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Original article

Chromatography analysis of seminal plasma proteins in buffalo semen samples with high and low cryotolerance

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Abstract

The aim of this study was to analyse and identify specific buffalo seminal plasma proteins (SPPs) responsible for sperm cryotolerance during low temperature storage. Computer Assisted Sperm Analysis (CASA) of the motility and viability of buffalo spermatozoa was performed before freezing and after thawing. Two sample groups were formed – ejaculates with high cryotolerance (group A) and low cryotolerance (group B). CASA demonstrated that the initial progressive motility after thawing of the spermatozoa in group A is significantly higher than in group B ($p<0.001$). Group B showed a significant increase in the percentage of static and non-progressive spermatozoa at 240 min, when compared to group A ($p<0.05$). SPPs, proteins in the cryoprotective medium (PM) and proteins in the mixture of PM and SP were separated by High Performance Liquid Chromatography (HPLC). Comparative analysis of the chromatographic profiles was performed to identify specific proteins related to sperm cryotolerance. SPPs profiles showed 5 distinct protein peaks in both groups, ranging from 500 kDa to 50 Da. Chromatograms of group A and group B showed quantitative and qualitative differences in protein content. Chromatograms of proteins in PM showed 11 well-expressed peaks. HPLC analysis of the mixtures of SPPs from the two groups and PM visualized the formation of a new bio-complex structure expressed by a protein peak specific for group A (7.674 min, AU 1.50). This protein peak can be referred as a phenotypic trait for buffalo ejaculates with high sperm cryotolerance.

Key words: buffalo, seminal plasma proteins, cryotolerance, HPLC

Introduction

The use of cryopreserved semen for the purposes of artificial insemination (AI) in buffaloes is relatively limited due to a number of biological factors. Such factors are the low cryotolerance and reduced fertility of buffalo spermatozoa, compared to those in bulls (Singh and Pant 2000, Kumaresan et al. 2005, Andrabi 2009, Minervini et al. 2012, Rasul et al. 2013, Ahmad et al. 2014). It should also be considered that there is still no effective cryobiotechnology to ensure successful cryopreservation of buffalo spermatozoa with well-preserved fertilization capacity. Resolving these issues is imperative for the implementation of gene banking

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from genetically valuable sires and for the improvement of meat and milk production.

Following the demand for new buffalo semen cryopreservation approaches, a number of studies have been conducted on the effects of the SPPs in relation to their role in cellular transformations after ejaculation. It is well known that some SPPs affect the motility and survival ability of the spermatozoa, thus influencing the sperm fertilization potential (Codognoto et al. 2018). Various groups of SPPs have been associated with fertility in horses, bulls and humans (Battut et al. 2005, Mogielnicka-Brzozowska 2011, Dar et al. 2018). The biological role of certain SPPs in the sperm capacitation process has been defined (Kim et al. 2015, Luna et al. 2015, Arangasamy and Singh 2017). Also, it has been assumed that the attachment to the sperm surface of certain SPPs (described as "decapacitation factor") may delay the capacitation and acrosome reaction, thus reducing the fertilization potential of the gametes *in vivo* (Gibbons et al. 2005, Bi et al. 2009, Kaur and Sharma 2012). Newly described "fertility-associated proteins" in bull and buffalo SP have been associated with sperm fertility in these species (Killian 2012, Ashrafzadeh et al. 2013, Karunakaran and Devanathan 2016).

In summary, SP contains various factors that affect the spermatozoa in specific ways. The present study aims to analyse the protein profiles of ejaculates with high and low cryotolerance and the interactions between SPPs and proteins from the PM in order to identify specific proteins related to buffalo sperm cryotolerance.

Materials and Methods

Semen samples (n=20) from eight elite buffalo sires, owned by the Executive Agency on Selection and Reproduction in Animal Breeding (EASRAB Sofia and Sliven, Bulgaria) were used in this study. Following the results of the initial motility after thawing, the semen samples were distributed into 2 groups – ejaculates with high cryotolerance (group A, n=12) and with low cryotolerance (group B, n=8). The distribution criterion for low cryotolerance was less than 30% initial progressive motility after thawing.

Computer-assisted sperm analysis of buffalo semen

Buffalo semen was frozen in straws using GH22L medium (Kichev and Danov 1975), following the standard procedure used in the EASRAB AI station. The thawing of the samples was performed in a water bath at 37°C. Motility and survival ability of spermatozoa after ejaculation and after thawing were analysed using the CASA system "Sperm Class Analyzer®" (Microptic®, Spain). Leja® 20 slides were used with 2 μl drop sample volume. The studies were conducted using the "Motility & Concentration" software module. Sperm motility and progression (static, progressive, non-progressive; %) were evaluated. A thermal resistance test was used to assess sperm survival rate (%) after thawing measured every 60 min up to 300 min. A minimum of 1000 spermatozoa and a minimum of 5 captured fields were analysed in each analysis.

Chromatographic analysis of proteins

Chromatographic protein separation was performed using a Binary HPLC Pump 1525 with UV/Visible Detector 2489 (Waters Company®) at λ=280. The separation column used was a TSK gel® G3000SW semi-preparative size-exclusion column, 21mm x 300mm, with a protein resolution of 10 to 500kDa (Tosoh Bioscience®). Gel Filtration Markers Kit for Protein Molecular Weights (MW) 12,000-200,000 Da ™ (Sigma-Aldrich®) was used to determine the MW of the proteins in the separated peaks. The resulting chromatographic protein peaks were characterized based on the molecular marker: ß – Amylase from sweet potato (8.982 min; 200 kDa); Bovine serum albumin (10.196 min; 66 kDa); Carbonic anhydrase from bovine erythrocytes (12.688 min; 29 kDa); Cytochrome C from horse heart (14.448 min; 12.4 kDa).

To obtain SP, the fresh ejaculates were centrifuged at 1000 ×g at room temperature for 5 min. In this way the sperm cells were settled without disrupting the cell integrity. The supernatant was gently removed and re-centrifuged at 10000 ×g at 4°C for 5 min. The following samples were subjected to HPLC separation: proteins from fresh SP, isolated immediately after ejaculation; proteins contained in the PM; proteins in the mixture of SP and PM samples (combined in 1:4 ratio). A sample volume of 150 μl was injected. Optimum operating parameters were adjusted: time (30 min), maximum pressure (375 psi) and flow rate (0.8 ml/min). Chromatograms were obtained and the separated protein fractions were eluated and collected for further analysis.

Results

The motility of fresh semen at 10min after ejaculation was: static 4.02 ± 1.56; non-progressive motile 5.45 ± 0.89; progressive motile 89.87 ± 2.75. The results of the CASA analysis of the motility and survival ability of sperm after thawing are presented in Table 1.

CASA demonstrated evident differences between

Table 1. Comparison of motility and survival ability of buffalo spermatozoa after thawing – 10 and 240 min.

Motility (%)	10min		240min	
	A	B	A	B
Static	15.75±7.8[a]	45.06±5.9[c]	39.01±6.9	59.02±4.2
Non-progressive motile	22.56±6.9	25.97±7.8	35.5±2.8	35.38±1.8
Progressive motile	55.23±8.2[a]	29.77±6.2[b]	25.58±7.3[a]	5.6±2.9[b]

A: spermatozoa with high cryotolerance; B: spermatozoa with low cryotolerance. Data are presented as mean ± SEM. Values in the same row with different superscripts (abc) are statistically different (a-b: $p<0.05$; a-c: $p<0.001$).

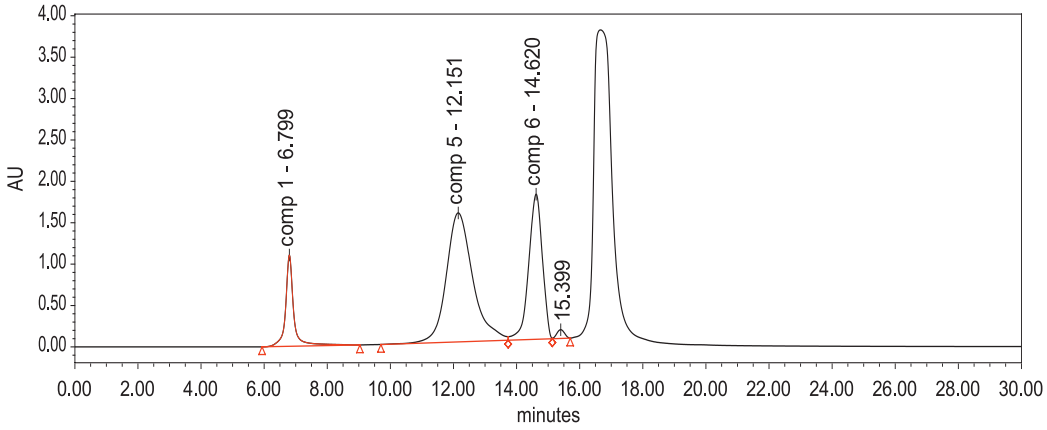

Fig. 1. Chromatographically separated SPPs from high cryotolerance ejaculates (group A).

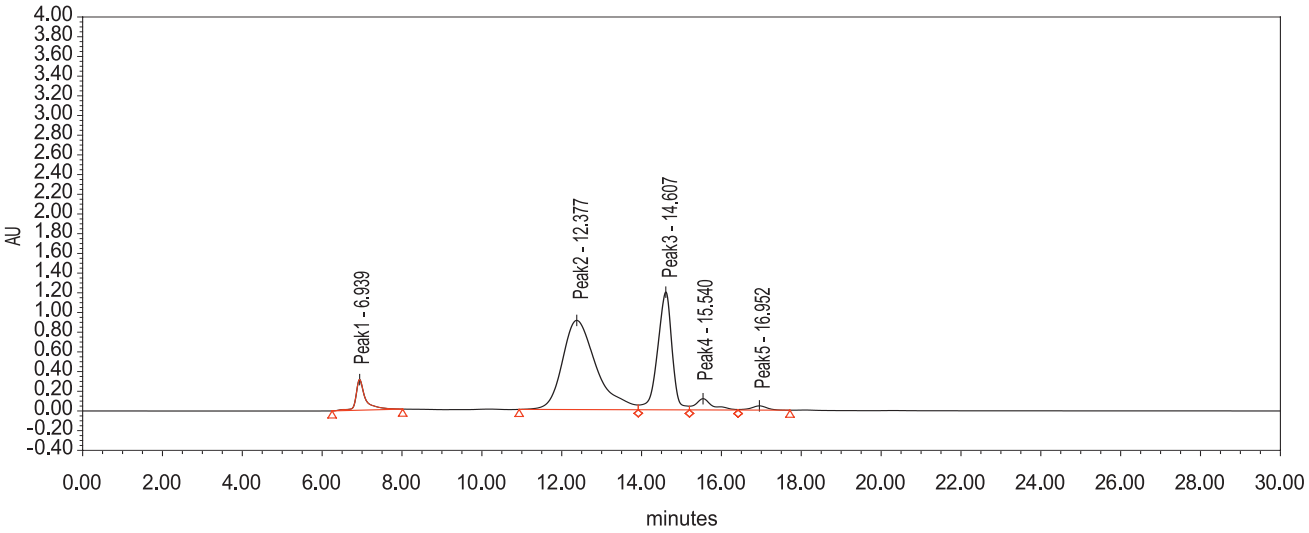

Fig. 2. Chromatographically separated SPPs from low cryotolerance ejaculates (group B).

the two groups regarding the percentage of cells with progressive motility, which in ejaculates with high cryotolerance is significantly higher, at the expense of the static or non-progressive spermatozoa. The thermal resistance test showed most significant differences between the two groups at 240 min after thawing. In ejaculates with high cryotolerance, spermatozoa with progressive motility were 25.58 ± 7.3, compared to 5.6 ± 2.9 in low cryotolerance samples ($p<0.001$).

The HPLC profiles of SPPs, proteins from the PM, and the combination of the two are shown in Figs. 1-6. Chromatograms of ejaculates with high cryotolerance (group A) and low cryotolerance (group B) showed apparent quantitative and qualitative differences in protein content. The proteins in groups with high and low cryotolerance ranged from 500 kDa to 50 Da. Five distinct peaks in buffalo SPPs were identified (Figs. 1 and 2). Specific differences in chromatographic profiles were detected. Group A showed well-expressed protein peaks at 12.151 min and 14.620 min (between 1.7 and 2.0 AU), while in group B these proteins had lower light absorption (between 1.0 and 1.4 AU), indi-

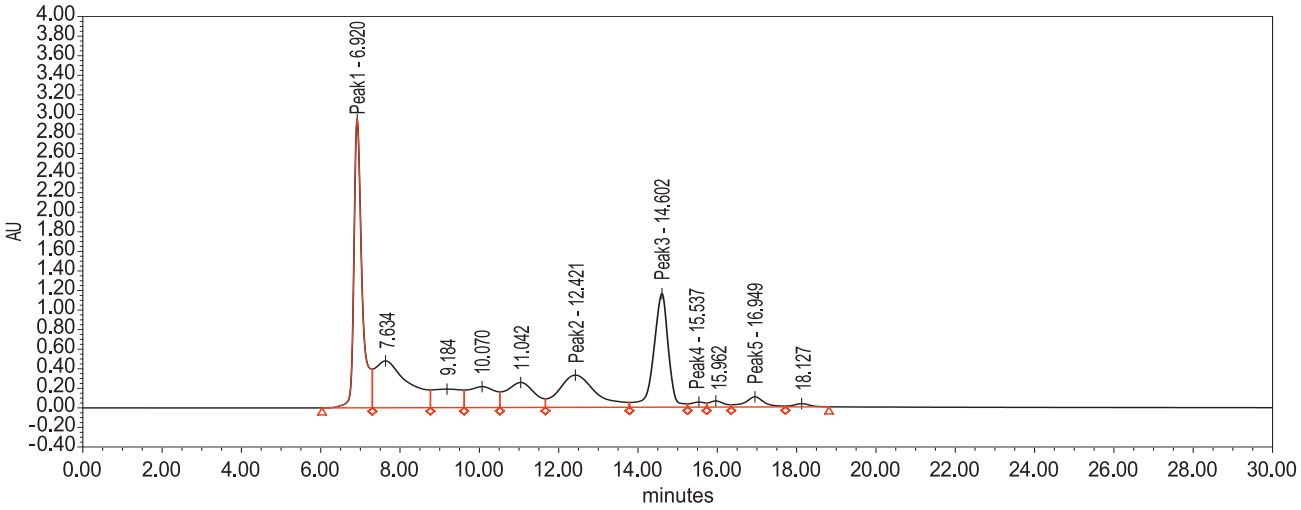

Fig. 3. Chromatographically separated proteins from PM.

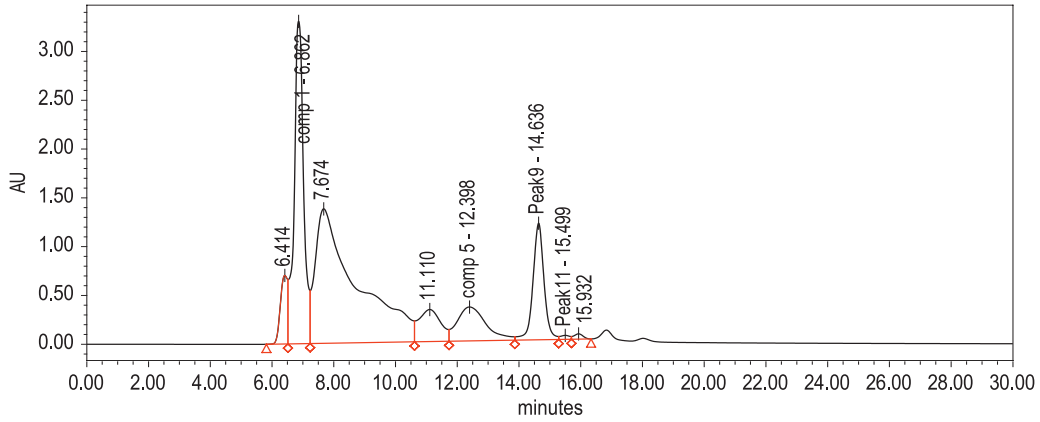

Fig. 4. Chromatographically separated proteins from the mixture of SP (group A) and PM.

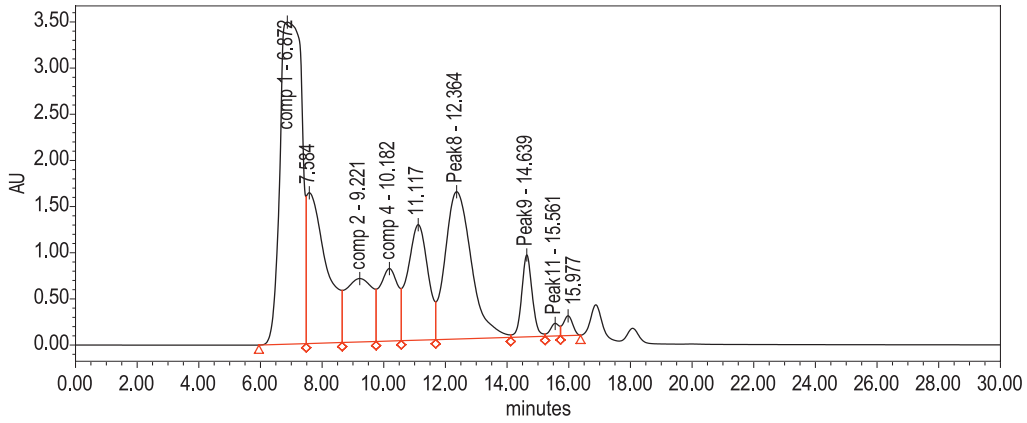

Fig. 5. Chromatographically separated proteins from the mixture of SP (group B) and PM.

cating lower concentration. Also, group A had a well-marked peak between 16 and 18 min (3.8 AU), which was almost absent in group B. This peak corresponds to proteins with relatively low MW between 14 and 6.4 kDa.

The PM chromatogram showed a wide variety of proteins. Eleven protein peaks were well visualized (Fig. 3). We suppose that the PM protein profile shows predominantly proteins originating from the egg yolk which is the main cryoprotector in the PM.

The combination of SPPs and PM proteins resulted in a chromatographic profile (Figs. 4 and 5) that demonstrated specific and very different quantitative and qualitative redistribution of proteins, when com-

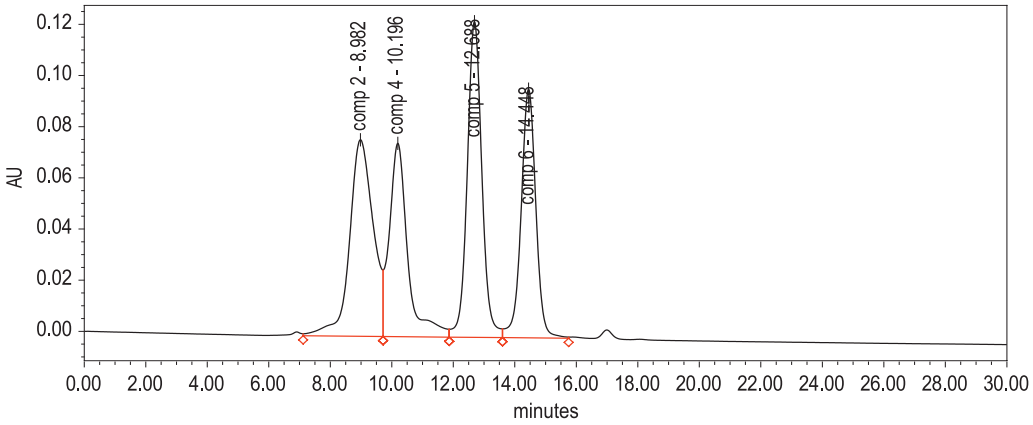

Fig. 6. Chromatographic profile of the mixture of marker proteins.

pared to the individual HPLC profiles of the SP and the PM. (Figs. 1, 2 and 3). Comparison of the chromatographic profiles of SPPs from group A (Fig. 1), proteins from the PM (Fig. 3) and the mixture of SPPs from group A and PM (Fig. 4) revealed a new peak at 7.674 min which was visualized only in the mixture (Fig. 4). This peak was absent in the profile of SPPs from group A (Fig. 1) and proteins from the PM (Fig. 3). Also, this peak was very poorly expressed in the profile of the mixture of SPPs from ejaculates with low cryotolerance (group B) and PM (Fig. 5).

The mixture of SPPs and PM proteins leads to a redistribution of the protein peaks in the chromatograms. It is noticeable that some protein groups that are present in the SP and in the PM are missing in the mixture. For example, the well-expressed protein peak at 16.952 min in ejaculates with high cryotolerance (Fig. 1) is almost absent in ejaculates with low cryotolerance (Fig. 2), in PM (Fig. 3), and in the mixtures (Figs. 4 and 5). At the same time, the protein peaks between 8 and 10 min are lacking in SP (Figs. 1 and 2), but are expressed in the mixtures of SP and PM (Figs. 4 and 5).

Discussion

In bulls, the BSP proteins are a major class of proteins contained in SP. Such proteins are: PDC-109 (BSP-A1/-A2), BSP-A3 and BSP-30-kDa (Dar et al. 2018). It is considered that some of these proteins bind to the sperm membrane phosphatidylcholine during ejaculation. Also, it is supposed that some SPPs bind to capacitation factors such as heparin and high-density lipoproteins (HDL) and support heparin- and HDL--induced capacitation (Lane et al 1999; Travis and Kopf, 2002; Plante and Manjunath 2015). The analysis of two proteins (26 kDa, pI 6.2 and 55 kDa, pI 4.5), which are predominant in fertile bull SP, and two other proteins (16 kDa, pI 4.1 and 16 kDa, pI 6.7) which are predominant in bulls with poor fertility, indicates that the role of SPPs is of great importance both for sperm function and fertilization potential (Chacur 2012, Gilani et al. 2018). The present study confirms that buffalo SP contains substances with a significant role in the biological properties of spermatozoa and are associated with their cryotolerance. Our results showed that ejaculates with high cryotolerance had a specific protein peak between 16 and 18 min (14-6.4 kDa; 3.8 AU), which was absent in ejaculates with low cryotolerance. The presence of these proteins could improve the fertilization potential of the gametes. There is data on a group of five heparin binding proteins (HBP) (14 to 31 kDa) that bind to the sperm surface. One such HBP, designated as a Fertility Associated Antigen (FAA), has been shown to increase sperm fertilization ability (Sprot et al. 2006, Kumar et al. 2016).

The present study showed that SPPs in buffalo ejaculates with high and low sperm cryotolerance had a specific qualitative and quantitative protein profile. It was demonstrated that the mixture of SPPs and PM proteins leads to the formation of new complex protein structures. This is registered by protein redistribution and the specificity of the chromatographic profiles. Specific peaks are found which are missing in the SP or PM, but are formed when they are mixed. We assume that these new structures result from the binding of lipids, phospholipids or protein components of the PM with the SPPs. It can be supposed that these new structures affect the sperm cryotolerance. Reasons for this assumption are the CASA results of the basic sperm parameters after thawing. In samples with small amounts of the newly registered peak (7.674 min), the progressive motility showed significantly lower values when compared to the samples where the said peak is clearly visible. We assume that the new peak is due to physicochemical redistribution of proteins between SP and the PM, resulting in the appearance of new and specific structures with MW over 200 kDa.

Conclusion

The HPLC profiles of separated buffalo SPPs show specific differences between ejaculates with high and low sperm cryotolerance. The combination of SPPs and PM proteins reveals redistribution of protein peaks and the formation of a new protein peak in high cryotolerance ejaculates. We believe that this peak is formed from new protein bio-complex structures. The presence of this protein peak at 7.674 min is a specific phenotypic trait for buffalo ejaculates with high sperm cryotolerance.

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