Daskalova et al., 2015; Kukov et al., 2012; Januskauskas at al., 2003; Martin et al.,2004; Moura 2005; O'Meara et al., 2007; Gradinarska et al., 2015).*

The lack of conclusive data on the mechanism of protection of the SPPs in sperm cryopreservation in these species, and scant information about SPP's role in the process of preservation and capacitation gave us a reason to make an attempt for separation and analysis of the SPPs with protective effects on the spermatozoa, in order to optimize the biotechnology for long-term sperm preservation *(Daskalova et al., 2012; Daskalova et al., 2014; Wysocki et al., 2015; Frazer and*

Strzezek, 2007; Strzezek et al., 2005; Thomas et al., 2006; Bailey et al., 2000; Kirilova et al., 2014; Ardon et al., 2013).

On the basis of this analysis and our resources, we turned our efforts towards the development of an effective biotechnology for long-term semen preservation based on native SPPs present only in ejaculates with high cryotolerance and good quality parameters of the spermatozoa. Based on these studies were created and proposed for practical application sperm diluents and methods for biological evaluation of the fertilization potential of spermatozoa from buffalo bulls, boars and dogs.

Materials and Methods

For the studies we used semen from elite breeding animals- 10 ejaculates from boars, 16 from Bulgarian Murrah buffalo bulls, 10 from dogs.

Buffalo bulls' semen is the property of Executive Agency on Selection and Reproduction in Animal Breeding (EASRAB) - Sofia and Sliven.

Boar ejaculates were collected using the gloved-hand technique from 10 Polish Larger White (average age of 2 years) used for breeding purposes in insemination centers in Olsztyn, Poland.

Dog semen was collected in cooperation with Central Veterinary Clinic – Sofia, Bulgaria. Ejaculates from clinically healthy dogs (4 to 11 years) were collected using the manual method.

Computer-assisted sperm analysis (CASA):

The motility and velocity of the spermatozoa were assessed by CASA System Sperm Class Analyzer® (Microptic®, Spain), analytical module „Motility and concentration".

- Buffalo bulls: 16 ejaculates were thawed and spermatological parameters were analyzed at the beginning of the experiment and at every hour until the 6th hour after thawing. CASA was performed using "Leja 20" chambers with 2 μl drop volume. A minimum of 1000 spermatozoa per sample were analyzed. Based on the received data, the ejaculates were classified into 2 groups– with high cryotolerance (group A) and low cryotolerance (group B) of the gametes.
- Dogs: CASA was performed on 10 fresh semen samples using cover slides (18x18 mm) with 8 μl drop volume. A minimum of 1000 spermatozoa per sample were analyzed. Based on the received data, the samples were distributed into 2 groups – with good (group 1) and poor (group 2) quality of the sperm.

Microscopic sperm motility evaluation:

- Boars: 10 ejaculates were thawed and divided into two groups: high cryotolerance (HCT) group with more than 40% motility after thawing and low cryotolerance (LCT) group with less than 5-10% motility after thawing.

Seminal plasma (SP) isolation:

- Buffalo Bulls: SP was isolated by centrifugation at 4ºC, 2000rpm for 10min and again at 12000rpm for 5min, where after it was filtrated through 0.22µm filter membrane Millipore®.
- Boars: SP was isolated from all samples by double centrifugation at room temperature at 3000x g for 5 min and again at 10000x g for 5 min.
- Dogs: SP was isolated from all samples by double centrifugation at 2500 rpm, 4°C, and 5 min and followed by 10000 rpm, 4°C, and 10 min.

High-Performance Liquid Chromatography (HPLC):

SPP separation was performed by High Performance Liquid Chromatography on Binary HPLC Pump 1525 with UV/Visible Detector 2489 (Waters Company®), with semi-preparative size exclusion chromatographic column TSK gel G3000SW, 21mm x 300mm, 10 to 500 kDa (Tosoh Bioscience®). Gel Filtration Markers Kit for Protein Molecular Weights 12,000-200,000 Da™ (Sigma-Aldrich®) was used for MW determination.
The distinct SPP fractions were collected for further analysis.

- Buffalo Bulls: Sample volume of 1000 µl was applied, at 20 min run time and 6 ml/min flow rate.
- Dogs: Sample volume of 150 µl was applied, at 20 min run time and 6 ml/min flow rate.

Fast Protein Liquid Chromatography (FPLC):

- Boars: Chromatography separation of SP from 5 boars with pre-researched HCT and 5 boars with LCT was performed. FPLC was performed using a Ceramic Hydroxyapatite Column type II (CHT) (Bio-Rad®) at 1 mL/min flow rate, 1000 µl sample volume and 5 mg/ml quantity of proteins.

Spectrophotometric analysis of protein concentration:

After the chromatography each collected protein fraction was analyzed spectrophotometrically for determination of protein concentration (Ultrospec 2000 UV/VIS Spectrophotometer, Pharmacia Biotech®).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

- Buffalo Bulls: The separated SPP fractions from group A were further characterized by 12% SDS-PAGE (TV100 Bio-Rad®). Standard protein marker (SigmaMarker™ wide range, 6.500-200.000 Da, Sigma-Aldrich®) was used for MW determination. Visualization of the protein bands was made by Coomassie Brilliant Blue staining.
- Boars: All obtained protein fractions were characterized by 15% SDS PAGE (Bio-Rad® Mini Protean tetra system, at 150 V DC). The gels visualization was done via the Coomassie brilliant blue (0.05%) method. SERVA® Protein Marker was used as standard.

Protein Identification by Mass spectrometry (MS):

- Boars: The proteins, characteristic only to ejaculates with HCT or LCT, were identified via MS. The protein bands of interest from the two different groups (LCT and HCT), were cut out from the SDS-PAGE gels and prepared for MS (Bruker-autoflex III smartbeam®) and identification. The results were compared against a database of protein sequences in the MASCOT application.

Results and Discussion

Buffalo bulls

Results from the HPLC analysis:

The results from the HPLC analysis show specific differences in the chromatographic profiles of the studied samples. Pronounced peaks are observed in all chromatograms that are more distinct at 280 nm wavelength. The proteins in those peaks vary from 5 to 500 kDa. The comparison of the results between ejaculates from group A and group B demonstrates differences in the chromatograms, which correspond to different quantitative and qualitative composition of proteins in the SP (figure 1). Groups of SPPs with pronounced peaks on 12 min (about 30 kDa) and 14 min (about 12 kDa) are found in ejaculates with proven high cryotolerance of the sperm. The same peaks, but with low light adsorption, are found in ejaculates with low cryotolerance of the gametes. This result speaks of a lower concentration of these proteins. Also, in group A there is a well pronounced peak between 16 and 18 min (molecular weight (MW) between 6 and 14 kDa), which is almost absent in group B.

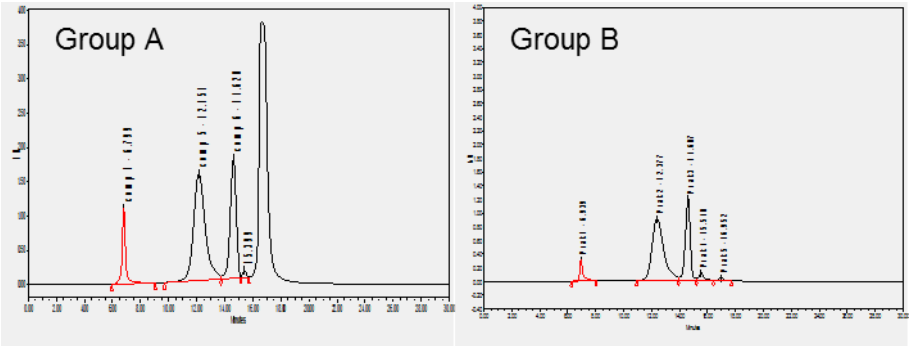

Figure 1. HPLC profile (λ=280 nm) of SPPs from Buffalo bull ejaculates with high sperm cryotolerance (group A) and ejaculates with low sperm cryotolerance (group B).

Results from the spectrophotometric analysis for protein concentration:

8 protein fractions were collected from the ejaculates with proven high cryotolerance the spermatozoa, which manifest into 8 well-defined peaks. The concentration of proteins varies from 1,076 mg/ml in fraction 4 to mg/ml 0,069 in fraction 8 (Table 2).

Table 2. Protein concentration in SPP fractions from Buffalo bull ejaculates with high cryotolerance of the spermatozoa

Quantity of proteins in SP from ejaculates with high cryotolerance			
Fraction 1	0,333 mg/ml	Fraction 5	0,465 mg/ml
Fraction 2	0,379 mg/ml	Fraction 6	0,223 mg/ml
Fraction 3	0,217 mg/ml	Fraction 7	0,183 mg/ml
Fraction 4	1,076 mg/ml	Fraction 8	0,069 mg/ml

Results from the SDS-PAGE of the SPPs from ejaculates with high cryotolerance of the gametes:

Proteins with high MW (200-150 kDa) are predominant in fraction 1. Low MW proteins (20-12 kDa) are predominant in fraction 4. It is noteworthy that lower concentrations of proteins are available in fractions 5 and 6. Also in fraction 1 protein bands with MW about 200 kDa are observed, as well as a small amount of proteins with lower MW, below 20 kDa. In faction 2 a protein band with MW about 90 kDa is seen. In faction 4 a large amount of proteins with low MW from 20 to 12 kDa and below 12 kDa can be seen. In fractions 5 and 6 protein bands with MW below 14 kDa are observable (figure 2).

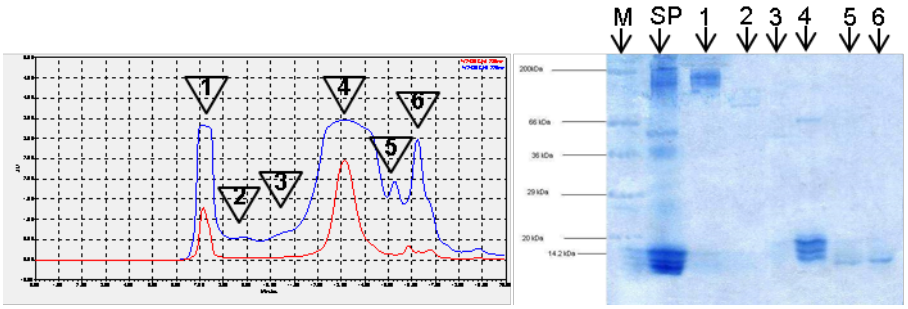

Figure 2. SDS-PAGE of the SPPs from Buffalo bull ejaculates after HPLC separation. M-Marker; SP- Seminal plasma; 1-6 – SPP fractions.

Boars

Results from the FPLC analysis:

The results obtained demonstrate differences in the protein profile of the SP from boar ejaculates with LCT and HCT of the spermatozoa (figure 3).

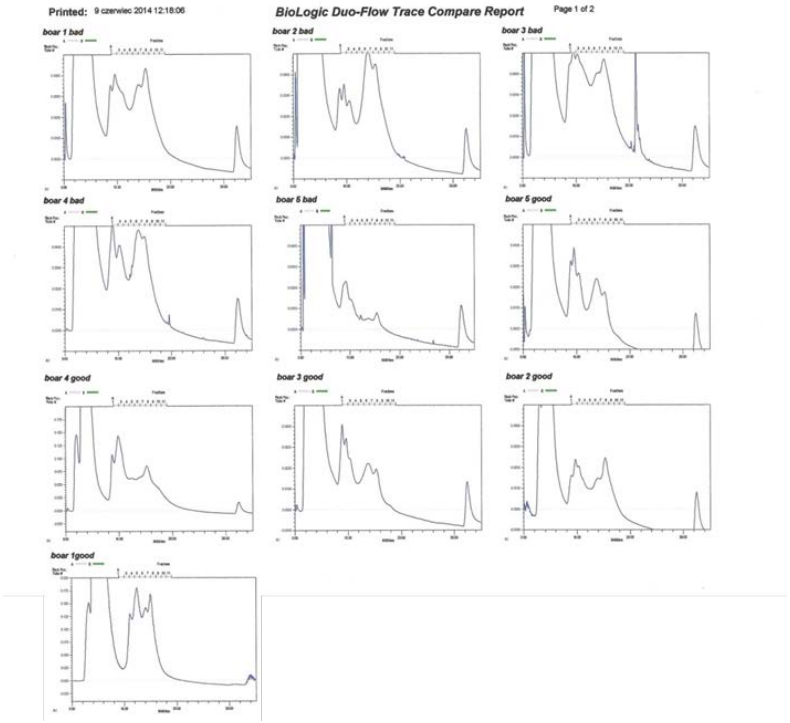

Figure 3. Chromatographic profiles of SP from 5 boars with LCT (bad) and 5 boars with HCT (good) of the gametes.

9 SPP fractions were collected from each SP after FPLC. The analysis of the chromatograms demonstrates significant differences between the chromatographic profiles of ejaculates with HCT and those with LCT.

Results from the spectrophotometric analysis of protein concentration:

5 HCT ejaculates and 5 LCT ejaculates were analyzed. The concentration of proteins varies from 5 mg/ml to 12.5 mg/ml (table 3).

Table 3. Protein concentration in SP from ejaculates with high and low cryotolerance of the gametes

Quantity of proteins in SP- HCT group:		Quantity of proteins in SP- LCT Group	
boar 1	5 mg/ml	boar 1	8.5 mg/ml
boar 2	7.75 mg/ml	boar 2	12.7 mg/ml
boar 3	11.5 mg/ml	boar 3	7.5 mg/ml
boar 4	5 mg/ml	boar 4	10.3 mg/ml
boar 5	12.5mg/ml	boar 5	10 mg/ml

Results from the SDS-PAGE of the SPPs with HCT of gametes:

All separated protein fractions were characterized by 15% SDS-PAGE. The gels of all tested animals were compared. The most significant differences were found between boars 1, 2 and 5 of the HCT group and boars 2, 3 and 5 of the LCT group.

The presence of protein bands specific to LCT and HCT was proven (figure 4). On gel 1(left) proteins identified solely in the SP from boars with LCT are framed in black, while on gel 2(right) are demonstrated protein bands established only in boar ejaculates with HCT of the spermatozoa.

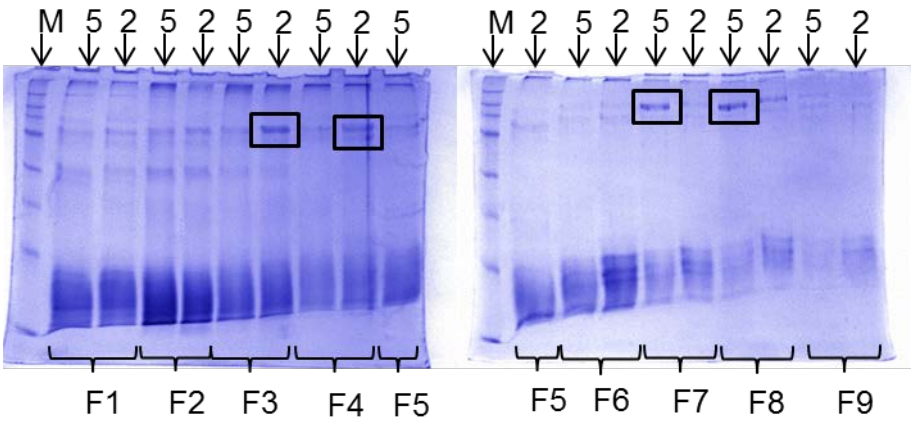

Figure 4. SDS-PAGE after FPLC separation of the SPPs from boar ejaculates with HCT (boar 5 good) and with LCT (boar 2 bad). M- Marker; F – SPPs fraction.

MS analysis for protein identification:

The proteins established in both groups were cut off from the gels and prepared for MS analysis. MS identified the protein found only in ejaculates with LCT as hexosaminidase B (HEXB) (score: 156 for GI/262072808, SUS SCROFA). A correlation was found between high levels of this protein in ejaculates with LCT and low motility of boar spermatozoa.

MS analysis showed that boar ejaculates with HCT have high levels of the protein Lactoferrin (LF) (score: 96 for GI/116488296, SUS SCROFA).

Dogs

Results from CASA:

Group 1 demonstrates significantly lower percentage of static spermatozoa, higher percentage of spermatozoa with progressive motility and significantly higher percentage of spermatozoa with rapid motility, when compared to group 2 (figure 5).

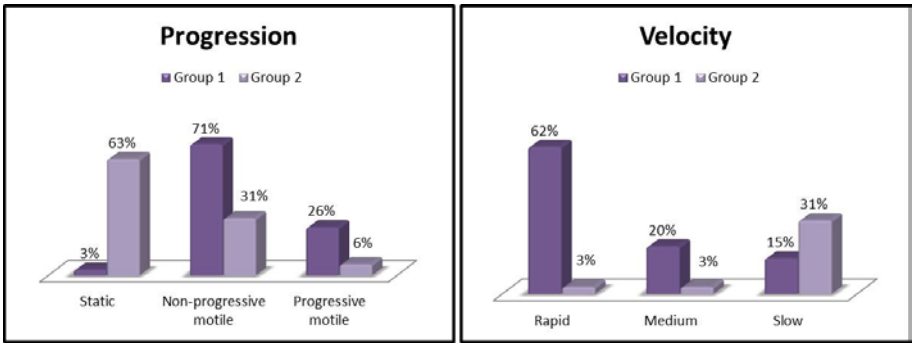

Figure 5. Comparative analysis of the motility and velocity of dog ejaculates with good and poor quality of the sperm

Results from the HPLC analysis:

Comparative HPLC analysis of the SPPs between the two groups establishes differences in the quantity of proteins contained in the separated fractions (figures 6-7).

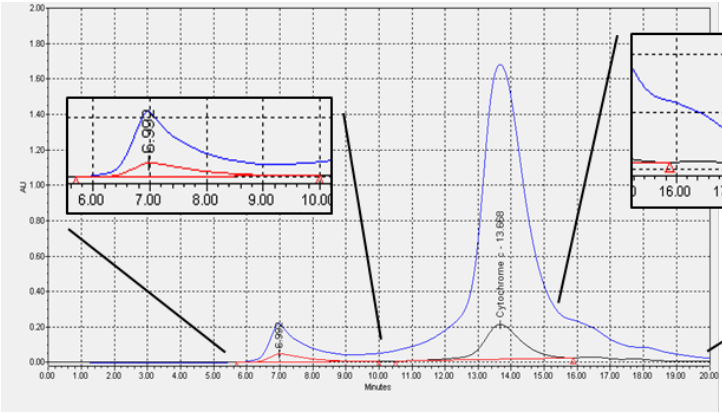

Figure 6. HPLC protein profile of SP from dogs with good quality parameters of the sperm (Group 1)

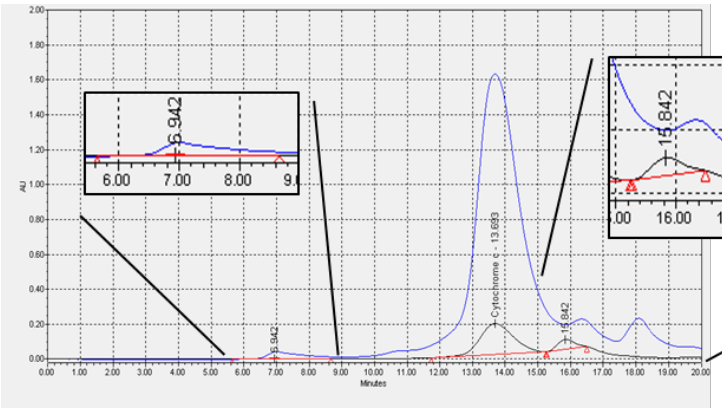

Figure 7. HPLC protein profile of SP from dogs with poor quality parameters of the sperm (Group 2)

The SPPs profile of group 1 demonstrates a well pronounced peak on 7th minute, which is almost absent in group 2. This peak contains proteins with MW over 200 kDa. It can be assumed that they belong to the group of zinc-binding proteins, which have an affinity for binding to the acrosomal region of the spermatozoa and exhibit a protective effect on the sperm plasma membrane.

Group 2 demonstrates a pronounced peak on 16th minute (below 12 kDa), which is less defined in group 1. Also there is a peak on 18th minute (below 10kDa), which is nearly absent in group 1.

Conclusion

Finding new phenotypic traits for gamete cryotolerance is an innovation that can be applied as a prognostic test in future practical use. A preliminary prognosis for ejaculates with high or low cryotolerance, related to specific SPPs, may be used in reproductive biotechnologies in animals.

The presence of the protein hexosaminidase B in boar SP is a phenotypic trait for spermatozoa with low cryotolerance. Lactoferrin is a phenotypic trait for high cryotolerance of boar semen.

The established specific chromatographic profile of SPPs from buffalo bull ejaculates with high cryotolerance of the spermatozoa can be applied in practice when assessing the quality of the semen in breeding animals.

The presence of proteins with MW over 200 kDa in dog SP is related to good motility and velocity parameters of canine spermatozoa. In ejaculates with poor quality parameters the presence of SPPs with low MW (under 12 kDa) is noticeable. The results obtained can be used in the field of reproductive biotechnology as a biological criterion for the quality of the semen.

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Reproduktivna biotehnologija u stočarstvu - trenutni status i budući izgledi

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Rezime

Do danas još uvek ne postoji optimalna biotehnologija koja osigurava maksimalno očuvanje funkcionalnih parametara spermatozoida bivola, svinja i pasa. Cilj ovog istraživanja je da se ispita biološki potencijal proteina semene plazme, koji su specifični samo za ejakulate sa visokim kriotolerancijom, i parametara kvaliteta spermatozoida. Parametri pokretljivosti i brzine spermatozoida su ocenjeni uz pomoć kompjuterske analize sperme. Proteini plazme sperme su razdvojeni ekskluzijom po veličini, tečnom hromatografijom i karakterisani poliakrilamidnom gel elektroforezom i masenom spektrometrijom.

Na osnovu dobijenih rezultata, stvoreni su diluenti sperme i metode za biološku evaluaciju potencijala oplodnje spermatozoida bivola, svinje i pasa i predloženi za praktičnu primenu.

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