RESEARCH ARTICLE

Canine seminal plasma – functions and interaction with capacitation

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ABSTRACT

Semen is a heterogeneous and complex cell suspension in a protein rich fluid with various functions, some of them well known, other still unexplained. The aim of the current research is focused on canine seminal plasma proteins and their functional relationship with capacitation of spermatozoa. Attempts were made to identify canine seminal plasma proteins with effect on sperm capacitation and motility. High-Performance Liquid Chromatography was done and 4 seminal plasma protein fractions were obtained and further characterized by electrophoresis (7.6 kDa – 200 kDa). Computer-assisted sperm analysis after incubation of spermatozoa with separated seminal plasma proteins revealed that Fraction 1, consisting of high molecular weight proteins (70 – 200 kDa), could increase the percentage of spermatozoa with progressive and rapid motility. Incubation with seminal plasma proteins from Fraction 1 leads to a significant increase of VCL (Curvilinear velocity) and decrease of LIN (Linearity) of spermatozoa, when compared to the other test samples and the control sample. Proteins with high molecular weight probably have the most significant influence on the process of hyperactivation and capacitation of canine spermatozoa.

Key words: chromatography, seminal plasma proteins, hyperactivation, capacitation, spermatozoa

Introduction

The semen consists of two major components – sperm cells (spermatozoa) and fluid part obtained after centrifugation called seminal plasma (SP). The spermatozoa originate from the semniferous tubules and are suspended in the SP. The SP is composed of secretions contributed by the testis, epididymis, seminal vesicles, ampullae, prostate and bulbourethral glands (Perumal, 2012). 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 (SPPs) partly originate from the blood plasma by exudation through the lumen of the male genital tract and partly are synthesized and secreted by various reproductive organs and are known as seminal plasma specific proteins. Several SPPs of blood origin (prealbumin, albumin, globulin, transferring, α-antitrypsin, β-lipoprotein, β-glycoprotein, orsomucoid, kininogen, peptide hormones, IgG, IgA and IgM) have been identified and characterized. A major part of SPPs originates from the testis, epididymis, vas deference, prostate, seminal vesicle and bulbourethral glands.

SP contains unique proteins necessary for sperm function and survival (Pilch & Mann, 2006; Davalieva et al., 2012). SPPs play a variety of roles: they help protecting the sperm by binding to the sperm surface during ejaculation and play a key role in sperm maturation, capacitation, acrosome reaction, hyperactivation and sperm-egg fusion (Primakoff & Myles, 2002; Varilová et al., 2006). They can also modulate immune response in male and female reproductive tracts, ensuring that the most competent spermatozoa meet the oocyte during fertilization (Kelly, 1995; Mogielnicka-Brzozowska & Kordan, 2011). Thus, seminal plasma proteins can serve as important biomarkers for male infertility (Sharma et al., 2013; Davalieva et al., 2012).

It has been shown that high levels of both boar SPPs with molecular weight (MW) of 26 kDa and isoelectric point (pI) of 6.2 and 55 kDa and pI 4.8 in boar's ejaculates are corresponding with the high rate of farrowing (Janakova, 2007) However, here are still many unclear questions about the role, effects, functionality and mechanisms of impact of proteins from SP. According to Luna et al. (2015), the

presence of two SPPs able to protect sperm against cold shock (RSVP14 and RSVP20) was evidenced in both SP and the ram sperm surface, and their influence in the fertilizing ability of spermatozoa capacitated in basal medium or with cAMP-elevating agents was determined. In research of Manjunath et al., 2002, it was found that bovine seminal vesicles secrete a family of similar proteins – bovine seminal plasma (BSP) proteins designated BSP-A1, BSP-A2, BSP-A3 and BSP-30-kDa. In their studies was shown that the BSP proteins interact specifically with heparin and high-density lipoproteins (HDL), the capacitation factors in bovine, and that the BSP proteins potentiate epididymal sperm capacitation induced by heparin and HDL. The BSP proteins stimulated cholesterol and phospholipid efflux from the sperm plasma membrane (PM). The lipid efflux from PM is dependent on BSP protein concentration and duration of incubation. The loss of membrane cholesterol is an important step in the capacitation process. These results together indicate that BSP proteins play an important role in sperm membrane lipid modification events that occur during capacitation of the spermatozoa. On the other hand, Spermadhesins have been the most studied SPPs in boar SP. As a family they consist of three members – Alanin-Glutamine-Asparagine protein (AQN), Alanine-Thryptophan-Asparagine proteins (AWNs) and the porcine seminal plasma proteins I and II (PSP- I and PSP- II) (Kelly at al., 2006). Spermadhesins are multifunctional 12-16 kDa glycoproteins whose biological activities depend on their sequence, grade of glycosylation or aggregation state on their ability to bind heparin. They have been associated with the multiple effects on spermatozoa including membrane stabilization, capacitation and interaction between sperm-oviduct lining and sperm zona pellucida binding (García et al., 2008; Mogielnicka-Brzozowska & Kordan, 2011).

However, the biological role, functions and effects of SPPs on spermatozoa are still not fully understood. Hence, this gives us direction for further estimation of the role of canine SPPs on sperm cells motility and capacitation.

The aim of our research was to identify SPPs specific to canine ejaculates with effect on motility and kinematic parameters during *in vitro* induced capacitation of spermatozoa.

For this purpose, after obtaining canine SP with normal indicators, a High Performance Liquid Chromatography (HPLC) was done. Four separated protein fractions were collected. The protein content in each SP fraction was visualized by 12% SDS-PAGE. Further research was to evaluate the effect after incubation of each separated SPPs fraction on the biological parameters of spermatozoa – survival, motility and kinematic parameters (VSL, VCL, LIN) during in vitro capacitation by Computer-Assisted Sperm Analysis (CASA).

The understanding of capacitation and the role of SPPs may explain many of the problems of reproduction and male fertility.

Materials and Methods

Preparation of samples

16 ejaculates were collected from 4 healthy dogs from different breeds with normospermia. Immediately after collection of semen, sperm motility and concentration, semen pH, survival rate were evaluated by CASA (Table 1 and Table 4). SP was yielded by centrifugation of canine semen at 2000 rpm, at 4ºC for 5 min. Afterwards supernatant was collected and again centrifuged at 10 000 rpm for 5 min, than filtered through a 0.22 µm membrane (Millipore) and stored on -80ºC.

Chromatography separation of seminal plasma

For HPLC 1 ml of the canine SP was loaded into a column TSK gel G3000SW, 21.5 mm x 300 mm, (TOSOH BIOSCIENCE) at a flow rate of 6ml/min and 1700 psi. Four separated SPPs fractions were obtained. Protein content in the collected samples was determined by specrtophotometry (Ultrospec-200, Pharmacia Biotech). All four protein fractions obtained were characterized by 12% SDS-PAGE (BioRad Mini Protean at 150 V DC). The gels visualization was done via the Coomassie brilliant blue (0.05%) method. BioRad Kaleidoscope Prestained Standards Protein Marker was used as standards.

Research of seminal plasma proteins and there effect during in vitro capacitation

To estimate the influence of SPPs on spermatozoa 16 ejaculates from 4 healthy dogs were used. Semen samples were centrifuged at 1000 rpm for 5 min at room temperature, to exclude the SP. Treated sperm cells were resuspended in capacitation medium (NaCl – 0.488g; KCl – 0.036g; $CaCl_2.2H_2O$ – 0.025g; KH_2PO_4 – 0.016g; $NaHCO_3$ – 0.316g; Na piruvarte – 0.003g; Na lactate – 0.24 ml; Glucose – 0.05g; BSA (Alb) – 0.4g) at concentration of 1:1. Resuspended samples were aliquated into equal volumes and 150 µl of SPPs from fractions 1, 2, 3 and 4 were added into the appropriate sample to a final volume of 500 µl. Thus prepared samples were incubated at 37ºC for 2h. Controls (C) were included with whole SP.

Evaluation of canine sperm motility and kinematic parameters after incubation with SPPs by CASA

Motility and kinematic parameters of canine sperm cells were monitored prior and after incubation with separated SPPs at 37ºC during *in vitro* induced capacitation conditions. The assessment was done by Sperm Class Analyzer (SCA) (Microptic, Spain). Measurements were conducted using the

Motility & Concentration Software and for each sample following parameters were recorded: motility parameters – static; non-progressive motile; progressive motile; rapid; medium and slow motility; kinematic parameters – curvilinear velocity (VCL); straight line velocity (VSL); average path velocity (VAP); linearity (LIN=VSL/VCL); straightness (STR=VSL/VAP); beat cross frequency (BCF); amplitude of lateral head displacement (ALH) and wobble of the sperm head about the average path (WOB). The velocity data were in μm/sec. For statistical analysis was used Student's t-test.

Results

Separation and characterisation of seminal plasma proteins

HPLC analysis revealed four major SPPs fractions in the canine SP. The protein concentration measured spectrophotometrically varied from 0.043 mg/ml to 0.295 mg/ml:

- Fraction 1 (Fr1) – 0.043 mg /ml
- Fraction 2 (Fr2) – 0.295 mg /ml
- Fraction 3 (Fr3) – 0.215 mg /ml
- Fraction 4 (Fr4) – 0.084 mg /ml

To establish baseline information about the pattern of canine SPPs, 12% gel SDS – PAGE analysis was performed after the HPLC separation of SP.

Proteins with MW ranging from 7.6 kDa to more than 200 kDa were visualized. Fraction 1 contained SPPs with high MW from 70 kDa, 78 kDa and more than 200 kDa. Fraction 2 demonstrated the most intense bands of low MW proteins (7.6 kDa and 10-12 kDa), and low content of high MW proteins (78 kDa). Fraction 3 also showed low MW proteins (7.6 and 12 kDa), but not as pronounced as in Fraction 2. Fraction 4 contained low intensity protein bands with low MW (Figure 1).

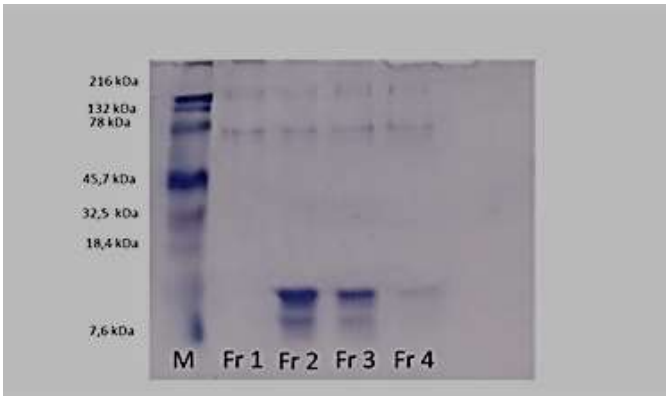

Figure 1. *12% SDS PAGE electrophoresis of canine proteins from seminal plasma after HPLC separation (Coomassie Brilliant Blue).*

Effect of separated canine seminal plasma proteins on sperm motility and kinematic parameters

In order to establish the impact of separated SPPs during *in vitro* induced capacitation conditions, CASA was used to register the changes in motility and kinematic parameters on the 1st and 2nd hour of incubation with the selected proteins.

Notably, sperm motility was influenced in different manner in the samples after incubation of canine spermatozoa with separated SPPs on the 1st and 2nd hour of incubation. In general, incubation in capacitation medium in addition with specific SPPs significantly increased the percent of rapid sperm cells in each sample in comparison with the control, as assessed by CASA.

Table 1. *CASA analysis of fresh ejaculate, n=16.*

Motility parameters	% ± SD
Static	2.87 ± 1.02
Non-progressive motile	74.32 ± 1.09
Progressive motile	22.80 ± 2.09
Rapid	27.02 ± 2.1
Medium	24.88 ± 1.10
Slow	45.21 ± 1.2

The results on the 1st hour of incubation showed significant differences between the control samples (5.61%) and the samples incubated with proteins from fraction1 (11.36%) ($p < 0.01$).

The most significant increase in the value of the progressively motile spermatozoa was registered in the samples incubated with proteins from Fraction 1 after the 2nd of incubation, compared to the other samples. Progressively motile spermatozoa in the samples incubated with proteins from Fraction 1 increased from 13.89% in the 1st hour to 19.94% in the 2nd hour of incubation. The data obtained are shown on Table 2 and Table 3.

An increase in the number of rapid spermatozoa (14.63%) was observed in the samples incubated with proteins from Fraction 1, as compared to samples with proteins from Fractions 2 (8.24%), 3 (11.11%), 4 (10.38%) and controls (8.00%). Decline in the percent of static sperm cells was observed in all samples after the 2nd hour of incubation with different SPPs, when compared to the 1st hour.

Data on kinematic parameters – VCL, VSL VAP, LIN, STR, WOB and ALH, after incubation with SPPs on 1st and 2nd hour are shown on Table 5 and Table 6. Analysis revealed that in all samples the mean values were changed significantly during *in vitro* induced capacitation and incubation with separated SPPs. The higher values of VCL were in samples incubated with proteins with high MW (Fraction 1).

Table 2. *Sperm motility parameters on 1st h after incubation with separated SPPs, n=16.*

Motility parameters	Control	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Static	21.67 ± 2.20	18.70 ± 3.14	17.15 ± 2.12	26.30 ± 3.13	20.34 ± 3.09
Non-progressive motile	70.82 ± 1.25	67.40 ± 4.24	68.25 ± 3.15	61.06 ± 2.14	66.94 ± 3.14
Progressive motile	7.51 ± 3.05[a]	13.89 ± 2.1[c]	14.59 ± 4.10[c]	12.63 ± 3.05[b]	12.71 ± 4.10[b]
Rapid	5.61 ± 2.04[a]	11.36 ± 1.1[c]	10.46 ± 1.09[b]	9.30 ± 1.06	8.86 ± 2.08
Medium	10.21 ± 4.03	4.93 ± 4.01	6.83 ± 2.02	6.60 ± 3.04	7.43 ± 3.05
Slow	62.51 ± 3.20	65.00 ± 3.23	65.56 ± 4.15	57.80 ± 4.13	63.36 ± 3.13

Legend: ± SD, Significant difference shown between a and b $p<0.05$; a and c $p<0.001$

Table 3. *Sperm motility parameters on the on the 2nd h after incubation with separated SPPs, n=16.*

Motility parameters	Control	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Static	15.71 ± 1.65	9.90 ± 1.10	14.24 ± 1.11	15.73 ± 1.07	15.39 ± 1.50
Non-progressive motile	73.02 ± 1.80	70.16 ± 1.55	74.24 ± 1.71	67.28 ± 1.11	69.08 ± 2.5
Progressive motile	11.26 ± 1.02[a]	19.94 ± 1.06[c]	11.51 ± 1.07	16.98 ± 1.02[b]	15.52 ± 2.05[b]
Rapid	8.00 ± 1.03[a]	14.63 ± 1.04[c]	8.24 ± 1.0	11.11 ± 1.03[b]	10.38 ± 1.02
Medium	12.85 ± 1.07	15.88 ± 1.085	9.34 ± 1.04	10.56 ± 1.05	15.01 ± 1.08
Slow	63.43 ± 1.13	59.58 ± 1.08	68.17 ± 1.12	62.58 ± 1.08	59.20 ± 5.1

Legend: ± SD, Significant difference shown between a and b $p<0.05$; a and c $p<0.001$

Table 4. *Sperm kinematic parameters of fresh ejaculate.*

Kinematic parameters	% ± SD
VCL	71.53 ± 1.8
VSL	31.50 ± 8.85
VAP	46.33 ±1.07
LIN	45.95 ± 4.35
STR	68.07 ± 1.03
WOB	66.26 ± 1.54
ALH	3.46 ± 1.84
BCF	9.27 ± 1.56

Samples incubated with proteins from Fraction 1 demonstrated greater increase in VCL at the 2nd hour of incubation (60.03%) in comparison with the other samples and the controls (Fraction 2 – 45.92%; Fraction 3 – 49.72%; Fraction 4 – 49.95%; control sample – 48.71%). Differences between samples with Fraction 1 and controsl were statistically significant ($p <0.001$).

As shown in Table 6, an increased number of spermatozoa with changed AHL was observed in the samples incubated with SPPs from Fraction 1 (6.82 ± 1.05) compared to the other samples. At the same time, a decrease of STR was found. The biggest difference in the values (ALH and STR) was registered in the samples with proteins from Fraction 1, compared with the other samples. The data showed that ALH on the 1st hour of incubation was 2.80% and on the 2nd – 6.82%. STR declined on the 2nd hour of incubation (68.12%) in comparison with 1st hour (82.58%).

The results obtained indicated that the incubation of spermatozoa with SPPs with high MW (Fraction 1) significantly increased the percentage of progressive and rapid spermatozoa. The supplementation of these proteins in the capacitation extenders changes the kinematic parameters of spermatozoa. These changes in the motility and kinematic parameters may be associated with hyperactivation of spermatozoa.

Discussion

CASA provides the means for an objective classification of a given population of spermatozoa. Using digital images of each sperm track, Sperm computer analyzer machines are able to analyse by processing algorithms, the motion properties of spermatozoa. The commonly reported CASA kinematic parameters – VCL, VSL, STR, LIN, ALH, BCF, VAP and WOB have been modelled and refined mathematically to describe best the motion parameters of each spermatozoon as in travels through a microscopic field (Cancel et al., 2000). In current research, using CASA, Cancel et al. analysed rats sperm incubated in different media for capacitation in a period of 4 hours. They use the analysis of several CASA parameters (LIN, VCL, STR, AHL, VSL and BCF) in order to provide a more accurate and objective definition of hyperactivation. The authors define the parameters VCL, ALH and BCF as indicators of high sperm vigour (those parameters can be characterized by hyperactive sperm movement) and a combination of other parameters (VSL, STR and LIN) characterize the progressive movement of the spermatozoa. It has been reported that sperm kinematic parameters VCL, ALH and BCF significantly increased during *in vitro* induced capacitation, while other parameters such as VSL, STR and LIN reduced. Finally, it was demonstrated that using of two CASA parameters, one for progression (LIN) and one for vigour (VCL) can be used to define hyperactivation track in an accurate manner. This subpopulation of spermatozoa probably represents percentage

Table 5. *Sperm kinematic parameters on 1st h after incubation with separated SPPs, n=16.*

Kinematic parameters	Control	Fraction 1	Fraction 2	Fraction 3	Fraction 4
VCL	45.35 ± 5.37	48.50 ± 5.21	42.87 ± 9.34	46.17 ± 5.37	43.53 ± 6.24
VSL	23.26 ± 5.27	35.68 ± 6.67	31.41 ± 1.37	30.09 ± 3.42	28.57 ± 6.93
VAP	31.68 ± 3.12	40.72 ± 5.71	36.02 ± 1.39	36.50 ± 3.97	34.60 ± 6.40
LIN	47.68 ± 8.79[a]	67.50 ± 6.71[b]	71.73 ± 1.50[b]	62.84 ± 7.96[b]	60.74 ± 4.97
STR	68.19 ± 4.13[a]	82.58 ± 1.70[b]	85.86 ± 5.78 [b]	80.45 ± 5.80	80.20 ± 1.05
WOB	67.70 ± 1.41	80.52 ± 1.09	83.14 ± 6.60	77.83 ± 4.26	76.60 ± 9.08
ALH	3.29 ± 1.70	2.80 ± 1.51	2.73 ± 1.63	2.72 ± 1.06	2.87 ± 1.32
BCF	8.54 ± 2.42	9.25 ± 2.11	9.85 ± 1.32	10.56 ± 1.07	9.89 ± 1.34

Legend: ± SD, Significant difference shown between a and b $p<0.05$; a and c $p<0.001$

Table 6. *Sperm kinematic parameters on 2nd h after incubation with separated SPPs, n=16.*

Kinematic parameters	Control	Fraction 1	Fraction 2	Fraction 3	Fraction 4
VCL	48.71 ± 6,88[a]	60.03 ± 3.14[c]	45.92 ± 6.19	49.72 ± 4.71	49.95 ± 7.42
VSL	26.38 ± 6,66	30.81 ± 9.77	28.57 ± 7.31	34.56 ± 5.67	30.62 ± 3.77
VAP	35.14 ± 3,07	45.06 ± 6.68	35.23 ± 9.00	40.58 ± 5.06	39.05 ± 4.64
LIN	46.63 ± 1,26	52.06 ± 5.71	60.89 ± 1.60	58.37 ± 8.18	56.41 ± 1.67
STR	74.24 ± 8,83	68.12 ± 5.00	78.90 ± 1.1	84.92 ± 5.84	78.66 ± 7.84
WOB	72.52 ± 7,02	78.14 ± 7.03	75.94 ± 9.13	81.44 ± 4.38	76.16 ± 7.64
ALH	2.97 ± 1,47[a]	6.82 ± 1.05[c]	3.03 ± 4.9	3.62 ± 1.21	2.80 ± 1.42
BCF	8.85 ± 1,48	9.20 ± 1.14	9.82 ± 1.79	9.89 ± 1.84	9.32 ± 1.89

Legend*:* ± SD, Significant difference shown between a and b $p<0.05$; a and c $p<0.001$

with fertilization ability (Cancel et al., 2000; Ferramosca & Zara, 2014).

A similar date and approach of using of these two CASA parameters has been demonstrated to evaluate hyperactivation of mouse spermatozoa (Neil et al.; 1987), bovine (McNutt et al., 1994), human, boar and ram sperm cells (Vulcano et al., 1998; Abaigar et al., 1999).

Our results coincide with the finding of other authors (Mortimer, 1997; Cancel et al., 2000) that address the relevance of specific SPPs for the function and influence on the capacitation and hyperactivation of spermatozoa (Ferramosca & Zara, 2014). We found that canine spermatozoa incubated in extenders which contain high MW SPPs (70 kDa, 78 kDa and more than 200 kDa) retain progressive and rapid motility, increase of VCL parameters, and decrease of LIN kinematic parameters. We suppose that proteins from Fraction 1 have the most significant effect on the course of the process of capacitation and hyperactivation on canine sperm (Annice et al., 2016). From our results it is confirmed that SP contains factors such as specific proteins favoring the process of capacitation of spermatozoa. SPPs with high MW, such as proteins from Fraction 1 with MW of 70-80 kDa and more than 200 kDa, improve the motility (progressive and rapid) and kinematic parameters (VCL) of canine sperm (Mogielnicka-Brzozowska & Kordan, 2011).

The studying of mechanisms, signaling pathways and biomolecules influence this process is crucial. Better understanding of process of capacitation and the role of some SPPs, probably could explain many of the problems of reproduction and male fertility.

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