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Addition of seminal plasma proteins effecting the *in vitro* kinetic properties of canine spermatozoa

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Abstract: The objective of this study is to evaluate the changes in the motility and kinetic patterns of canine spermatozoa, capacitated and decapacitated, after the addition of seminal plasma protein fractions with different molecular weight. It has been proposed that proteins in seminal plasma support the survival of the spermatozoa and exert a dual effect: capacitation and decapacitation. The seminal plasma from fresh ejaculates was subjected to chromatographic separation and four protein fractions were obtained. Computer-assisted sperm analysis was used to determine the sperm subpopulations with specific motion and kinetic characteristics after incubation with each of the four protein fractions. Two-dimensional electrophoresis of the fractions that exhibit a significant effect on the capacitation and decapacitation was performed. By sperm class analyser, capacitation changes were observed in the sperm subpopulation with a high curvilinear velocity and amplitude of lateral head displacement incubated with the seminal plasma protein fraction with a high molecular weight, which was also reflected in the decreased linearity, straightness, and progressive motility. The sperm subpopulation incubated with the seminal plasma protein fraction with a low molecular weight seemed to undergo a process of decapacitation (decreasing of the curvilinear velocity, increasing of the linearity, straightness and showing progressive motility). Despite their ample panorama of actions, the role of seminal plasma proteins regarding capacitation and decapacitation is still undetermined.

Keywords: capacitation; decapacitation; kinetics; motility

Seminal plasma (SP) is a complex fluid, containing proteins necessary for spermatozoa and serves as a medium to carry and protect the sperm after ejaculation, but also serves as a modulator of the sperm function. Some of the seminal plasma proteins (SPP) have a significant impact on the acrosome reaction, recognition of the oocyte and fertilisation (Rodriguez-Martinez et al. 2011). Certain proteins act as signals for the female immune system to modulate the sperm rejection or tolerance and influencing the relative fertility of the male by attaining a status of maternal tolerance towards the embryo and placental development (Rodriguez-Martinez et al. 2011; Ickowicz et al. 2012). Some SPPs are relevant to the process of capacitation, interaction and fusion of the gametes (Ickowicz et al. 2012). Spermadhesines isolated from the SP and identified as PSP-I and AWN-1 are associated with the successful outcome of fertilisation. Spermadhesines are proteins with low molecular weight (MW) (14–18 kDa) and exhibit multiple effects on the spermatozoa including plasma membrane stabilisation, capacitation and have the ability to attach to the zona pellucida (Kraus et al. 2005). Another group of SPPs are called osteopontins (OPNs) and are positively associated with fertility in bulls (55 kDa, pI 4.5) and stallions (SP-1,

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72 kDa, pI 5.6) (Cancel et al. 1999). Some SPP influence the fertilising ability of sperm cells by increasing the motility and progressive movement after ejaculation in bulls, mice, and boars (Bjorkgren and Sipila 2019). Interestingly, specific SPPs have been described as decapacitation factors in humans and mice and are assumed to protect the spermatozoa from early capacitation, thus reducing the possibility of sperm-oocyte binding. Hyperactivated motility is known to appear simultaneously with capacitation and is critical to the success of fertilisation, because it enhances the ability of the sperm to detach from the wall of the oviduct, to penetrate mucous substances and to penetrate the zona pellucida of the oocyte (Harayama 2013). During hyperactivation, the movement and vigour of the spermatozoa undergo dramatic changes, which are best characterised by movements in a non-progressive circular path, described as "whiplash".

The computer-assisted sperm analysis (CASA) parameters: linearity (LIN), curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) could be used for the detection of the spermatozoa's hyperactivation (Cancel et al. 2000). The aim of our research was to establish a certain SPP with an effect on the movement patterns of canine spermatozoa related to the processes of capacitation and decapacitation.

MATERIAL AND METHODS

Seminal plasma preparation, evaluation and size exclusion chromatography (SEC)

Ejaculates were collected from 21 healthy dogs at the age of 3–6 years (*Canis familiaris*) by manual stimulation in the presence of female dog as a stimulus. The ejaculates were subjected to a CASA-analysis (Microptic®, Madrid, Spain). The semen was measured for the total concentration, motility (static; non-progressive and progressive) and velocity indicators: 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). The SP was obtained by double centrifugation (Labofuge 400, Hanau, Germany) at 300 *g* at 4 °C for 5 min and after obtaining the supernatants, they were centrifuged again at 10 000 *g* at 4 °C for 5 minutes. SEC was performed for the separation of the canine SP. One millilitre (1 ml) of the SP was loaded into a column (Watson; Tosoh Bioscience, Madison, USA) at a flow rate of 6 ml per min and 1 700 psi was used. Fractions of the SP with different MWs were obtained and called: fraction 1 (fr. 1); fraction 2 (fr. 2); fraction 3 (fr. 3) and fraction 4 (fr. 4).

Effects of the addition of the SPP on the sperm motility and kinetic parameters

The effect of each SEC canine SPP fractions (fr. 1, fr. 2, fr. 3 and fr. 4) on the sperm motility and kinetic indicators was analysed by CASA. After the initial measurement with a sperm class analyser (Microptic®; Barcelona, Spain), the semen samples were centrifuged at room temperature for 5 min and at 2 000 *g* to exclude the SP. The samples were re-suspended in a capacitation medium (NaCl – 0.244 g, KCl – 0.018 g, $CaCl_2 \times 2H_2O$ – 0.013 g, KH_2PO_4 – 0.008 1 g, $NaHCO_3$ – 0.015 8 g, $C_3H_3NaO_3$ – 0.001 4 g, $NaC_3H_5O_3$ (50%) – 0.242 ml, glucose – 0.025 g, BSA – 0.4%; d.d. H_2O up to 50 ml, pH 7.8) in a ratio of 1 : 1 and aliquoted into volumes of 750 µl and 250 µl. Each separated SPP fraction was added into the appropriate vials to a final volume of 1 000 µl, the final number of samples was four. Control (C) samples with whole SP were used. All the samples were incubated at 37 °C for 2 hours. On the 1st and 2nd hour of incubation, each sample with the different SPP fraction was measured for changes in motility and for the CASA kinetic parameters. A *T*-test in Excel was used for the statistical analysis where *, ** and *** are statistically different at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. The *T*-test compares the sperm motility and kinetic parameters between the control and each fraction (fr. 1, fr. 2, fr. 3 and fr. 4) and also the SPP fractions with each other during incubation.

2D-PAGE of the SPP

The performed experiments demonstrated that the proteins contained in fr. 1 and fr. 2 show the most significant effects on the processes of capacitation and decapacitation compared to fr. 3 and fr. 4. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was conducted to identify the proteins contained in SPP fr. 1 and fr. 2. To deter-

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mine the isoelectric point (Ip) of the proteins, immobilised pH gradient (IPG) strips for isoelectric focusing (IEF) were used (Immobiline™ DryStrip; GE Healthcare®, Chicago, USA) with an immobilised non-linear gradient, pH range: 3–11 NL and length: 11 cm. The strips were placed in an Ettan™ IPGphor™ 3 IEF device (GE Healthcare®, Chicago, USA) and was set to perform four steps with different voltages so that the total number of volt-hours (V/h) was equal to 12 000 V/h. 15% SDS-PAGE for subsequent separation based on MW was performed and the gels were stained with a PlusOne Silver Staining Kit (GE Healthcare®, Chicago, USA). The Kaleidoscope™ Prestained SDS-PAGE Standard (BIO-RAD®, Hercules, USA) was used to determine the MW of the protein spots, and the pI – Broad range pI marker (pH 3–10) (GE Healthcare®, Chicago, USA) was used to determine their pI. The gels were scanned with a GS-900™ Densitometer (GE Healthcare®, Chicago, USA) imagining system.

RESULTS

After the ejaculates were collected, the sperm motility and kinetic parameters were measured by sperm class analyser (SCA) – total concentration of 200–400 × 10^6 cells/ml; static: 5.69 ± 1.00; non-progressive: 70.18 ± 2.18; progressive: 24.13 ± 1.33; VCL: 70.66 ± 3.5; VSL: 33.77 ± 2.11; VAP: 43.51 ± 3.18; STR: 66.69 ± 3.21; LIN: 47.13 ± 2.11; BCF: 8.18 ± 1.01; WOB: 62.21 ± 3.33 and ALH: 5.26 ± 1.01.

Seminal plasma preparation, evaluation and size exclusion chromatography (SEC)

The chromatography of the canine SP was performed. Four SPP fractions with different MWs were obtained – fr. 1, fr. 2, fr. 3 and fr. 4.

Effects of the SPP addition on the sperm motility and kinetic variables

The effect of the four chromatograph-separated seminal protein fractions on the spermatozoa motility and kinetics was observed until the 2nd hour of incubation. The changes to the motility and kinetic variables during the 1st and 2nd hour of incubation with the four fractions are presented in Table 1 and Table 2. In all the samples, changes in the static sperm subpopulations are observed. More significant differences in all the CASA parameters were observed during the 2nd hour of incubation. In the SPP fr. 2 samples, an increase in the progressive spermatozoa

Table 1. Sperm motility and kinetic indicators during the 1st hour after incubation with the chromatograph-separated seminal plasma protein fractions

Indicators	Control	SPP fr. 1	SPP fr. 2	SPP fr. 3	SPP fr. 4
Static (%)	23.18 ± 1.44	19.18 ± 2.14	19.99 ± 1.08	24.66 ± 3.13	20.99 ± 1.15
Non-progressive (%)	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

Values within the row with different superscripts (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$) are statistically different; $n = 21$; Motility and kinetic indicators between the C sample and each fraction (fr. 1, fr. 2, fr. 3 and fr. 4) and also the SPP fractions with each other during incubation

ALH = amplitude of lateral head displacement; BCF = beat cross frequency; C = control sample; LIN = linearity; SPP fr. 1 = seminal plasma protein fraction 1; SPP fr. 2 = seminal plasma protein fraction 2; SPP fr. 3 = seminal plasma protein fraction 3; SPP fr. 4 = seminal plasma protein fraction 4; STR = straightness; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight-line velocity; WOB = wobble

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Table 2. Sperm motility and kinetic indicators during the 2nd hour after incubation with the chromatograph-separated seminal plasma protein fractions

Indicators	Control	SPP fr. 1	SPP fr. 2	SPP fr. 3	SPP fr. 4
Static (%)	17.03 ± 2.60*	10.01 ± 1.88*	15.18 ± 1.06	17.99 ± 2.18	17.88 ± 2.01
Non-progressive (%)	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

Values within the row with different superscripts (*P < 0.05, **P < 0.01 and ***P < 0.001) are statistically different; n = 21; Motility and kinetic indicators between the C sample and each fraction (fr. 1, fr. 2, fr. 3 and fr. 4) and also the SPP fractions with each other during incubation

ALH = amplitude of lateral head displacement; BCF = beat cross frequency; C = control sample; LIN = linearity; SPP fr. 1 = seminal plasma protein fraction 1; SPP fr. 2 = seminal plasma protein fraction 2; SPP fr. 3 = seminal plasma protein fraction 3; SPP fr. 4 = seminal plasma protein fraction 4; STR = straightness; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight-line velocity; WOB = wobble

was observed, compared to the C and SPP fr. 1 samples. In the SPP fr. 1 sperm subpopulation, a significant increase in the non-progressive spermatozoa was observed compared to the other samples. The most significant changes in the kinetic parameters were observed in all the samples and the C sample during the 2nd hour of incubation. A significant increase in the VCL and ALH and a decrease in the STR and LIN parameters were indicated in the samples incubated with proteins from SPP fr. 1 compared to the other samples and the C sample. These changes in the kinetic parameters are a sign of initiated hyperactivation. The samples incubated with proteins contained in the SPP fr. 2 during the 2nd hour of incubation demonstrated a significant decrease in the VCL and an increase in the LIN and STR, compared to the SPP fr. 1 and C samples, and could have an influence as a decapacitation factor.

2D-PAGE of SPP

After the obtained CASA results, identifying the proteins in SPP fr. 1 and fr. 2 was performed

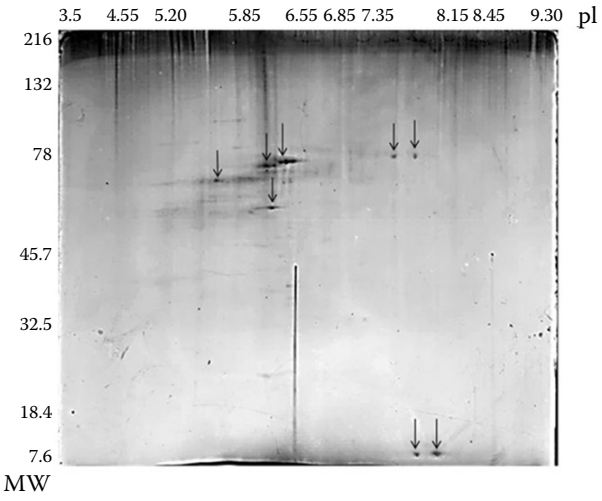

Figure 1. 2D-PAGE separation of the proteins in SPP fr. 1

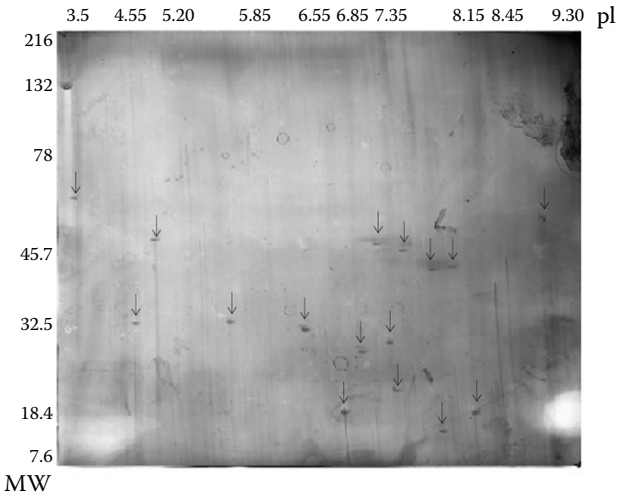

Figure 2. 2D-PAGE separation of the proteins in SPP fr. 2

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by 2D-PAGE. In SPP fr. 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, 6 kDa and pI 7.58 and 6 kDa and pI 7.95 (Figure 1).

In SPP fr. 2, 16 proteins spots were detected as follows: proteins 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 protein spots with 52 kDa and 3 pI (Figure 2).

DISCUSSION

The target of this study was to evaluate the capacitation and decapacitation in canine spermatozoa by tracking the changes in the CASA parameters for the motility and kinetics after the addition of the SPP fractions with different MWs. The characterisation of the proteins in fr. 1 and fr. 2, which demonstrated the most significant effect on the above-mentioned processes, was performed using 2D-PAGE. We observed that the proteins in fr. 1 with a predominantly high MW between 65–80 kDa may have a beneficial effect on the hyperactivation. According to other authors, changes in the motility (increase in the non-progressive movement) and kinetic parameters (increase in the VLC and AHL, and decrease in the LIN) are a sign of hyperactivity of the spermatozoa (Suarez and Ho 2003; van der Horst et al. 2018). The only sperm functional test in humans and many animal species that shows a positive relationship with the fertilisation success, clinical pregnancy and live birth outcome is hyperactivation. Hyperactivation is mainly required for three reasons: 1) The large "search radius" of the sperm improves the chances of meeting the oocyte. 2) Most spermatozoa seem to attach to the villi of the cells of the oviduct and then detach when ovulation occurs. This detachment can only happen when the sperm becomes hyperactivated. 3) Finally, the force with which the spermatozoa make contact with the oocyte allows not only attachment, but also initiates the acrosome reaction (van der Horst et al. 2018). The SPP with a MW of 26 kDa and pI 6.2 and 55 kDa and pI 4.5 are present in highly fertile bulls (Killian et al. 1993). Briefly, proteins with a high MW identified as OPN were positively correlated with fertility in stallions (72 kDa and pI 5.6) and bulls (55 kDa and pI 4.5) (Hao et al. 2006). The SPP of mammals play a significant role in the sperm motility and functionality by regulating the capacitation, gamete interaction and fusion. Individual proteins from the same family carry out their functions in a species-specific manner (Rodriguez-Martinez et al. 2011). A certain protein from a Bali bull SP, reported as phospholipase A_2 (PLA_2) with a MW of 50–55 kDa is thought to be involved in the sperm capacitation. Also, the SPPs, identified as heparin, binding seminal plasma proteins (HBP) with a MW of 61.5 kDa are involved in the acrosome reaction on canine spermatozoa (de Souza et al. 2007).

Capacitation is a basic event for spermatozoa, involving the loss of the surface SPP known as the decapacitation factor and the consequent acquisition of the fertilising ability. Decapacitation factors are components of the seminal plasma which modulate the fertilising ability of the spermatozoa (Araki et al. 2016). Decapacitation arises from the interaction between the cholesterol, phospholipids, seminal plasma proteins and a fibronectin-like substance and is delivered via small vesicles in the seminal plasma (Killian et al. 1993; Araki et al. 2016). Its function is to prevent the premature onset of the cascade mechanism known as capacitation and this is achieved through spermatozoa membrane stabilisation by maintaining a physiological cholesterol/phospholipid ratio. Therefore, it is known that an SPP with a low MW of 16 kDa and pI 4.1 and 16 kDa and pI 6.7 correlate with low fertility in bulls. Generally, these factors are believed to inhibit the sperm capacitation, acrosome reaction and ultimately to interfere with fertilisation (Killian et al. 1993). Also, specific SP components have been described as decapacitation factors in humans and other species, and are assumed to protect the sperm from factors in the female tract that could trigger early capacitation, thus reducing the possibility of sperm-oocyte binding (Huang et al. 2007). The SPP originating from seminal vesicle secretions, detected as SVS4 with a low MW (10 kDa) inhibits the initiation of the sperm capacitation (capacitation inhibition) and reduces the fertility of the capacitated spermatozoa (decapacitation) (Araki et al. 2016). On the other hand, in our research, it was demonstrated that proteins in SPP fr. 2 with a MW of 15–52 kDa may have a decapacitation effect on the canine sperma-

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tozoa. The process can be detected by observing the changes that the spermatozoa undergo, regarding their CASA kinetic parameters, more precisely the VCL, ALH and LIN. A decrease in the VCL and ALH, followed by a gradual increase in the LIN can be used as a detection marker of the occurring decapacitation of the gametes.

Nonetheless, the SPP have a broad spectrum of action and their effect on fertility is still an object of further research. With the obtained results of our research, we supplement new data to the knowledge on the role and effects of SPPs to the canine spermatozoa. The specific protein from the SP with different MWs are relevant for the sperm function, particularly the SPP with a high MW seems to be related to the hyperactivation and subsequent capacitation and lower MW proteins seem negatively related to the motility and some kinetic parameters, and have decapacitation effects regarding male gametes.

In conclusion, it could be stated that seminal plasma is a source of protein that may influence the sperm physiology. Nonetheless, there are still gaps in the existing knowledge on the functional attributes of seminal plasma proteins. In conclusion, our research appends the growing evidence that demonstrates that SPP plays different roles in regard to the sperm functionality. In this study, we describe some SPPs with different MWs and pIs which are related to the processes of the hyperactivation and decapacitation of canine spermatozoa. The roles of SPP are still to be established. Future studies should evaluate the protein isoforms and how they interact with sperm to ascertain their biological functions.

Conflict of interest

The authors declare no conflict of interest.

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