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Effect of seminal plasma zinc–binding proteins on motility and membrane integrity of canine spermatozoa stored at 5°C

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Abstract

Sperm surface binding sites for non-zinc-binding proteins (nZnBPs) and zinc-binding proteins (ZnBPs) were studied by the fluorescence technique with biotin-labelled proteins. The nZnBPs binding pattern was unspecific, no characteristic sites on plasmalemma were found. ZnBPs were attached mainly to the acrosomal region of sperm head and to the sperm flagellum. ZnBPs added to the incubation mixture of the canine spermatozoa allowed the preservation of higher values for total motility, progressive motility, curvilinear line velocity, straight line velocity, and beat cross frequency ($P < 0.05$), both at time 0 and after 1 h incubation at 5ºC. The addition of nZnBPs to the incubation mixture caused only weak positive effects when compared with control sample (PBS). A higher percentage of canine-ejaculated spermatozoa with intact membranes were observed when ejaculate was incubated with ZnBPs in comparison to control sample stored with PBS ($P < 0.05$) or nZnBPs ($P < 0.05$). Spermatozoa diluted with ZnBPs and nZnBPs exhibited a higher percentage of cells with active mitochondria when compared with control, both at time 0 and after 1 h; however, no statistical differences were observed. Our results emphasise the role of seminal plasma protein in securing the correct quantity and availability of zinc ions as a component regulating the motility of canine spermatozoa. The protective effect of ZnBPs against the cooling effect may be due to their ability of preventing sperm membrane damage.

Key words: dog, zinc-binding proteins, spermatozoa, motility.

Introduction

Zinc contents of the prostate gland, seminal fluid, and ejaculated sperm are very high and testicular zinc is essential for spermatogenesis (17). Zinc is also involved in a number of functions, which are crucial for sperm physiology. It increases the stability of membrane lipid bilayers (27). A high content of zinc in the sperm nuclei contributes to the stability of the quaternary structure of the chromatin (4). Zinc affects sperm motility and acrosome reaction (13, 23). Since bioavailable zinc binds to low and high molecular weight proteins, the seminal plasma zinc binding protein-ligands seem to be significantly important in the effect of zinc on sperm functions (4). The data on seminal plasma zinc binding protein-ligands is sparse and concern only humans (24, 26) and boars (5, 14, 19, 29), while there is a complete lack of information regarding dogs. Our findings have demonstrated for the first time the characteristics of zinc-binding proteins (ZnBPs) secreted by the canine prostate, which might be used in the reproductive processes (18). The aim of this study was to examine the influence of ZnBPs on motility characteristics, membrane integrity, and mitochondrial status of ejaculated canine spermatozoa stored at 5°C.

Data concerning canine seminal plasma proteins will enable better understanding of the reproductive processes in this species and can contribute to the improvement of the technology of canine sperm preservation.

Material and Methods

Chemical agents. Chemicals and biochemicals were of the highest purity grade available and were purchased from Sigma (USA) unless otherwise stated.

Animals and semen collection. Semen (12 ejaculates, three ejaculates from each dog, one ejaculate weekly) was collected from four crossbred dogs aged 3 to 5 years using digital manipulation. The dogs were clinically healthy and kept in individual cages with access to water (*at libitum*) and fed commercial canine feed. Seminal plasma was separated from spermatozoa by centrifugation at 700 × g, 15 min at room temperature and the recovered seminal plasma was further re-centrifuged at 10 000 × g for 10 min at room temperature and stored at -80°C until further analysis. Sperm pellets were diluted in 1 mL of PBS and mixed, re-centrifuged (700 × g, 6 min at room temperature) to remove all seminal plasma proteins, and then were immediately used for analysis. All experiments were conducted in accordance with the guidelines set out by the Local Ethics Committee for Experimentation with Animals.

Protein determination. Total protein content was measured according to Lowry *et al.* (15) using bovine serum albumin (BSA, Serum and Vaccine Manufacturing Plant, Cracow, Poland) as a standard.

Isolation of non-zinc-binding proteins (nZnBPs) and zinc-binding proteins (ZnBPs) of canine seminal plasma. Separation of proteins from seminal plasma was conducted using the method described by Mogielnicka-Brzozowska *et al.* (18). Both nZnBPs and ZnBPs fractions were dialysed against deionised H_2O and stored at -80°C until further analysis.

Preparation of biotin-labelled nZnBPs and ZnBPs. According to the method described by Manaskova and Jonakova (16) with modifications, 1 mL of *N*-hydroxysuccinimidobiotin dissolved in 50 µL of *N,N*-dimethylformamide was added to the protein solution (2 mg/mL) in 0.5 M $NaHCO_3$, pH 8.8 (4 mL). After stirring for 30 min at room temperature in the dark, the solution was dialysed against PBS overnight at 4°C.

Attachement of biotin-labelled nZnBPs and ZnBPs to ejaculated spermatozoa. Freshly ejaculated canine spermatozoa were washed four times with PBS, according to the method described by Manaskova and Jonakova (16) with modifications. Sperm suspension diluted 1:10 with PBS was incubated with biotin-labelled nZnBPs and ZnBPs for 1 h at 37°C. After washing and centrifugation, avidin coupled with FITC (100 µg/mL) was added and incubated for 1 h. After washing, the suspension of spermatozoa was diluted 1:10, smeared on slide and incubated for 15 min with 1.5 µg/mL of DAPI (4,6 diamidino-2-phenylindole). Samples were analysed and photographed using confocal laser microscope (Zeiss LSM-700) and its software (Zeiss LSM Image Browser). As a control, spermatozoa were incubated with avidin-FITC; no interaction was observed (data not shown).

The effect of nZnBPs and ZnBPs on canine spermatozoa stored at 5°C. Previously, the influence of ZnBPs and nZnBPs in concentrations ranging from 0.4% to 1% of canine seminal plasma total protein content added to canine-ejaculated spermatozoa on motility parameters and sperm membranes (data not shown) was examined. The best results were obtained when the concentration of ZnBPs and nZnBPs was 0.8% of the total seminal plasma protein content, and only this concentration was used for further experiments. Control - sperm pellet was diluted in PBS to a concentration of 30 x 10^6 cells. Group A –sperm pellet was diluted in PBS with 0.8% nZnBPs (sperm concentration 30 × 10^6 cells). Group B –sperm pellet was diluted in PBS with 0.8% ZnBPs (sperm concentration 30 × 10^6 cells). Aliquots of each sample were taken for analysis (motility, membrane integrity, and mitochondrial status) from fresh and treated semen samples immediately after dilution (time 0), and after 1 h incubation at 5°C (refrigerator). In this study, only semen samples with at least 70% motile spermatozoa and less than 20% morphologically abnormal spermatozoa were used.

Assessment of motility of spermatozoa using CASA. Sperm motility characteristics were evaluated using the Hamilton-Thorne Sperm Analyzer IVOS version 12.3 (Hamilton-Thorne Biosciences, USA). The settings of IVOS were as follows: frame acquired - 30, frame rate - 60 Hz, minimum cell contrast - 75, minimum cell size - 6 pixels, straightness threshold - 75%, low VAP cut-off - 9.9 µm/s^{-1}, low VSL cut-off - 20 µm/s^{-1}, static size gates - 0.80-4.93, static intensity gates - 0.49-1.68, static elongation gates - 22–84. Three microliters of aliquots of semen were placed in a Makler counting chamber (Sefi-Medical Instruments Ltd., Israel) at 38ºC and evaluated. Six fields randomly selected by a computer were analysed for each semen sample.

The following parameters were analysed: total motility (MOT, %), progressive motility (PMOT, %), curvilinear line velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), linearity coefficient (LIN, %), and straightness (STR, %).

Acrosome integrity. Semen samples were evaluated for acrosome integrity as described by Watson (32). For each sample, approximately 200 spermatozoa were counted in a random order under a bright light microscopy (1000 × magnification) and categorised as intact acrosomes or non-intact (damaged acrosomes). Briefly, canine spermatozoa with intact acrosomes exhibited a uniform Giemsa staining pattern overlying the acrosomal region, whereas spermatozoa with non-intact acrosomes displayed a clear patchy staining pattern, with damaged apical ridges or loose

acrosomal caps. The percentage of spermatozoa with intact acrosomes and non-intact acrosomes was recorded.

Plasma membrane integrity. Sperm plasma membrane integrity was assessed with dual fluorescent probes, SYBR-14 and propidium iodide (PI) (Live/Dead Sperm Viability Kit; Molecular Probes, USA), according to the method of Garner and Johnson (12), with slight modifications. For this assay, 10 mL of 1 mM SYBR-14 solution in HEPES-BSA (pH 7.4) and 10 mL of 2.4 mM PI in Tyrode's salt solution were added to 1 mL aliquot of diluted semen samples (30 × 10^6 spermatozoa/mL) and incubated for 10 min at 37°C. Aliquots of stained sperm cells were examined at 600 × magnification under an epifluorescence microscopy (Olympus CH 30, Japan). Membrane-intact spermatozoa stained bright green with SYBR-14, whereas PI showed red fluorescence for membrane-damaged cells. Double-stained (SYBR-14/PI) moribund spermatozoa were also considered as membrane-damaged cells. A minimum of 200 cells per slide was examined in each aliquot.

Mitochondrial activity. The percentage of live spermatozoa with active mitochondria was assessed using a combination of fluorescent stains. The sperm mitochondrial function was assessed using dual staining with fluorescent probes, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes, USA) with PI, according to Dziekonska *et al.* (11). Aliquots of sperm samples (30 × 10^6 spermatozoa/mL) were incubated with JC-1 solution (1 mg of JC-1/mL of dimethylsulfoxide, DMSO) for 15 min at 37°C. Following incubation, sperm samples were stained with PI (10 µL of PI solution in 0.5 mg/mL of phosphate buffered solution) for 10 min at 37°C, washed and centrifuged (600 × g, 5 min at room temperature), and the sperm pellets were re-suspended in a HEPES buffered solution (10 mM HEPES, 0.85% NaCl, 0.1% bovine serum albumin, pH 7.4). Stained sperm samples were placed on microscopic slides, covered with coverslips (22 × 22 mm), and examined under a fluorescence microscope (Olympus CH 30 RF-200). Viable spermatozoa with active mitochondria emitted orange-red fluorescence. Two slides were evaluated per sample and 100 spermatozoa were counted per slide.

Statistical analysis. All results are expressed as mean ± SEM. The data was analysed by ANOVA followed by the Duncan *post hoc* test ($P < 0.05$) for multiple comparisons. The Statistica software package (StatSoft Incorporation, USA) was used to analyse the data.

Results

Attachement of biotin-labelled nZnBPs and ZnBPs to ejaculated spermatozoa. Sperm surface binding sites for nZnBPs and ZnBPs were analysed by a fluorescence technique with biotin-labelled proteins. The binding pattern for nZnBPs was unspecific and no characteristic sites on the sperm plasmalemma were identified (Fig. 1A). The ZnBPs bound mainly to the acrosomal region of sperm head and flagellum (Fig. 1B).

Sperm motility characteristics. Spermatozoa total motility (MOT) decreased ($P < 0.05$) in the group A (nZnBPs), group B (ZnBPs), and in the control after 1 h storage at 5°C (Table 1). However, the smallest decline was observed in group B. Spermatozoa stored in group B displayed a higher ($P < 0.05$) values of MOT and PMOT, VCL, VSL, and VAP parameters when compared with control and group A after 1 h storage. The addition of nZnBPs caused only a weak positive effect ($P > 0.05$) on the sperm motility parameters when compared with control. No significant differences ($P > 0.05$) were observed among groups A and B and control either for ALH, LIN or STR. The values for BCF in group B were higher ($P<0.05$) compared to control after 1 h of storage. Differences in the sperm MOT, PMOT, VCL, VSL, and VAP after 1 h storage were statistically significant between group A and group B ($P < 0.05$).

Acrosome integrity. Spermatozoa stored with ZnBPs (group B) exhibited a higher ($P < 0.05$) percentage of cells with undamaged acrosome when compared with control and group A (Table 2). No statistical differences in the sperm acrosome integrity were observed between group A and control.

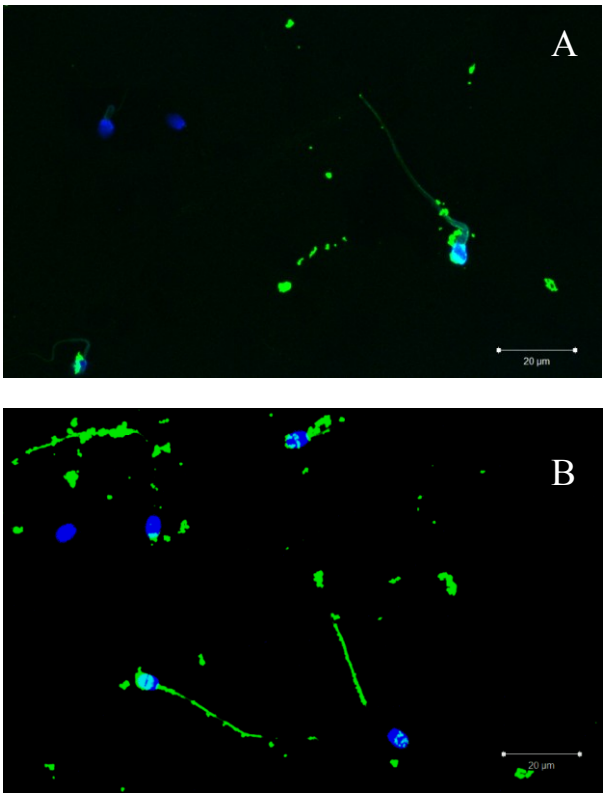


Fig. 1. The attachment of biotin-labelled seminal plasma nZnBP (A) and ZnBP (B) to ejaculated spermatozoa, detected by the fluorescence technique. Green colour: fluorescence presenting the binding of nZnBPs and ZnBPs (biotin/avidin-FITC system); Blue colour: staining of the cell nucleus with DAPI. Scale bar 20µm

Table 1. Percentage of spermatozoa exhibiting total motility (MOT), progressive motility (PMOT), curvilinear velocity (VCL), straight line velocity (VSL), average velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linear coefficient (LIN), progressive motile spermatozoa (STR,%). Group A - spermatozoa incubated with 0.8% mixture of nZnBPs isolated from canine seminal plasma; Group B – spermatozoa incubated with 0.8% mixture of ZnBPs isolated from canine seminal plasma (n = 12)

Incubation variants	MOT %		PMOT %		VCL µm/s		VSL µm/s		VAP µm/s		ALH µm		BCF Hz		LIN %		STR %	
Incubation time	0 h	1 h	0 h	1 h	0 h	1 h	0 h	1 h	0 h	1 h	0 h	1 h	0 h	1 h	0 h	1 h	0 h	1 h
Control	46.3 ± 4.6^{ax}	28.0 ± 3.7^{ay}	4.2 ± 1.9^{ax}	2.6 ± 0.9^{ay}	103.6 ± 12.3^{a}	93.3 ± 9.5^{a}	50.3 ± 6.7^{a}	51.7 ± 5.2^{a}	64.3 ± 8.8^{a}	64.3 ± 6.8^{a}	5.7 ± 1.0	5.6 ± 1.4	5.1 ± 0.7^{a}	9.3 ± 1.7^{a}	50.3 ± 2.2	58.1 ± 2.3	78.7 ± 2.5	80.8 ± 1.2
Group A	48.0 ± 6.6^{ax}	31.2 ± 6.5^{ay}	4.3 ± 1.3^{a}	3.2 ± 1.4^{a}	109.9 ± 11.0^{a}	109.2 ± 10.5^{a}	54.8 ± 5.4^{a}	50.5 ± 4.8^{a}	69.7 ± 7.2^{ab}	66.3 ± 6.7^{a}	6.2 ± 0.7	6.3 ± 1.3	14.2 ± 0.4^{b}	12.9 ± 2.1^{ab}	51.3 ± 2.7	48.8 ± 2.4	78.5 ± 3.1	76.5 ± 1.5
Group B	50.0 ± 2.1^{a}	48.7 ± 8.3^{b}	9.4 ± 3.1^{a}	10.6 ± 3.4^{b}	132.7 ± 10.6^{b}	132.4 ± 8.9^{b}	69.3 ± 5.2^{b}	66.5 ± 4.7^{b}	83.5 ± 6.5^{b}	84.1 ± 5.6^{b}	6.7 ± 0.5	6.9 ± 0.5	16.8 ± 1.4^{b}	14.5 ± 1.8^{b}	53.4 ± 1.3	52.6 ± 1.0	82.4 ± 1.5	79.1 ± 1.2

Values are expressed as mean ± SEM
^{ab}Different superscripts denote statistical difference ($P \leq 0.05$) within columns
^{xy}Different superscripts denote statistical difference ($P \leq 0.05$) within rows

Table 2. Percentage of spermatozoa with undamaged acrosome, intact plasmalemma, and active mitochondria in fresh semen after dilution (time 0) and after 1 h incubation at 5°C. Control – spermatozoa incubated with PBS; Group A - spermatozoa incubated with 0.8% mixture of nZnBPs isolated from canine seminal plasma; Group B – spermatozoa incubated with 0.8% mixture of ZnBPs isolated from canine seminal plasma (n = 12)

Incubation variant	Undamaged acrosome %		Intact plasmalemma (SYBR/PI dual staining) %		Active mitochondria (JC-1/PI dual staining) %	
Incubation time	0 h	1 h	0 h	1 h	0 h	1 h
Control	91.0 ± 3.2^{a}	89.6 ± 3.6^{a}	70.5 ± 4.3^{a}	68.3 ± 5.4^{a}	75.5 ± 3.5	76.8 ± 7.1
Group A	93.7 ± 2.6^{ab}	94.0 ± 1.0^{a}	82.8 ± 2.8^{ab}	76.3 ± 5.2^{ab}	81.0 ± 2.7	82.6 ± 5.8
Group B	96.4 ± 1.4^{b}	96.1 ± 1.4^{b}	84.3 ± 3.1^{b}	86.0 ± 2.5^{b}	81.4 ± 2.5	86.4 ± 5.4

Values are expressed as mean ± SEM
^{ab}Different superscripts denote statistical difference ($P \leq 0.05$) within columns

Membrane integrity. Spermatozoa stored with ZnBPs (group B) exhibited a higher ($P < 0.05$) percentage of cells with intact plasmalemma when compared with control at time 0 and after 1 h storage (Table 2). In group A the addition of nZnBPs had a slight positive effect on the sperm plasma membrane.

Mitochondrial activity. Spermatozoa stored with ZnBPs (group B) and nZnBPs (group A) exhibited a higher percentage of cells with active mitochondria when compared with control at time 0 and after 1 h; however, no statistical differences were observed (Table 2).

Discussion

The prostate is the only accessory sex gland of the canine reproductive system and prostatic secretory fluid is practically the only component of the seminal plasma in this species. Proteins are the main constituent of prostatic secretion which is the source of high quantities of zinc ions (17). It is known that zinc ions fulfil their functions in the reproductive system due to binding different (low and high) molecular weight ligands (5, 26). The findings of our previous study demonstrated the presence of high molecular weight zinc-binding protein ligands in boar and canine seminal plasma (18, 19). Under the influence of denaturing and reducing agents, they dissociate to low molecular weight polypeptides that together with sperm-adhesive properties are also present in large quantities in boar, bull, and stallion seminal plasma (8, 9, 31). The sperm-adhesins' structure and function are well-documented, in contrast to canine seminal plasma proteins with almost unknown properties. To complement the lack of information on canine seminal plasma ZnBPs, we aimed to establish their influence on the motility and membrane integrity of liquid stored canine spermatozoa using sensitive diagnostic methods.

Sperm storage is often associated with the use of low temperature conditions. But it is well-known that low temperatures alter the function of spermatozoa. Cold shock results in the destabilisation of sperm membranes and impairment of sperm function. There is an information that the addition of seminal plasma proteins to spermatozoa before cooling and freezing minimizes cryoinjury effects (3, 6, 20). In canine, research demonstrated both negative (25) and positive effect (21) of prostatic fluid on the fertilising capacity of frozen-thawed spermatozoa. The use of prostatic fluid to dilute thawed canine spermatozoa resulted in higher fertility in bitches after intravaginal insemination (22). The findings of our study demonstrated that the addition of ZnBPs, isolated from canine seminal plasma, to incubation mixture of spermatozoa stored at 5ºC caused the maintenance of motility parameters and protected the sperm membrane against cold shock damage. Compared to fresh semen, the percentage of motile spermatozoa decreased considerably in samples washed with PBS. This resulted from the substantial dilution of spermatozoa (30×10^6 cells), which is connected with the removal of adsorbed plasma proteins with a protective action on spermatozoa membranes. Canine prostatic fluid proteins coat spermatozoa and mask progesterone receptors, delay capacitation (28), and protect sperm membranes (30). By using fluorescence technique we showed that ZnBPs are mainly attached to the acrosome and flagellum of ejaculated canine spermatozoa, thus they can exert the positive effect on these structures preventing from their damage. In other mammal species, spermadhesins coat spermatozoa, which serve as acrosome membrane-stabilising factors (31). There are several proteins with sperm-coating and zinc-binding properties, which also have a positive impact on the parameters of the stored spermatozoa. The main boar seminal plasma zinc-binding spermadhesin - PSPI/PSPII, exerted positive effects on highly-diluted boar spermatozoa viability, motility and mitochondrial activity (7, 10). ZnBPs of boar seminal plasma allowed preservation of a higher percentage of cells exhibiting linear motility and a higher percentage of intact acrosomes when stored at 4ºC (19). A positive relationship between the quantity of high molecular weight ZnBPs and sperm motility was also found in humans (1). Some positive effects exerted by canine ZnBPs may occur due to albumins. They belong to the zinc-binding protein family. Albumin can absorb lipid peroxides, which contributes to its protective effect on the sperm membrane and motility (2). In our study, nZnBPs exhibited only weak protecting effect (no statistical differences) on spermatozoa motility parameters and membrane integrity. Such occurrence indicates the importance of zinc-binding properties of seminal plasma proteins in terms of the protective effect.

Under cold shock conditions spermatozoa absorb eight times more zinc than at physiological temperature (5). Moreover, excessively high zinc content in spermatozoa exerts inhibitory effect on their motility (13). Thus, it is possible that one of the mechanisms of the ZnBPs action on canine spermatozoa motility is their ability to chelate free or loosely-bound zinc ions, which prevents their excessive absorption by spermatozoa under cold shock conditions. Such a property was observed in human seminal plasma ZnBPs (4). On the other hand, ZnBPs can also coat membranes (especially the acrosome and flagellum), which prevents their damage and changes in their permeability.

Presented results emphasise the significant role of seminal plasma proteins in securing the correct quantity and availability of zinc ions as a component regulating the motility of canine spermatozoa. However, a detailed explanation of the mechanisms of plasma ZnBPs' action on canine spermatozoa (under physiological conditions and during their storage) requires further research.

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