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Original article

PROTEIN FACTORS OF CAPACITATION AND DECAPACITATION IN CANINE SEMINAL PLASMA

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Summary

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The aim of the present study was to determine the proteins in canine seminal plasma with direct effect on specific sperm parameters during fertilisation. The influence of seminal plasma proteins was focused on three processes: capacitation, hyperactivation and decapacitation. Size exclusion chromatographic fractional separation of proteins from seminal plasma based on their molecular weights was performed. In addition, two-dimensional electrophoresis of two seminal protein fractions was done. The effect of protein molecules on the motion and kinetic parameters of the male gametes in *in vitro* conditions was monitored by computer-assisted sperm analysis. The activity of alkaline phosphatase, creatine kinase and alanine aminotransferase in whole seminal plasma and each chromatography fraction was measured spectrophotometrically using the BA-88A semi-automatic chemistry analyzer. The protein fractions with different molecular weights affected the *in vitro* motility and kinetic characteristics (curvilinear velocity, straightness, linearity and amplitude of lateral head displacement) of spermatozoa, leading to hyperactivation or initiating decapacitation kinetics changes. The detailed study of the seminal plasma proteome would add fundamental information about the processes associated with physiological changes occurring in spermatozoa before fertilisation. The study of the canine seminal plasma proteome could add relevant information about its effects on the fertilising ability of the male gametes and the changes occurring in them before fertilisation.

Key words: chromatography, proteins, spermatozoa, two-dimensional electrophoresis

INTRODUCTION

The semen is a complex fluid which carries and protects spermatozoa after ejaculation and serves as a modulator of the functions of the gametes. Seminal plasma (SP) is a mixture of secretions of several male accessory glands, including prostate, seminal vesicles, epididymis etc. (Sharma *et al.*, 2013). The seminal plasma proteome has an ample panorama of functions and some of them appear responsible for establishing fertilisation. Seminal plasma proteins (SPP) contained in SP are relevant for the function of spermatozoa and sperm interactions with the various

environments along the female genital tract towards the oocyte vestments (Rodríguez-Martínez *et al.*, 2011). The interaction of the SP with spermatozoa induces binding of SPP onto the sperm surface and membrane remodelling potentially impacting the sperm transport, survival and fertilising ability.

The SP contains proteins involved in the inflammatory and immune response of the female tract (Davalieva *et al.*, 2012). Therefore, the seminal plasma proteome has been investigated in a large range of species, including mammals, birds, fish and insects. The association of the SP with semen preservation or fertility is identified as variety of protein markers are already described in many domestic animal species (Druart & de Graaf, 2018). In humans, specific seminal proteins have been identified that are over- or underexpressed in the SP of men with poor sperm quality. The distinct presence of some of the SPP may serve as potential biomarkers and provide insight into the role played by these proteins in male infertility (Sharma *et al.*, 2013; Druart & de Graaf, 2018). However, several seminal proteins are important for capacitation, interaction and fusion of the sperm and oocytes (PSP-I and AWN-1) (Leahy *et al.*, 2019). The SPP influence the fertilising ability of spermatozoa by increasing progressive movement and affectshyperactivation in bull, mouse, ram and boar spermatozoa (Ickowicz *et al.*, 2012; Leahy *et al.*, 2019). It was found that low molecular weight SPP (12–16 kDa) have an effect on sperm cells, plasma membrane stabilisation, capacitation and fusion of the spermatozoa and the oocytes (Henao, 2018). The presence of sperm-plasma motility inhibitor proteins (semenogelin I and II) in human SP has inhibitory effect on sperm movement (Yoshida *et al.*, 2003). Proteins called SVS2 are decapacitation factors and regulate sperm fertilising ability of mouse sperm (Araki *et al.*, 2016).

This study aimed to add information on the diverse role of the proteomics of SP. Proteomic assessment of the SP could provide valuable information for the greater understanding of SPP physiological function. Understanding the protein profile of SP is important because it has a profound impact on sperm physiology and thus may affect sperm functioning (Sharma *et al.*, 2013). So, the research was conducted to find how the seminal proteins act on motility and kinetic parameters connected with the processes of capacitation and decapacitation on domestic dog (*Canis lupus familiaris*) spermatozoa.

MATERIALS AND METHODS

A chromatographic separation, more specifically size exclusion chromatography (SEC) of canine SP was performed. For this purpose, ejaculates from 21 dogs were obtained by masturbation in the area of the bulbus glandis, observing all the norms of ethical treatment of animals. After the collection, the ejaculates were measured by computer-assisted sperm analysis (CASA) and Sperm Class Analyser (SCA) (Micropticum, Spain). The total concentration, motility and kinetic parameters of ejaculates were determined. Before SEC, the SP was separated from the ejaculate by centrifugation at 2000 rpm for 5 min and after obtaining the supernatants the latter were centrifuged again at 10,000 rpm for 5 min. The chromatography of SP was done with 1 mL SP loaded into a column (Watson, Tosoh Bioscience) at a flow rate of 6 mL/min

and 1700 psi. The SEC separated the protein molecules by differences in their size.

After SEC of seminal plasma, the effect of seminal proteins with different molecular weights (MW) on the motion and kinetic parameters of the gametes leading to sperm hyperactivation and capacitation in *in vitro* conditions was monitored by SCA. For this purpose, after initial evaluation, the fresh semen samples were centrifuged at 300×g for 5 min to exclude the SP. After centrifugation the semen samples were re-suspended with equal amount of capacitation medium (NaCl 0.244 g, KCl 0.018 g, $CaC_{l2}\times 2H_2O$ 0.013 g, KH_2PO_4 0.0081 g, $NaHCO_3$ 0.0158 g, $C_3H_3NaO_3$ 0.0014 g, $NaC_3H_5O_3$ (50%) 0.242 mL, Glucose 0.025 g, BSA 0.4%; dd H_2O up to 50 mL, pH 7.8). A control sample (C) with whole SP was used. Re-suspended samples were aliquoted into volume of 750 µL, after that 250 µL of each chromatography separated protein fractions were added into the sample to a final volume of 1000 µL. Controls and samples were incubated for 2 h at 37 ^{o}C.

On the 1st and 2nd h of incubation with the different seminal plasma proteins, changes in sperm motility (progressive, non-progressive and static spermatozoa in the individual samples incubated with SPP with different MW) and kinetic parameters: curvilinear velocity (VCL), straight line velocity (VSL); average path velocity (VAP); straightness (STR), linearity (LIN), beat cross frequency (BCF); wobble of the sperm head about the average path (WOB) and amplitude of lateral head displacement (ALH) were measured using SCA.

The influence of SEC separated SPP fractions as decapacitation factors was evaluated. After initial evaluation, all ejaculates were centrifuged at 300×g for 5 min at for the removal of the SP. Control sample (C) with whole SP was used. A capacitation medium for dogs was added to the already purified SP samples (1:1). The samples were aliquoted in volumes of 750 µL, and 250 µL of different chromatographically separated SPP fractions were added to each sample to a final sample volume of 1000 µL. All samples were incubated at 37 ^{o}C for 1 h. Changes in sperm motility and kinetic parameters were measured using SCA and CASA analysis at the 30th min and 1st hour of incubation with SEC separated SPP fractions.

In addition, the protein fractions that have exhibited an effect on capacitation and decapacitation were subjected to a two-dimensional electrophoresis (2D-PAGE). Immobilised pH gradient (IPG) strips for isoelectric focusing (IEF) with immobilised nonlinear gradient, pH range: 3–11 NL and length: 11 cm were used for determining the isoelectric point (Ip). For the implementation of the protocol for isoelectric focusing the strips were placed in the IEF device Ettan™ IPGphor™ 3 (GE Helathcare®). The device was set to perform four consecutive steps with different voltages so that the total number of volt-hours (V/h) was 12,000 V/h. After IEF, 15% SDS-PAGE was performed for separation of the protein molecules based on their MW. After conducting the SDS-PAGE the gels ware stained with PlusOne Silver Staining Kit (GE Helathcare®). For molecular weight detection of the protein spots the Kaleidoscope ™ Prestained SDS-PAGE Standard (BIO-RAD®) was used. In order to determine the Ip of the spots, pI-Broad range pI marker (pH 3-10) (GE Healthcare®) was used. Gels were scanned on imagining system GS-900TM Densiometer (GE Healthcare®).

Furthermore, the enzyme activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and creatine kinase (CK) in all SPP fractions after SEC were investigated. To determine the activity of the enzymes, a semi-auto chemistry analyzer (Mindray BA-88A) was used. The apparatus was equilibrated with dH_2O. Reagents (Chema Diagnostica) for determination of the enzyme activity of ALP, CK and ALT were used and the enzymes were measured in U/L.

Data were statistically analysed by means of t-test in Excel at level of significance $P<0.05$.

RESULTS

After the collection of 21 ejaculates from healthy dogs, sperm motility and kinetic parameters were measured by SCA. The initial specifications were: concentration 200–400 $\times 10^6$ cells/mL; static spermatozoa 5.69±1.00%; non progressive spermatozoa 70.18±2.18%; progressive spermatozoa 24.13±1.33%, VCL 70.66±3.5 μm/s; VSL 33.77±2.11 μm/s; VAP 43.51±3.18 μm/s; STR 66.69±3.21%; LIN 47.13±2.11%; BCF 8.18±1.01 Hz; WOB 62.21±3.33% and ALH 5.26±1.01 μm/s.

The SEC of SP was performed and four seminal protein fractions with different MW were obtained and characterised by 15% SDS PAGE. It was found that the molecular weights of proteins in the four SPP fractions varied, more specifically: proteins in SPP fraction 1 contained high MW molecules (60 kDa – 80 kDa), SPP fraction 2 contained proteins with 17 kDa – 50 kDa, while proteins in SPP fraction 3 with 7–15 kDa and SPP fraction 4 (3 kDa – 10 kDa) were those with the lowest MW. The role of the chromatographic

Table 1. Capacitation changes in motility and kinetics of spermatozoa after 1st h of incubation with SEC separated SPP fractions (mean ± SD, n=11)

	Control sample	Sample incubated with			
		SPP fraction 1	SPP fraction 2	SPP fraction 3	SPP fraction 4
Static (%)	23.18±1.44	19.18 ± 2.14	19.99±1.08	24.66±3.13	20.99±1.15
Nonprogressive (%)	68.73±2.25	64.87± 2.45	67.19 ± 2.62	63.35 ±1.12	65.22 ±2.11
Progressive (%)	8.09 ±2.22*	16.18±1.81**	13.77±3.01**	11.99±4.09	13.86±2.10
VCL (μm/s)	45.35±1.02	48.5± 1.84	42.87±3.34	46.17±4.57	43.53±5.24
VSL (μm/s)	23.26±3.27*	35.68±2.67*	31.41±2.78*	30.09±3.42	28.57±1.93
VAP (μm/s)	31.68±3.12	40.72±2.51	36.02±1.39	36.5±3.97	34.6±6.4
STR (%)	68.19±4.13*	82.58±1.7**	78.90±3.55**	80.45±3.8	80.2±1.05
LIN (%)	47.68±1.79**	67.5±3.73**	60.89±1.5*	62.84±1.96**	60.74±4.97
BCF (Hz)	8.54±1.42	9.25±2.11	9.85±1.32	10.56±1.00	9.89±1.69
WOB (%)	67.7±1.41*	80.52±1.09**	83.14±4.6**	77.83±1.26	76.6±4.07
ALH (μm)	3.29±0.70	2.8±1.01	2.73±1.63	2.72±1.06	2.87±1.32

VCL: curvilinear velocity, VSL: straight line velocity; VAP: average path velocity; STR: straightness, LIN: linearity, BCF: beat cross frequency; WOB: wobble of the sperm head about the average path, ALH: amplitude of lateral head displacement. Statistically significant differences within a row: $P<0.05$: between * and **; $P<0.001$: between * and ***.

Table 2. Capacitation changes in motility and kinetics of spermatozoa after 2nd h of incubation with SEC separated SPP fractions (mean ± SD, n=11)

	Control sample	Sample incubated with			
		SPP fraction 1	SPP fraction 2	SPP fraction 3	SPP fraction 4
Static (%)	17.03±2.60*	10.01±1.88*	15.18±1.06	17. 99±2.18	17.88±2.01
Nonprogressive (%)	71.69 ±1.99	74.69±2.18	69.01±1.06	66.89±1.11	68.21±3.88
Progressive (%)	12.08±1.11*	11.13±1.09*	28.98±2.00***	15.12±2.22	16.79±2.99
VCL (μm/s)	48.71±1.88*	60.03±2.14**	35.92±2.19***	49.72±2.71	49.95±2.42
VSL (μm/s)	26.38±1.33	30.81±2.77	28.57±1.31	34.56 ±1.67	30.62±3.77
VAP (μm/s)	35.14±2.07	45.06±3.68	35.23±3.01	40.58±1.06	39. 05±2.64
STR (%)	74.24±3.83	68.12±2.06*	85.87±1.11**	84.92±2.84	78.66±3.84
LIN (%)	46.63±1.26*	52.06±2.71**	71.73±1.61***	58.37±4.18	54.41±1.66
BCF (Hz)	8.85±1.08	9.65±1.79	9.96±1.14	9.89±1.08	9.32±1.09
WOB (%)	72.52±4.02	78.14±1.69	75.94±3.33	81.44±2.38	74.16±4.64
ALH (μm)	2.97±0.70*	6.82±1.05*	3.03±1.03	3.62±1.21	2.80±1.00

VCL: curvilinear velocity, VSL: straight line velocity; VAP: average path velocity; STR: straightness, LIN: linearity, BCF: beat cross frequency; WOB: wobble of the sperm head about the average path, ALH: amplitude of lateral head displacement. Statistically significant differences within a row: P<0.05: between * and **; P<0.001: between * and ***.

separated seminal protein fractions on sperm motion and kinetics leading to capacitation was observed. The changes leading to capacitation were observed at 1st and 2nd h of incubation with the four SPP fractions (Tables 1 and 2). Changes in the static sperm subpopulations in control sample and all observed samples were established. In all samples, including the control, a decrease in static sperm subpopulation was observed. A significant change in specific CASA sperm parameters was also found, such as VCL, LIN and ALH in all observed samples and the control. The most significant changes in the sperm parameters were found at the 2nd h of incubation with seminal protein fractions with different molecular weight in the controls and all other samples.

The results demonstrated that the changes on CASA parameters leading to sperm hyperactivation were observed in samples incubated with SPP fraction 1. Sperm subpopulations with high VCL and ALH were found in samples incubated with proteins containing SPP fraction 1 with MW 60–80 kDa. Another sign for ongoing hyperactivity – decreasing of LIN was indicated in the same samples. Significant differences were observed in progressive and non-progressive sperm subpopulation in all samples and the control. The highest percentage of non-progressive spermatozoa (sign for ongoing hyperactivation) was established in samples with SPP fraction 1 compared to the control and other samples by the 2nd h of incubation with proteins with different MW. The sample with added SPP fraction 1 showed significant decrease of progressiveness which was associated with the beginning of hyperactiviation, compared to other samples and the control. Significant changes in SPP fractions with lowest MW (SPP fractions 3 and 4) were not detected unlike motility deviations registered with SPP fraction 1, and were not relevant for the ongoing process of hyperactivation.

Besides, the influence of the chromatography separated seminal protein fractions on motion and kinetics leading to decapacitation effects on canine spermatozoa was researched through changes on sperm motility and kinetic parameters by the 30th minute and 1st hour of incubation with the four SPP fractions were measured by SCA (Tables 3 and 4).

Using CASA analysis, changes in the several CASA sperm parameters was observed in all samples. The analysis of the data showed significant changes in progressive and non-progressive movement of spermatozoa in all samples, especially on the 1st hour of incubation with different SPP fractions. The most significant increase in progressive sperm subpopulation and decrease in non-progressive movement of spermatozoa was observed in samples incubated with SPP fraction 2. In all samples, significant changes of the static sperm cells was measured after 1 hour incubation with different seminal plasma proteins. A significant decrease was observed in VCL and LIN kinetic parameters of samples incubated with SPP fraction 2 by the 1th hour, in comparison to all other samples and controls. Also, in samples with added seminal plasma protein from fraction 2, significantly decreased STR and WOB were observed after 1 hour incubation, demonstrating an effect opposite to capacitation, e.g. decapacitation. In samples incubated with SPP fractions 3 and 4, the decapacitation changes were not significant compared to the samples with SPP from fraction 2.

After SCA and CASA analysis, the separated protein fractions that showed significant effect (SPP fractions 1 and 2) on specific sperm parameters linked to capacitation and decapacitation, were characterised by 2-D electrophoresis. In SPP fraction 1 the following protein spots were detected: 68 kDa and 5.65 pI, 75 kDa and 6.8 pI, 76 kDa and 6.91 pI, 77 kDa and 7.58 pI and 78 kDa and 7.77 pI,

Table 3. Decapacitation changes in motility and kinetics of spermatozoa after 30 min of incubation with SEC separated seminal plasma protein (SPP) fractions (mean ± SD, n=10)

	Control sample	Sample incubated with			
		SPP fraction 1	SPP fraction 2	SPP fraction 3	SPP fraction 4
Static (%)	22.11±2.11	19.99±1.34	19.66±2.00	22.06±1.13	20.00±1.99
Nonprogressive (%)	67.37±1.44	65.77±2.91	71.19±2.62	63.00±3.22	64.88 ±1.04
Progressive (%)	8.88±1.02*	17.01±1.11**	14.77±1.11**	12.01±1.88	13.00±1.11
VCL (μm/s)	47.99±1.34	50.5±1.84	41.66±1.67	47.71±3.99	48.59±2.67
VSL (μm/s)	22.99±2,22	37.02±3.55	30.98±1.33	31.55±2.89	29.99±3.33
VAP (μm/s)	32.07±2.55	42.00±1.99	37.99±2.87	35.89±2.90	34.44±1.89
STR (%)	67.86±3.33*	83.22±3.33**	85.86±3.55**	81.00±1.00	81.00±1.88
LIN (%)	47.77±1.11*	59.55±3.73**	64.73±1.5***	61.84±1.96**	60.01±2.00
BCF (Hz)	8.33±1.11	9.00±1.41	9.00±2.00	11.04±1.22	9.08±1.00
WOB (%)	77.7±1.41	79.99±1.45	83.00±2.03	76.99±1.98	77.00±3.70
ALH (μm)	3.99±1.0	3.8±1.01	2.79±1.87	2.89±1.00	2.99±1.00

VCL: curvilinear velocity, VSL: straight line velocity; VAP: average path velocity; STR: straightness, LIN: linearity, BCF: beat cross frequency; WOB: wobble of the sperm head about the average path, ALH: amplitude of lateral head displacement. Statistically significant differences within a row: P<0.05: between * and **; P<0.001: between * and ***.

Table 4. Decapacitation changes in motility and kinetics of spermatozoa after 1[h] of incubation with SEC separated seminal plasma protein (SPP) fractions (mean ± SD, n=10)

	Control sample	Sample incubated with			
		SPP fraction 1	SPP fraction 2	SPP fraction 3	SPP fraction 4
Static (%)	16.93±1.22*	10.10±1.00**	16.00±2.01	18.09±2.22	18.00±1.12
Nonprogressive (%)	71.77±3.01**	79.01±1.06***	61.69±2.18*	69.89±1.11*	67.00± 2.67
Progressive (%)	13.65±1.97*	11.98±2.00*	20.13±1.09**	15.99±3.00	17.00±1.33
VCL (µm/s)	48.71±1.88*	60.03±2.14*	35.92±2.19***	49.72±2.71	49.95±2.42
VSL (µm/s)	26.99±2.34	29.01±1.00	29.89±3.33	34.00±1.00	31.01±1.23
VAP (µm/s)	36.03±1.87	46.99±1.55	35.77±1.99	41.03±3.89	40. 00±1.22
STR (%)	47.00±2.33**	51.98±3.00*	71.89±2.67***	59.00±1.11	55.03±3.89
LIN (%)	80.00±1.56*	88.12±2.33**	72.90±2.58**	84.05±2.00	77.00±1.25
BCF (Hz)	8.88±1.00	9.00±1.00	9.99±2.03	9.76±1.00	9.00±2.23
WOB (%)	75.52±1.44	78.00±3.74	72.94±1.67	81.00±1.52	73.98±1.18
ALH (µm)	2.98±1.00*	6.93±1.00**	3.18±1.01	3.55±1.00	2.69±1.11

VCL: curvilinear velocity, VSL: straight line velocity; VAP: average path velocity; STR: straightness, LIN: linearity, BCF: beat cross frequency; WOB: wobble of the sperm head about the average path, ALH: amplitude of lateral head displacement. Statistically significant differences within a row: P<0.05: between * and **; P<0.001: between * and ***.

Table 5. Enzyme activity (mean± SD, n=21) of alkaline phosphatase (ALP), creatine kinase (CK) and alanine transaminase (ALT) in SEC-separated seminal plasma protein (SPP) fractions

Enzyme	ALP, U/L	CK, U/L	ALT, U/L
SPP fraction 1	106±1.79	13±1.16	6±1.31
SPP fraction 2	60±1.01	20±1.44	2±1.00
SPP fraction 3	46±1.95	18±1.91	3±1.13
SPP fraction 4	40±2.02	18±1.99	3±1.04

6 kDa and pI 7.58 and 6kDa and pI 7.95 The 2-D PAGE of proteins contained in SPP fraction 2 showed 16 proteins spots: with 15 kDa and 7.9 pI, 18 kDa and 8.3 pI, 19 kDa and 6.85 pI, 21 kDa and 7.4 pI, 29 kDa and 7.1 pI, 30 kDa and 7.3 pI, 32 kDa and 6.3-6.5 pI, 33 kDa and 4.7 pI, 42 kDa and 7.5 pI, 43 kDa and 7.9 pI, 46 kDa and 7.4 pI, 47 kDa and 7.1 pI, 50 kDa and 4.9 pI, 51 kDa and 9.0 pI and finally, 52 kDa and 3 pI.

The analysis of enzyme activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and creatine kinase (CK) in each SEC-separated SPP fraction demonstrated that the enzyme levels varied significantly (Table 5). The highest ALP was found in SPP fraction 1, equal to 106 U/L. The enzyme values decreased significantly in the subsequent fractions. This finding correlated to above mentioned results for capacitation and hyperactivation of spermatozoa incubated with high molecular weight SPP fraction 1. In terms of CK levels, they were highest in SPP fraction 2 (20 U/L), which exhibited a decapacitation effect on spermatozoa in *in vitro* conditions. The levels of ALT

were the highest in SPP fraction 1 (6 U/L) compared to the other fractions, which supported the opinion on the involvement of the enzyme in capacitation changes of the gametes *in vitro*. The obtained results from enzyme assays in each of the chromatographic fractions indicated their participation in the processes of capacitation, hyperactivation and decapacitation of the spermatozoa.

DISCUSSION

Seminal plasma is the natural environment for sperm cells and contains a vast number of components, in particular seminal proteins important for successful sperm maturation and fertilisation (Bubenikova *et al.*, 2020). In addition, SP is a highly complex biological fluid containing proteins, amino acids, enzymes, fructose and other carbohydrates, lipids, major minerals and trace elements. Seminal plasma proteins partly originate from the blood plasma by exudation through the lumen of the male genital tract and partly are synthesised and secreted by various reproductive organs and are known as seminal plasma specific proteins (Sharma *et al.*, 2013). The protein-rich fluid has different functions, some of them well known, others still obscure (Rodríguez-Martínez *et al.*, 2011). However, despite the physiological and medical interest in SPP and many previous studies trying to cover large numbers of proteins there are still many unknown facts about the SPP role and influence (Pilch & Mann, 2003). The present study has analysed the effects of the selected seminal plasma protein group that might play an important role in hyperactivation, capacitiation and decapacitation of canine spermatozoa, using SEC, SCA and 2D-PAGE. Seminal proteins with higher MW were found in SPP fraction 1: a protein with 68 kDa and pI 5.65, one with 75 kDa and pI 6.89, a protein spot with 76 kDa and pI 6.91 kDa, a spot with MW 77 kDa and pI 7.58,1 with 78 kDa and pI 7.77 and 2 protein spots with 6 kDa and pI 7.87 and 6 kDa and pI 7.95. These proteins in SSP fraction 1 with MW between 65–80 kDa may have an effect on hyperactivation and lead to capacitation changes on canine sperm cells. According to Cancel *et al.* (2000), the increase in sperm non-progressive movement, changes in specific kinetic parameters, namely the increase of VLC, AHL and decreased LIN are signs of sperm hyperactivation. The functional test showed a positive correlation between fertilisation success, clinical pregnancy, live birth outcome and hyperactivity of spermatozoa in human and many other spices (Cancel *et al.*, 2000; Ho & Suarez, 2001; Burkman, 2020). Sperm plasma proteins with 55–60 kDa and pI 4.5 were found in highly fertile bulls. Seminal plasma protein identified as spermadhesine have been related to multiple roles on sperm cells, including membrane stability, capacitation and sperm-zona pellucida interplay (Rodriguez-Martinez *et al.,* 2011). The proteins PSP-I/ PSP-II are seminal plasma proteins, able to preserve the sperm viability, motility and mitochondrial activity of in many species (Batruch *et al.*, 2011). According to other authors, there is a positive relationship between the quantity of PSPI/ PSPII and sperm motility in humans (Aquino-Cortez *et al.,* 2016). In addition, heparin binding seminal plasma proteins with MW 61.5 kDa can lead to sperm acrosome reaction and subsequent capacitation in dogs (Souza *et al.,* 2007; Talukdar *et al.*, 2015). SPP with 55–72 kDa in boars, bulls and stallions were positively correlated with male fertility. These proteins are identified as members of the

osteopontin family in the animal species mentioned above. It is considered that osteopontin may have a significant effect regarding sperm motility and capacitation, interaction and fusion with the oocyte (Rodríguez-Martínez *et al.*, 2011). It was found that phospholipase A2, with 50–55 kDa is involved to sperm capacitation in Bali bulls. The biochemical analysis of SPP fraction 1, revealed the presence of high levels alkaline phosphatase, compared to the other samples and the control. It is known that ALP catalyses the detachment of phosphate residues from different substrates. Its activity has been demonstrated in SP and spermatozoa from porcine and other mammalian species, however, the role of ALP in male reproduction has not been clarified yet, but it is considered that ALP has a function in boar sperm capacitation and *in vitro* fertilisation (Bucci *et al.*, 2013). A basic event for sperm cells, involving the loss of surface plasma proteins known as decapacitation is important in the consequent acquisition of fertilising ability. A relation between ALP and different fertility sperm parameters has been reported in fresh semen in stallions (Mc Donnell & Turner, 2003).

This research has also tried to identify seminal protein factors having a decapacitating effect on canine spermatozoa. The role of decapacitation is to prevent the premature onset of the cascade mechanism known as capacitation and this is achieved through sperm plasma membrane stabilisation by maintaining a stable physiological cholesterol/phospholipid interrelation. The decapacitation factors in SP serve to prevent early capacitation and acrosome reaction. Several seminal proteins with 16 kDa, pI 4.1 and 16 kDa and pI 6.7, are observed as decapacitation factors in bull seminal plasma. Seminal plasma protein components have been described as decapacitation factor in boar, goat, humans and etc. species. These proteins protect spermatozoa from factors in the female tract that could trigger early capacitation, thus reducing the possibility of sperm-oocyte binding (Huang *et al.,* 2007). According to Araki *et al.* (2016), 10 kDa proteins in SP have a decapacitation effect on mouse spermatozoa. Our research demonstrated that SPP with low MW inhibits the capacitation in canine spermatozoa. SPP fraction 2 contains proteins with MW between 15–51 kDa and they showed an effect on changes in specific CASA kinetic parameters. Samples incubated with SPP fraction 2 showed decreased VCL and AHL, followed by gradual increase in LIN and STR and increased progression in sperm motility. In addition, SPP with low MW have been found and identified as phosphatidylethanolamine-binding protein 1 in mouse and human spermatozoa. Phosphatidylethanolamine-binding protein is located on the acrosomal cap, the post-acrosomal region and the flagellum and may have inhibition effect on initiation of capacitation (Gibbons *et al.*, 2005). The presence and decapacitating action of the seminal protein SPINK3 in mouse sperm has been recently found. The SPINK3 protein inhibited Src kinase, a modulator of the potassium channel responsible for plasma membrane hyperpolarization. Lack of hyperpolarisation affected calcium channels activity, impairing the acquisition of acrosomal responsiveness and blocking hyperactivation (Zalazar *et al.*, 2020). Interestingly, the levels of CK were higher in SPP fraction 2 compared to other chromatography protein fractions. The enzyme CK is indispensable for sperm function because it catalyses the regeneration of ATP from the chemical shuttle

between creatine and creatine phosphate (Banihani & Abu Alhayjaa, 2005). However, the role of CK in sperm function remains unclear and contra-dictory. In a recent research, low semen creatine levels is associated with reduced sperm motility, while high CK activity is associated with poor sperm quality (Nasrallah *et al.,* 2020). These data are in line with our results, because reduced fertility can be reported as keeping gametes in an inactivated decapitated state. On the other hand, elevated CK levels are associated with severe oligospermia, irrespective of the clinical diagnosis. CK may be a sensitive indicator of sperm quality and maturity in the follow-up of patients treated for male factor infertility (Hallak *et al.,* 2001). According to earlier studies the inverse relationship between CK level, sperm concentration and morphological forms suggests that CK levels can be a reliable marker for semen quality in subfertile men, which may be associated with low levels of capacitation (Nasrallah *et al.*, 2020). Elevated CK levels and their correlation with lipid peroxidation levels may reflect biochemically immature spermatozoa. CK is crucial for adequate sperm motion and enhance sperm motility may be modulated by increasing CK activity, which may explain the rise of sperm subpopulation with progressive movement. The role of ALP in sperm capacitation and overall fertility has been discussed only in some animal species. ALP plays a certain role in capacitation and decapacitation: in fact, the high levels of ALP activity found in seminal plasma (especially in the sperm-rich fractions) could be responsible for maintaining the sperm stabile movement pattern and for preventing the capacitation that could impair the fertilising ability (Bucci *et al.*, 2013). A link between sperm capacitation and ALP activity was observed when ALP levels decrease in response to exposure to pentoxifylline, caffeine and theophylline: substances that have been demonstrated to be capacitation enhancers via inactivation of phosphodiesterase (Glogowski *et al.*, 2002). According to other authors the enzyme activity decreases under capacitating conditions and the addiction of ALP reduces both the number of capacitated cells and fertilisation rate after insemination (Bucci *et al.*, 2013). Regarding the enzyme activity in ALT, significant changes were not observed in our research. According to some authors the activity of ALT is a good indicator of semen quality, so the lower the ALT activities, the better the quality of semen (Pratap *et al.*, 1999). On the contrary, recent experiments demonstrated that the increased ALT activities might be due to acrosome reaction, leading to changes in the mitochondrial sheath with loss of protein from the midpiece and increase in cell membrane permeability with or without rupture of cell membrane in buffalo spermatozoa (Taldukdar *et al.*, 2015). There is contradictory information about the effect of the proteins in the SP, and their influence on the sperm motility and kinetic parameters is still disputed. Many aspects concerning the role of seminal plasma proteins in sperm motility, capacitation and decapacitation remain unexplored.

Seminal plasma comprises many of and unique proteins necessary for function of sperm cells. Our research found several seminal plasma proteins which probably have a role in the hyperacivation, subsequent capacitation and decapacitation. Protein fractions containing high molecular weight molecules clearly affect the capacitance and hyperactification of male gametes. Low molecular weight fractions have a clear effect on the suppression of

the hyperactive movement profile and lead to decapacitation changes in sperm cells. The processes of capacitation and decapitation are markedly influenced by the activity of basic enzymes in the SP. Further studies in the field of the SP proteome would contribute to understanding the nature of major processes affecting male gametes during fertilisation. This in turn would give prerequisites for the successful manipulation of the gametes in artificial insemination techniques and the selection of suitable breeders.

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REFERENCES

Araki, N., A. Kawano, N. Kang, W. Miyado, K. Yoshida & M. Yoshida, 2016. Seminal vesicle proteins SVS3 and SVS4 facilitate SVS2 effect on sperm capacitation. *Reproduction*, **4**, 313–321.

Aquino-Cortez, A., L. Silva, M. Araújo, A. Menezes & D. Moura, 2016. Proteins of the canine seminal plasma. *Ciência Rural*, **5**, 901–908.

Banihani, S. & R. Abu-Alhayjaa, 2016. The activity of seminal creatine kinase is increased in the presence of pentoxifylline. *Andrologia*, **48**, 603–608.

Batruch, I., I. Lecker, D. Kagedan, R. Smit, B. Mullen, E. Grober, K. Lo, P. Diamandis & K. Jarvi, 2011. Proteomic analysis of seminal plasma from normal volunteers and post-vasectomy patients identifies over 2000 proteins and candidate biomarkers of the urogenital system. *Proteome Research*, **10**, 941–953.

Bubenikova, F., P. Postlerova, O. Simonik, J. Sirohi & J. Sichtar, 2020. Effect of seminal plasma protein fractions on stallion sperm cryopreservation. *Molecular Science*, **21**, 64–71.

Bucci, G., G. Isani, E. Giaretta, M. Spinaci, C. Tamanini, E. Ferlizza & G. Galeati, 2013. Alkaline phosphatase in boar sperm function. *Andrology*, **1**, 100–106.

Burkman, L., 2020. Hyperactivated motility of human spermatozoa during in vitro capacitation and implications for fertility. In: Controls of Sperm Motility: Biological and Clinical Aspects, Taylor & Francis, Florida, USA, pp. 27–33.

Cancel, A., D. Lobdell, P. Mendola & S. Pereault, 2000. Objective evaluation of hyperactivated motility in rat spermatozoa using computer-assisted sperm analysis, *Human Reproduction*, **3**, 1322–1328.

Davalieva, K., S. Kiprijanovska, P. Noveski, T. Plaseski, B. Kocevska, C. Broussard & D. Plaseska-Karanfilska, 2012. Proteomic analysis of seminal plasma in men with different spermatogenic impairment. *Andrologia*, **44**, 256–264.

Druart, X. & S. de Graaf, 2018. Seminal plasma proteomes and sperm fertility. *Animal Reproduction Science,* **194**, 33–40.

Gibbons, R., S. Adeoya-Osiguwa & L. Fraser, 2005. A mouse sperm decapacitation factor receptor is phosphatidylethanolamine-binding protein 1. *Reproduction*, **4**, 497–508.

Glogowski, J., D. Danfort & A. Ciereszko A, 2002. Inhibition of alkaline phosphatase activity of boar semen by pentoxifilline, caffeine, and theophylline. *Andrology*, **23**, 783–792.

Hallak, I., K. Sharma, F. Pasqualotto, P. Ranganathan, A. Thomas & A. Agarwal, 2001. Creatine kinase as an indicator of sperm quality and maturity in men with oligospermia. *Urology*, **3**, 446–451.

Henao, F. 2018. Relationship between plasma proteins and boar semen freezability. *Health Science,* **1**, 1–6.

Ho, H. & S. Suarez, 2001. Hyperactivation of mammalian spermatozoa: Function and regulation, *Reproduction,* **4**, 519–526.

Huang, Y., H. Chen, Y. Lin, C. Ciou, Y. Kuo, S. Chen & C. Chang, 2007. Suppression effect of seminal vesicle autoantigen on platelet-activating factor induced mouse sperm capacitation. *Journal of Cellular Biochemistry,* **4**, 941–951.

Ickowicz, D., M. Finkelstein & H. Breitbart, 2012. Mechanism of sperm capacitation and the acrosome reaction: Role of protein kinases. *Asian Journal of Andrology,* **14**, 816–821.

Leahy, T., J. Rickard, N. Bernecic, X. Druart & S. Graaf, 2019. Ram seminal plasma and its functional proteomic assessment. *Reproduction,* **6**, 243–256.

Nasrallah, F., M. Hammami & S. Oma, 2020. Semen creatine and creatine kinase activity as an indicator of sperm quality. *Clinical Laboratory,* **66**, 9–13.

McDonnell, S. & M. Turner, 2003. Alkaline phosphatase in stallion semen: characterization and clinical applications. *Theriogenology,* **60**, 1–10.

Pilch, B. & M. Mann, 2006. Large-scale and high-confidence proteomic analysis of human seminal plasma. *Genome Biology,* **7**, 40–46.

Pratap, N., V. Reddy, A. Sharma, G. Honnappa, M. Devraj, A. Krishnaswamy & K. Arrora, 1999. Estimation of transaminases (AST and ALT) in cryopreserved *Murrah buffalo* semen. *Indian Journal of Animal Reproduction*, **20**, 159–160.

Rodríguez-Martínez H., U. Kvist, J. Ernerudh, L. Sanz & J. Calvete, 2011. Seminal plasma proteins: what role do they play? *American Journal of Reproductive Immunology,* **1**, 11–22.

Sharma, R., A. Agarwal, G. Mohanty, R. Jesudasan, B. Gopalan, B. Willard, S. Yadav & E. Sabanegh, 2013. Functional proteomic analysis of seminal plasma proteins in men with various semen parameters. *Reproductive Biology and Endocrinology,* **11**, 38–42.

Souza, F., F. Barreto & C. Lopes, 2007. Characteristics of seminal plasma proteins and their correlation with canine semen analysis. *Theriogenology,* **1**, 100–106.

Talukdar, D., K. Ahmed, S. Deori & G. Chandra, 2015. Heparin-induced *in vitro* capacitation changes of swamp buffalo spermatozoa. *Turkish Journal of Veterinary and Animal Sciences,* **39**, 629–633.

Yoshida, K., T. Yamasaki, M. Yoshiike, S. Takano, I. Sato, T. Iwamoto, 2003. Quantification of seminal plasma motility inhibitor/semenogelin in human seminal plasma. *Andrology,* **6**, 878–884.

Zalazar, L., C. Stival, A. Nicolli, G. De Blas, D. Krapf & A. Cesari, 2020. Male decapacitation factor SPINK3 blocks membrane hyperpolarization and calcium entry in mouse sperm. *Frontiers in Cell and Developmental Biology,* **3**, 18–23.

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