Theriogenology 197 (2023) 159–166

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Inhibition of the cholesterol transporter ABCA1 by probucol decreases capacitation and tyrosine phosphorylation of dog spermatozoa, and is dose dependent

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ARTICLE INFO

Article history:
Received 18 September 2022
Received in revised form
28 November 2022
Accepted 28 November 2022
Available online 5 December 2022

Keywords:
Dogs
Sperm membrane
Cholesterol
Capacitation
ABCA1

ABSTRACT

The ATP binding cassette (ABC) transporter molecule ABCA1 participates in the cholesterol transport within and through cell membranes. We recently demonstrated that in dog spermatozoa, capacitation could be decreased with probucol (PRO), an ABCA1 specific antagonist. In this study, a dose-effect relationship of PRO on dog sperm capacitation, tyrosine phosphorylation and cholesterol efflux from the sperm plasma membrane was investigated. A total of 16 ejaculates from dogs of different breeds, aged 2–4 years were used. Sperm motility and membrane integrity in the main fraction was determined by CASA. Samples were stained with a boron dipyrromethene difluoride (BODIPY) fluorophore (P9672, Sigma- Aldrich, A) diluted in DMSO at a final concentration of 0.4 μM. All samples were divided into 5 aliquots, with 0, 100, 250, 500 and 1000 μM of PRO. After incubation at 37 °C for 2 h, PI was added and flow cytometry performed. All aliquots were examined for capacitation and acrosome reaction by using the CTC assay and tyrosine phosphorylation (TP). Membrane integrity was measured in all aliquots to investigate the effect of PRO on cell membranes. Membrane integrity did not differ between controls (0 μM), and 100, 250 and 500 μM PRO, but decreased with 1000 μM PRO ($p < 0.05$). Increasing PRO concentration decreased the percentage alive cells with cholesterol efflux per PRO group (0 μM: 77.8 ± 10.6%, 100 μM: 63.7 ± 11.7%, 250 μM: 52.1 ± 12.9%, 500 μM: 37.7 ± 11.6%, 1000 μM: 33.1 ± 14.4%; $p < 0.05$), decreased head and entire tail phosphorylated cells (0 μM: 34.6%, 1000 μM: 5.1% $p < 0.05$); and decreased the percentage capacitated cells (maximum with PRO 500 μM: capacitated vs. control: 54.2 ± 17% vs 25 ± 7.7%, $p < 0.05$). Conclusion: PRO decreased the cholesterol efflux, and decreased tyrosine phosphorylation and capacitation in a dose-dependent manner. This suggests a strong involvement of the ABCA1 transporter in different functional aspects of sperm capacitation in dogs.

1. Introduction

The ATP binding cassette (ABC) transporters are membrane molecules regulating the cholesterol and phospholipid transport within and through cell membranes of many different cells, including spermatozoa [1–3]. The ABC transporters have been detected in spermatozoa membranes of many species; ABCG2 for example was found in mature spermatozoa from mice, rats, bulls and humans [4]. In bull spermatozoa, ABCG2 was found to regulate cholesterol efflux; however, in epididymal sperm only [5]. In mice, expression of ABCA1, A7, A17 and G1 was detected in spermatozoa membranes [1,6]. Expression of ABCA1 was also assessed in human spermatozoa membranes and a role in spermatozoa development was hypothesized [7]: Recently we detected ABCA1 expression in adult and prepubertal stallion spermatozoa [8] and in membranes of canine epididymal and ejaculated spermatozoa [9]. ABCA1 is a molecule that regulates the unidirectional lipid transport within and through membranes and mainly the cholesterol efflux [10]; it is strongly expressed in mouse testicular tissue [11]. In ram, ABCA1 is supposed to play a role in reverse cholesterol transport; however, inhibition only reduced the cholesterol efflux by 8–15% [12]. Cholesterol efflux is an essential step during capacitation of spermatozoa. The ABC transporters have been suggested to contribute to the active transport of cholesterol and phospholipids to certain micro-domains (rafts) in the outer spermatozoa membrane, mainly

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https://doi.org/10.1016/j.theriogenology.2022.11.046

located in the apical region of the head, where the molecules are accumulating and presented to acceptor molecules [13]; in case of ABCA1, to lipid-poor apolipoproteins [14]. The ABC transporters are thus providing a facilitated pathway but the procedure is active. The acceptor molecules can then transport cholesterol to sites of need like the oviduct epithelia [13]. However, there seem to species-specific differences concerning which ABC transporters mainly participate in the cholesterol efflux and there are few studies investigating the situation in canine spermatozoa.

In a former study we demonstrated expression of ABCA1 in membranes of canine epididymal and ejaculated spermatozoa, mainly in the acrosome and midpiece [15]. To investigate the function of this molecule, we performed follow up studies using antagonists. The lipid-lowering drug probucol (PRO) was found to significantly inhibit cholesterol efflux in human skin fibroblasts and J774 mouse macrophages [16–18]. Therefore, in a recent study, we tried to antagonize the ABCA1 molecule in dog spermatozoa membranes by using different concentrations of this specific ABCA1 inhibitor [19]. With a final concentration of 100 μM PRO, we observed a decrease in capacitated sperm as determined by CTC, and in addition a decrease of cholesterol efflux as assessed with a cholesterol efflux assay. However, the effect was low grade. We therefore hypothesized that the effect might be dose-dependent. The aim of this study was to investigate a dose-effect relationship between different doses of PRO and 1) membrane integrity, 2) cholesterol efflux, 3) capacitation as examined by CTC and 4) movement of lipids during membrane remodeling as examined by tyrosine phosphorylation.

2. Materials and methods

2.1. Animals and semen collection

A total of 16 semen samples were collected by digital manipulation. All dogs were breeding dogs of different breeds and either animals belonging to the University of Veterinary Medicine Vienna (3 Beagles) or healthy patients of the University of Veterinary Medicine Vienna, referred for semen analysis (Schnauzer, Flat Coated Retriever, Golden Retriever, Berger Blanc Suisse). The university ethics committee was informed; however, an approval was not required. At the beginning of the project, one Beagle was repeatedly collected on consecutive days (n = 8) to establish the method. Only semen of good quality and only the sperm rich fraction was used for the experiment.

2.2. Semen quality analysis

2.2.1. Kinematic data

Motility (M), progressive motility (P) and further kinematic parameters were examined by CASA (CASA, Sperm Vision®, Minitüb, Tiefenbach, Germany) after dilution to 100 × 10^6 spermatozoa/mL with a TRIS-citric acid-fructose buffer [17–19]. The temperature of analysis was 37 °C. A 3 μL droplet was placed on a glass slide and covered with a cover slip. Seven fields were evaluated per sample with 100 cells per field and a frame rate of 60/s, always by the same examiner. The following motility parameters were assessed: Curvilinear velocity (VCL, μm/s), Linear velocity (VSL, μm/s), Mean velocity (VAP, μm/s), Mean coefficient (STR, %), Linear coefficient (LIN, %), Wobble coefficient (WOB, %), Frequency of head displacement = beat cross frequency (BCF, Hz), Amplitude of lateral head displacement (ALH, μm), Distance curved line (DCL, μm), Distance average path (DAP, μm). Total motility was defined as VCL >15 μm/s, progressive forward motility as VCL >15 μm/s and LIN >0.9%. Linear motility was defined as STR >0.9% and LIN >0.5%.

2.2.2. Membrane integrity

The membrane integrity was assessed by using SYBR-14/PI double staining [19] visualized by an epifluorescence microscope connected to the CASA; briefly, 100 μL of sperm sample were placed in a vial with 2 μL of SYBR-14/PI and incubated for 10 min at room temperature (around 21 °C) in darkness. One droplet was placed on a glass slide, covered with a glass coverslip and evaluated via fluorescence microscopy at magnification ×400 (Olympus AX70, Olympus Optical Co., Ltd., Japan; U-MWB filter block, BP420-480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500). The heads of viable spermatozoa show bright green color, damaged membranes are stained red. Both colors are recognized automatically by a video camera. Each sample was evaluated once, but at least 500 spermatozoa were evaluated per sample and the mean calculated by the software provided by SpermVision®. Results are given as mean percentage of spermatozoa with intact (green) membranes.

2.2.3. Concentration measurement and morphology

The concentration of the sperm samples was determined using a Nucleocounter SP-100 (ChemoMetec A/S, Allerod, Denmark) according to the manufacturer's instructions. The percentage of spermatozoa with abnormal morphology and acrosome damages were assessed after fixation in Hancock's solution (37% formalin [19]) by analyzing 200 cells per sample under a phase contrast microscope at ×1000 magnification (oil immersion).

2.2.4. Experimental design

A detailed description of the experimental design in provided in Fig. 1.

2.2.5. Incubation with bodipy and probucol

After the basic examinations for normal semen characteristics, the raw semen was washed twice with PBS by centrifugation at 700*g* for 5 min. The supernatant was discarded and the pellet rediluted with PBS to 100 × 10^6/mL. Out of this pool, one aliquot was taken and diluted to 5 × 10^6/mL for the flow cytometry control samples with propidium iodide (PI) and probucol (PRO). Further aliquots were taken for membrane integrity examination after addition of PRO (0, 100, 250, 500 and 1000 mM in 0, 2, 5, 10 and 20 μL of DMSO). These samples were then used for the CTC assay and the tyrosine phosphorylation staining.

To the pool with 100 × 10^6 spermatozoa/mL, a boron dipyrromethene difluoride (Bodipy) fluorophore (P9672, Sigma- Aldrich, Vienna, Austria) was added at a final concentration of 0.4 μM, as described by Bernecic et al. [20]. For this purpose, a stock solution was prepared (1 mM Bodipy in DMSO) and 1 μL of the Bodipy stock solution added to 999 μL of semen; the suspension was incubated for 10 min in the dark at 37 °C, and then washed with PBS to eliminate superfluous Bodipy. The pellet was resuspended with PBS to a concentration of 5 × 10^6/mL. From this Bodipy marked pool, aliquots were taken for control samples (one for Bodipy only and four with DMSO only), furthermore five aliquots for the different concentrations of probucol (PRO; Sigma Aldrich, P9672) were taken. For the PRO samples, a 50 mM stock solution was prepared (25.8 mg in 1 ml 100% DMSO). From this stock solution, 0, 2, 5, 10 and 20 μL were added ad 1 mL of semen for a final concentration of 0, 100, 250, 500 and 1000 μM of PRO. All samples (membrane integrity samples, experimental samples and controls) were incubated for 1 h at 37 °C in a water bath in the dark. Ten minutes before the end of incubation, PI was added at a final concentration of 5 μg/mL (2.5 μL/mL of semen), then the experimental and control samples prepared for flow cytometry.

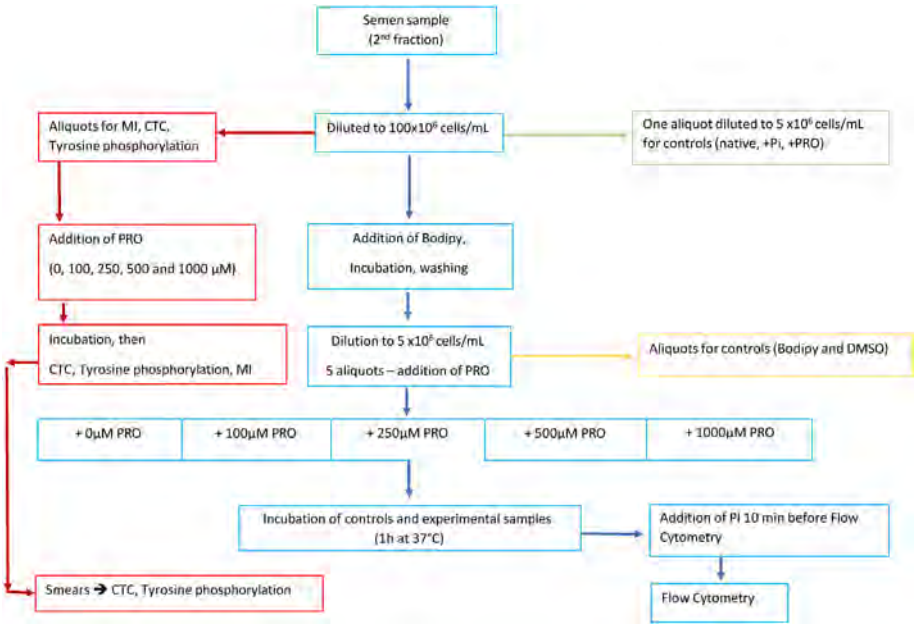

Fig. 1. Experimental design. CTC = chlortetracycline assay, MI = membrane integrity, PI = propidium iodide, PRO = probucol.
One native sample was first diluted to 100 × 10^6 spermatozoa/mL. From this pool, samples were taken for analysis of membrane integrity (MI), tyrosine phosphorylation and chlortetracyclin assay (CTC); see left side - red arrows. These samples did not receive Bodipy; however, PRO was added and the samples incubated as those for flow cytometry. The smears for tyrosine phosphorylation were prepared after incubation. The CTC samples were diluted 1 + 1 to 50 × 10^6 spermatozoa/mL, to obtain enough volume (200 µL per sample). From the pool of 100 × 10^6 spermatozoa/mL, one more aliquot was taken and diluted to 5 × 10^6 spermatozoa/mL to prepare controls for later flow cytometry: native semen, semen with PI and semen with PRO; see right side - green arrow. Then Bodipy was added to the pool with 100 × 10^6 spermatozoa/mL, the sample incubated in a water bath at 37 °C for 10 min in the dark and then washed to remove excess Bodipy (middle, blue arrows). After washing, the sample was diluted to 5 × 10^6 spermatozoa/mL. From this pool, aliquots were taken for flow cytometry analysis of controls (one with Bodipy and four with 2,5,10 and 20 µL of DMSO; right side - yellow arrow). The remaining pool with 5 × 10^6 spermatozoa/mL was split in aliquots and 0,100,250, 500 and 1000 µM PRO added. The samples were incubated in a water bath for 1 h at 37 °C in the dark. 10 min before the end of incubation time, PI was added to all experimental samples, and the controls with PI and DMSO. For each sample, flow cytometry was performed, first with controls (native semen, PI, PRO, Bodipy, DMSO) and then with the experimental samples. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.2.6. Chlortetracyclin (CTC) assay

From the pool without Bodipy, samples were taken after incubation with PRO and a CTC assay was performed as described by Hewitt [21] and Witte et al. [22] with experimental and control samples. All chemicals were obtained from Sigma-Aldrich (Hoechst 33258: H6024; glutaraldehyde: G6403; DABCO: D2522, chlortetracycline CTC: 94498; Cysteine: 30095; polyvinylpyrrolidone PVP: 81400). CTC-solution: 4 mg CTC Chloride +8.8 mg Cysteine in 10 mL CTC buffer: 24 mg TRIS +76 mg NaCl/10 mL aqua bidest. PVP 2% in PBS: 40 mg PVP/2 mL PBS. The CTC buffer and PVP solution were aliquoted and stored at −18 °C, the CTC solution was freshly prepared before each assay.

After 2 h of incubation, 0.1386 µg Hoechst 33258 (0.7 µg/mL) were added to both of the 200 µL aliquots and the samples incubated for 2 min. Afterwards, 2 mL PVP 2% in PBS were added and the mixture centrifuged at 900 *g* for 5 min. The sperm pellet was mixed with 45 µL of CTC-solution (freshly made before analysis) and incubated for 20 s. After addition of 8 µL of a glutaraldehyde-solution 12.5%, 10 µL of each semen sample were put on a glass slide together with 1 droplet of DABCO 0.22 M in glycerol, mixed, the smear covered and sealed with clear varnish. The smears were stored at 4 °C in darkness and evaluated the next day. The percentage of uncapacitated (U), capacitated (C) and acrosome reacted (AR) sperm was assessed using a fluorescence microscope at magnification ×1000 (oil immersion, filter cube U-MWB; Fig. 2a,b,c).

2.2.7. Tyrosine phosphorylation (indirect immunofluorescence)

For tyrosine phosphorylation, samples were taken from the same pool without Bodipy as for the CTC and after incubation with PRO, indirect immunofluorescent staining was performed. A semen smear was prepared on a glass slide, fixed with 4% paraformaldehyde (PFA; w/v) for 30 min at room temperature and air dried on slides. Spermatozoa were then permeabilized using 0.5% Triton X-100 (v/v) for 15 min at room temperature. After rinsing with PBS, slides were incubated with 1% BSA in PBS (w/v; PBS-BSA) for 1 h at room temperature and incubated overnight at 4 °C with mouse anti-phosphotyrosine antibody (1:100 dilution; clone 4G10®; Merck Millipore, Darmstadt, Germany) in PBS-BSA. Prepared slides were washed three times with PBS-BSA and incubated for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:100 dilution; Sigma Aldrich, Vienna, Austria). Spermatozoa nuclei were counterstained with 10 µM Hoechst33342 (Sigma Aldrich, Vienna, Austria). After extensive washing with PBS, slides were mounted with Entellan® (rapid mounting medium, 107960, Merck Millipore, Vienna, Austria) and covered with a coverslip. All slides were microscopically examined using a wide field fluorescence-imaging microscope (Zeiss Axio Imager Z2, magnification ×630, oil immersion, NA 1.4). Alexa Fluor 488 and Hoechst 33342 were excited using a 450–490 nm and 300–400 nm spectrum and the fluorescence detected using 500–550 nm and 420–470 nm, respectively (Carl Zeiss Microscopy, LLC, Filter Set 38 and 49). Images were evaluated for tyrosine phosphorylation as described by Petrunkina et al. [23,24]. Briefly, six patterns were seen (Fig. 2): 1) non-phosphorylated sperm with marked fluorescence of the equatorial zone; 2) sperm with midpiece protein tyrosine phosphorylation, and 3) with entire tail protein tyrosine phosphorylation; 4) sperm with head protein tyrosine phosphorylation; 5) sperm with head and midpiece or 6) with head and tail protein tyrosine phosphorylation. A total of

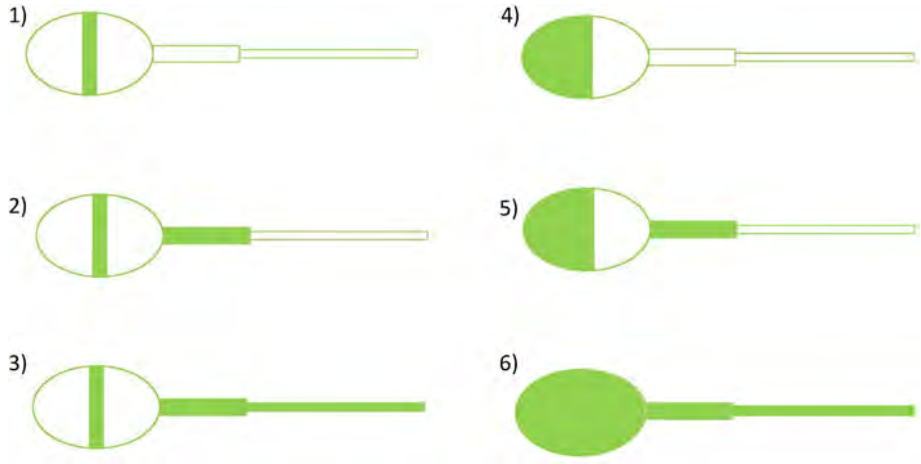

Fig. 2. Tyrosine phosphorylation patterns of dog spermatozoa
The evaluation scheme as described by Ref. [23] was used. The green color indicates fluorescence. 1) No phosphorylation; 2) Midpiece phosphorylated, head not phosphorylated; 3) Entire tail phosphorylated, head not phosphorylated; 4) Tail not phosphorylated, head phosphorylated; 5) Midpiece and head phosphorylated; 6) Entire tail and head phosphorylated. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

200 cells was evaluated per sample and the percentage of each pattern assessed.

2.2.8. Flow cytometry analysis of cholesterol efflux

Flow cytometry analysis was performed using a BD FACSCANTO II flow cytometer (Becton Dickinson Biosciences). Bodipy cholesterol and PI were excited at a wavelength of 488 nm. Bodipy-cholesterol fluorescence was detected at 530 nm and PI fluorescence at 585 nm. A sperm cell specific population was gated based on a forward light scatter and sideward light scatter profiles. 50000 cells were recorded per samples for further analysis in FlowJo (Version 10, FlowJO, LLC). Cells were gated as follows: Q1 = non-viable cells (PI+) without cholesterol efflux, Q2 = non-viable (PI+) cells with cholesterol efflux, Q3 = viable (PI-) alive cells with cholesterol efflux, Q4 = viable cells (PI-) without cholesterol efflux. Before each measurement, the control samples (raw sperm, sperm with Bodipy only, sperm with DMSO only, sperm with PI only) were evaluated first. Results are given as percentage alive cells with cholesterol efflux per PRO group.

3. Statistical analyses

All analyses were calculated using the IBM SPSS statistics version 24 (SPSS Ltd, Hong Kong). Data were analysed for normal distribution using the Kolmogorov Smirnov test. Analysis for significant differences between groups was done using Sirdak's multivariate analysis, comparison between two groups was performed using the Mann Whitney *U* test. To exclude a possible dog effect, the dog was chosen as random factor, and all others as fixed variables. The mixed model analysis revealed that the dog effect was negligible. All data are given as x ± SD, if not stated otherwise. A p value of $\leq$0.05 was considered statistically significant.

4. Results

4.1. Spermatozoa concentration and kinematic data

The spermatozoa concentration averaged 227.1 ± 178.5 × 10^6/mL, the average motility was 90.0 ± 8.2% and the average progressive motility 85.3 ± 12.8%. Further kinematic data of 14 dogs as measured by CASA were recorded and are shown in Table 1.

4.2. Membrane integrity

Membrane integrity of raw samples was 91.7 ± 5.1% (n = 16). After dilution and incubation of samples with PRO, values were significantly decreased, but did not differ between controls (0 μM), and 100, 250 and 500 μM PRO, respectively; however, membrane integrity decreased with 1000 μM PRO in comparison to the controls with 0 μM PRO ($P < 0.05$, Fig. 3).

4.3. Cholesterol efflux (flow cytometry)

For flow cytometry, n = 13 samples were available and evaluated. Without addition of PRO, the majority of the alive sperm population showed cholesterol efflux. This population shifted markedly towards the quadrant of alive cells without efflux (Q4), when the ABCA1 antagonist PRO was added, especially with 500 μM. The effect was not increased with 1000 μM, as is shown in the exemplary scatter plot (Fig. 4a and b).

After addition of PRO and incubation for 1 h at 37 °C, the cholesterol efflux decreased with increasing PRO concentration by 14–40% (in comparison to 0 μM: 77.8 ± 10.6%; 100 μM: 63.7 ± 11.7%, $p < 0.05$; 250 μM: 52.1 ± 12.9%, $p < 0.01$; 500 μM: 37.7 ± 11.6%, $p < 0.01$; 1000 μM: 33.1 ± 14.4%, $p < 0.05$ in comparison to 250 μM; Fig. 5) and the percentage of alive cells without efflux increased

Table 1
Kinematic data.

	N	Minimum	Maximum	X	SD
DAP μm	14	24.42	58.21	46.52	12.81
DCL μm	14	47.91	89.56	72.45	14.57
VAP μm/s	14	15.42	54.09	41.70	14.06
VCL μm/s	14	53.36	132.62	104.80	29.82
VSL μm/s	14	103.95	206.63	162.60	35.06
STR %	14	33.89	121.18	94.03	32.30
LIN %	14	0.63	0.94	0.86	0.09
WOB %	14	0.32	0.68	0.55	0.12
ALH μm	14	0.47	0.73	0.63	0.08
BCF Hz	14	3.08	5.71	4.38	0.75

DAP = Distance average path, DCL = Distance curved line, VAP = Mean velocity, VCL=Curvilinear velocity, VSL=Linear velocity, STR = Mean coefficient, LIN = Linear coefficient, WOB=Wobble coefficient, ALH = Amplitude of lateral head displacement, BCF=Frequency of head displacement = beat cross frequency. X = mean, SD = standard deviation.

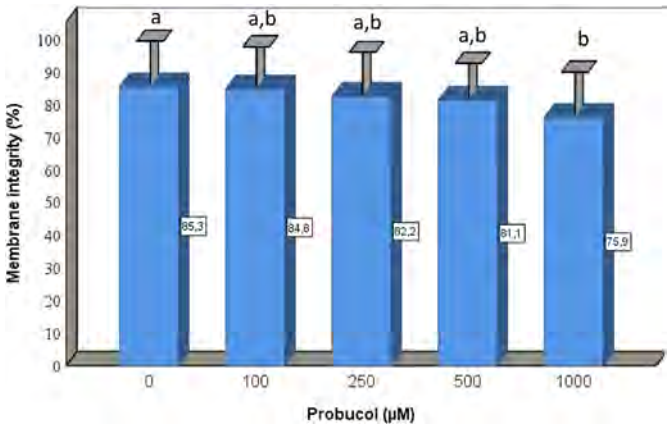

Fig. 3. Membrane integrity in the samples with different PRO concentrations
Membrane integrity was measured after staining with CYBR-14/PI using a fluorescence microscope; the average of 500 automatically evaluated cells was calculated by SpermVision® and the result given as percentage alive/viable cells (x ± SD); a,b = $p < 0.05$.

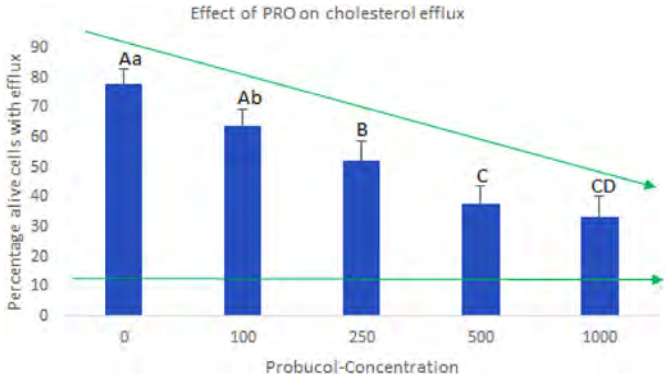

Fig. 5. Effect of PRO on cholesterol efflux
Only alive cells were evaluated. a,b = $p < 0.05$; A,B; A,C = $p < 0.01$; B,D = $p < 0.05$, PRO concentration is given in µM. Values are x ± SD.

(Fig. 6). With 1000 µM PRO, cells showed an increase in deviation from the average value, probably due to marked morphological changings of the head.

4.4. Tyrosine phosphorylation

Tyrosine phosphorylation revealed typical fluorescence patterns, as previously described [23]. An intense fluorescence over the equatorial region was seen in all cells. Significant differences between PRO groups were found for non-phosphorylated (Fig. 7a), and head and entire tail phosphorylated cells (Fig. 7b). PRO in increasing concentrations increased the percentage of non-phosphorylated cells and decreased the head and entire tail phosphorylated cells significantly (Fig. 8). Samples with 1000 µM PRO did not increase the effect seen with 500 µM PRO; however, showed a high percentage of morphologically abnormal spermatozoa with changes in head size and shape.

4.5. Chlortetracycline assay (CTC)

The CTC assay revealed a dose dependant increase in non-capacitated cells and a decrease in capacitated cells with increasing PRO. Largest effects were determined with PRO 500 µM: capacitated vs. control (0 µM): 54.2 ± 17% vs 25 ± 7.7% ($p < 0.05$, Fig. 9). The percentage of acrosome reactions did not change with changing PRO concentrations.

5. Discussion

In this study, the effect of increasing concentrations of the ABCA1 antagonist probucol (PRO) was striking. The effect of a decrease in cholesterol efflux on capacitation, tyrosine phosphorylation and on membrane integrity was investigated and the effect on all investigated parameters was dose-dependent. Different dosages were required to recognize effects on membrane integrity, calcium redistribution during the CTC assay or the shift in membrane phospholipids during the tyrosine phosphorylation assay. When looking at the results of the membrane integrity assay which was done using CYBR-14/PI, a significant decrease was observed with 1000 µM PRO diluted in DMSO at a concentration of 20 µL/mL of semen; at this concentration, we additionally recognized an effect on sperm morphology, especially the heads. DMSO is believed to have caused these morphological changes that probably only concerned a subpopulation of highly sensitive cells and consecutively decreased membrane integrity. Interestingly the control

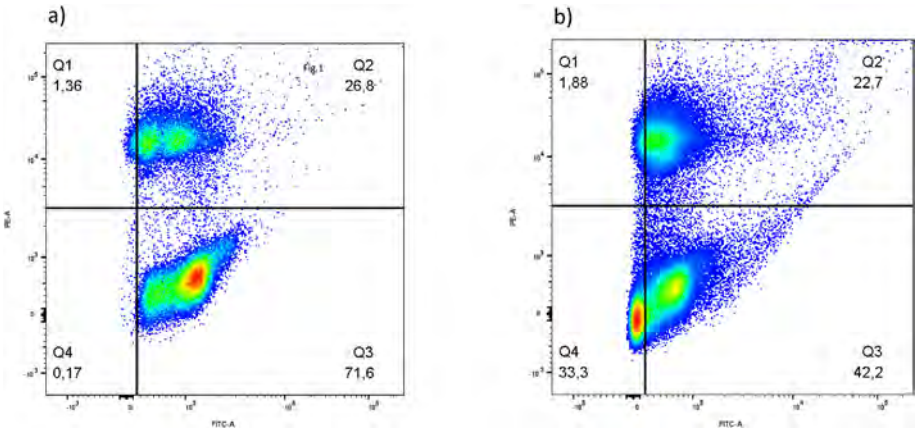

Fig. 4. Flow cytometry of sperm samples after staining with a Bodipy-fluorophore
Examples for flow cytometry scatter plots after gating using the FlowJo software. a) 0 µM PRO, b) 1000 µM PRO. PI = propidium iodide, Q1 = non-viable cells (PI+) without cholesterol efflux, Q2 = non-viable (PI+) cells with cholesterol efflux, Q3 = viable (PI-) alive cells with cholesterol efflux, Q4 = viable cells (PI-) without cholesterol efflux. The numbers in the quadrants indicate the final percentages of 50.000 evaluated cells. With 1000 µM PRO, more cells outside the main population were seen showing a marked shift to the right upper corner of the quadrant. The pattern was consistent among individuals.

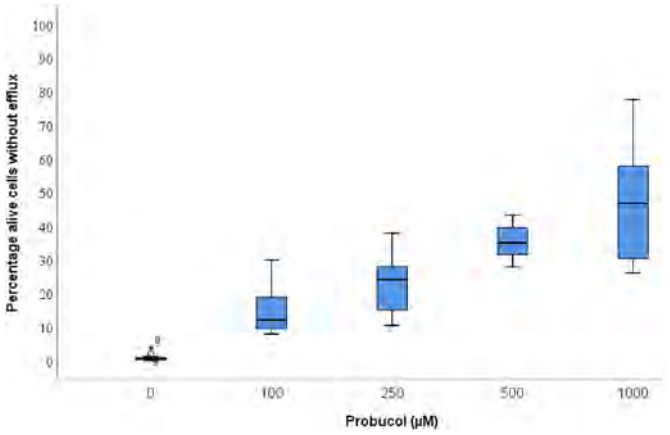

Fig. 6. Box and Whisker plots of alive cells without efflux in samples with increasing PRO concentration
The figure impressively shows the increase in the percentage of alive cells without efflux with increasing PRO; however, in addition the increase in deviation – the increase in DMSO and/or PRO caused changes in head size and shape, and an increase in cell damages in the samples with 1000 μM, as was seen microscopically.

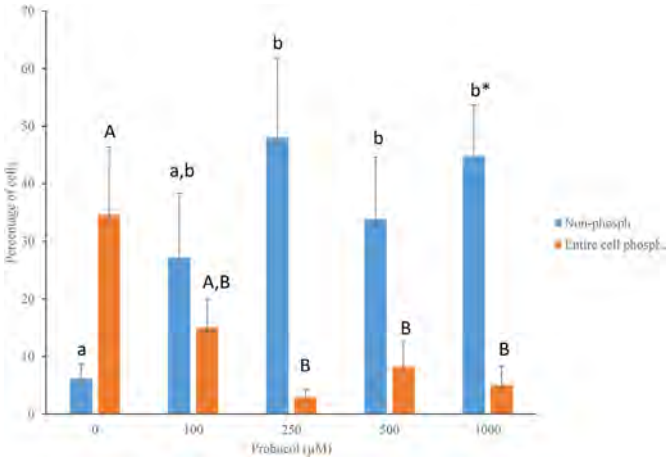

Fig. 8. Effect of PRO on tyrosine phosphorylation
Only two phosphorylation patterns out of six are shown since these patterns showed significant changes with increasing PRO concentrations.
Non-phosp. = percentage of cells without tyrosine phosphorylation; Entire cell phosph. = percentage of cells with complete tail and entire head tyrosine phosphorylation. Data are given as x ± SF (n = 8).
a,b = $p < 0.05$, a,b* = $p < 0.01$ (Non-phosph.); A,B = $p < 0.05$ (Entire cell phosph.).

sample with DMSO only (20 μL/mL of semen) did not change the percentage of cells with cholesterol efflux as seen with flow cytometry; the effect was restricted on membrane integrity. This effect obviously is dose-dependent, since DMSO at lower concentrations is known to exert an antioxidative effect on dog spermatozoa [25]. When goat spermatozoa were diluted with a TRIS-egg yolk extender containing 1.75% v/v of DMSO, the percentage of normal acrosomes significantly decreased in comparison to 1% v/v of DMSO [26]. The final concentration of DMSO was much lower than in our experiment, emphasizing the differences between species concerning the sensitivity of the membrane when exposed to DMSO. These results show that the DMSO concentration is a crucial parameter whereas the PRO concentration used in our study was acceptable and probably not causing damages per se. However, DMSO is required to solubilize PRO and higher concentrations of PRO in lower volumes of DMSO would be interesting. Therefore, before the start of the study, different concentrations of PRO in DMSO were investigated; stock solutions with 100 and 500 mM were prepared. The solution of DMSO was only useful when a maximum concentration of 50 mM was used; higher concentrations caused clotting.

The PRO concentrations used in this study effectively decreased the cholesterol efflux, which was significant with all concentrations; however, there was no difference between 500 and 1000 μM PRO. In a former study, we used 100 μM of PRO to measure the cholesterol efflux from canine spermatozoa membranes by using another assay, namely the Amplex® Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR, USA); the suppressive effect was weak and the significance only one-sided [19]. The Bodipy-cholesterol fluorophore combined with flow cytometric measurement highly increased the significance of our previous results, emphasizing the fact that ABCA1 blockade effectively decreased cholesterol efflux. We therefore suppose that the ABCA1 molecule at least supports unilateral cholesterol efflux; still nothing is known about the mechanism of cholesterol redistribution in canine spermatozoa. Further studies using other antagonists and labels like filipin [20] are probably required to investigate cholesterol redistribution. In a recent study with ram spermatozoa, the sulfonylurea compound glibenclamide, which is a drug for treatment of type 2 diabetes mellitus, was used to antagonize ABCA1 as well as other ATP binding cassette molecules [12]. The experiment was done with 100 μM glibenclamide, which is known to be the lowest effective dose and only reduced the cholesterol efflux by 8–15%. In our study, the cholesterol efflux was decreased by 14%, when the

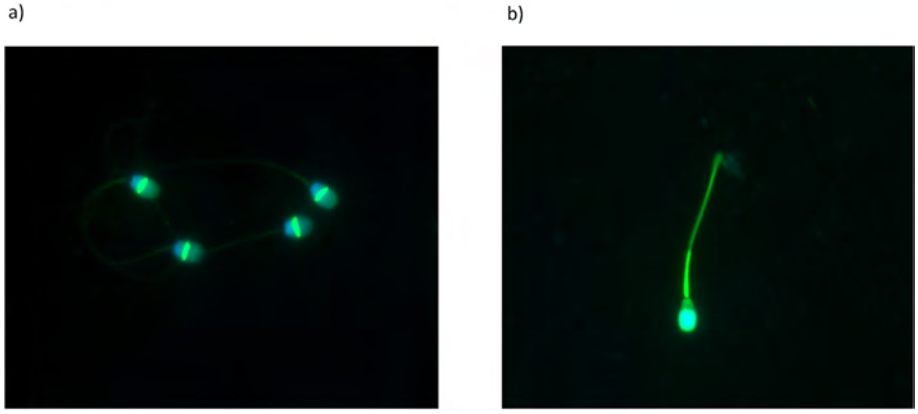

Fig. 7. Tyrosine phosphorylation: non-phosphorylated cell (a) and head and entire tail phosphorylated cell (b). Note the marked fluorescence in the equatorial region that was seen in all cells.

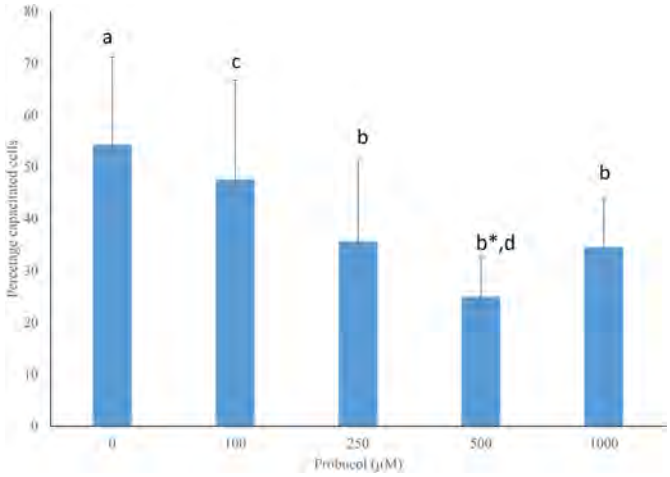

Fig. 9. Effect of PRO on capacitation
The figure shows the decreasing effect of different PRO concentrations on the CTC pattern indicating capacitation, as described by Ref. [21]. Values are given as x ± SD (n = 8). a,b = $p < 0.05$, ab* = $p < 0.01$, c,d = $p < 0.05$.

lowest effective PRO concentration of 100 μM was used and by 44% with 1000 μM PRO. The effect therefore seems to be comparable in both species, but we clearly showed that in dogs, it is dose-dependent and the impact of ABCA1 on cholesterol efflux is high. In the study of Bernecic et al. [12], the importance of the acceptor molecule is nicely shown, which was not examined in our study. In this study, we diluted the semen in PBS, which consists of sodium chloride, potassium chloride and phosphate. We did not add apolipoprotein or any other known acceptor of cholesterol; in dogs, apolipoprotein is believed to be the main acceptor of cholesterol. It will be interesting to examine the cholesterol efflux in dog spermatozoa after inhibition of more ATP binding cassette molecules and using selected acceptors as was done with ram spermatozoa previously [12].

Intense remodeling of the spermatozoa plasma membrane is a prerequisite for capacitation and the fertilizing ability of the cell. Cholesterol efflux is an important step during the molecular cascade leading to capacitation, and is preceded by Ca^{2+} ion influx and redistribution of Ca^{2+} within sperm membrane compartments. Translocation and phosphorylation of other membrane phospholipids occur later during the procedure. In this study, we wanted to examine the effect of a decrease in cholesterol efflux on different steps of capacitation, and found significant effects on both Ca^{2+} redistribution and tyrosine phosphorylation, and both effects were dose-dependent. The decrease in capacitation was observed previously [19]; however, in this study, we found that the effect was maximum with 500 μM PRO, whereas with 1000 μM no further inhibition was possible. The latter is supposed to be caused by the detrimental effect of DMSO on spermatozoa membranes.

The tyrosine phosphorylation of spermatozoa was evaluated as described by Petrunkina et al. [23,24]; however, some patterns were summarized since it was not possible to differentiate between the different grades of head fluorescence; we therefore only evaluated 6 patterns. Interesting is the observed significant decrease of cells with entire tail and head tyrosine phosphorylation and the increase in cells without apparent fluorescence that was seen in samples with 250–1000 μM PRO. These effects may reflect the importance of the amount of effluxed cholesterol for the phospholipid translocation procedure. The tyrosine phosphorylation of dog spermatozoa changes over time and obviously with the degree of cholesterol efflux; the sequential phosphorylation of the mid-piece, principle piece and end piece is followed by phosphorylation of the head [24]. The time course of tyrosine phosphorylation was described to differ between individuals; however, with increasing incubation time, fluorescence of the entire cell occurred [24]. Unfortunately we could not compare the degree of fluorescence or capacitation observed in our study with the results of [24] since other incubation media were used. In this study, significant changes occurred after addition of 250 μM PRO. The latter of course maybe a question of incubation time and medium which were standardized in this study. The impact of time and medium are interesting, but incidental in this study, since the aim was to investigate the effect of different dosages of an ABCA1 antagonist; and the effect of a decrease in cholesterol efflux on capacitation events. We chose a minimum incubation time of 1 h to be able to see differential patterns, before the continuing cell destabilization would mask all changings.

6. Conclusions

The specific antagonization of ABCA1 decreased the cholesterol efflux of dog spermatozoa with all PRO concentrations investigated; in parallel, the tyrosine phosphorylation of the entire tail and head and the CTC pattern indicating capacitation were significantly reduced in a dose-dependent manner. This suggests a strong involvement of the ABCA1 transporter in sperm capacitation in dogs. However, the present study arises new questions, especially concerning the impact of other ATP binding cassette molecules and the redistribution of cholesterol in dog spermatozoa.

Funding

This article/material has been supported by the Polish National Agency for Academic Exchange under Grant No. PPI/APM/2019/1/00044/U/00001.

Ethics

This study was notified to the Ethical Committee of the University of Veterinary Medicine Vienna; no ethical approval was required. All owners of the dogs gave their consent to the use of the semen.

CRediT authorship contribution statement

S. Schäfer-Somi: Conceptualization, Methodology, involved in the clinic and laboratory part of the project, interpretation of data and writing of the manuscript, Supervision, Visualization. **S. Claassen:** involved in the laboratory part of the project and editing of the manuscript. **D. Lechner:** involved in the laboratory part of the project, interpretation of data and editing of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

References

[1] Morales CR, Marat AL, Ni X, Yu Y, Oko R, Smith BT, et al. ATP-binding cassette transporters ABCA1, ABCA7, and ABCG1 in mouse spermatozoa. Biochem Biophys Res Commun 2008;376(3):472–7. https://doi.org/10.1016/j.bbrc.2008.09.009.
[2] Aye ILMH, Singh AT, Keelan JA. Transport of lipids by ABC proteins: interactions and implications for cellular toxicity, viability and function. Chem Biol Interact 2009;180(3):327–39. https://doi.org/10.1016/j.cbi.2009.04.012.
[3] Quazi F, Molday RS. Differential phospholipid substrates and directional transport by ATP-binding cassette proteins ABCA1, ABCA7, and ABCA4 and disease-causing mutants. J Biol Chem 2013;288(48):34414–26. https://doi.org/10.1074/jbc.M113.508812.

[4] Scharenberg C, Mannowetz N, Robey RW, Brendel C, Repges P, Sahrhage T, et al. ABCG2 is expressed in late spermatogenesis and is associated with the acrosome. Biochem Biophys Res Commun 2009;378(2):302–7. https://doi.org/10.1016/j.bbrc.2008.11.058.
[5] Caballero J, Frenette G, D'Amours O, Dufour M, Oko R, Sullivan R. ATP-binding cassette transporter G2 activity in the bovine spermatozoa is modulated along the epididymal duct and at ejaculation. Biol Reprod 2012;86(6):181. https://doi.org/10.1095/biolreprod.111.097477.
[6] Morales CR, Ni X, Smith CE, Inagaki N, Hermo L. ABCA17 mediates sterol efflux from mouse spermatozoa plasma membranes. Histol Histopathol 2012;27(3):317–28. https://doi.org/10.14670/HH-27.317.
[7] Calderón B, Huerta L, Casado ME, González-Casbas JM, Botella-Carretero JI, Martín-Hidalgo A. Morbid obesity-related changes in the expression of lipid receptors, transporters, and HSL in human sperm. J Assist Reprod Genet 2019;36(4):777–86. https://doi.org/10.1007/s10815-019-01406-z.
[8] Merkl M, Ertl R, Handschuh S, Aurich C, Schäfer-Somi S. The cholesterol transporter ABCA1 is expressed in stallion spermatozoa and reproductive tract tissues. Theriogenology 2016;85(6):1080–9. https://doi.org/10.1016/j.theriogenology.2015.11.019.
[9] Palme N, Becher AC, Merkl M, Glösmann M, Aurich C, Schäfer-Somi S. Immunolocalization of the cholesterol transporters ABCA1 and ABCG1 in canine reproductive tract tissues and spermatozoa. Reprod Domest Anim 2014;49(3):441–7. https://doi.org/10.1111/rda.12294.
[10] Vaughan AM, Oram JF. ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. JLR (J Lipid Res) 2003;44(7):1373–80. https://doi.org/10.1194/jlr.M300078-JLR200.
[11] Wellington CL, Walker EKY, Suarez A, Kwok A, Bissada N, Singaraja R, et al. ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. Lab Invest 2002;82(3):273–83. https://doi.org/10.1038/labinvest.3780421.
[12] Bernecic NC, Graaf SP de, Leahy T, Gadella BM. HDL mediates reverse cholesterol transport from ram spermatozoa and induces hyperactivated motility. Biol Reprod 2021;104(6):1271–81. https://doi.org/10.1093/biolre/ioab035.
[13] Leahy T, Gadella BM. New insights into the regulation of cholesterol efflux from the sperm membrane. Asian J Androl 2015;17(4):561–7. https://doi.org/10.4103/1008-682X.153309.
[14] Yancey PG, Bortnick AE, Kellner-Weibel G, La Llera-Moya M de, Phillips MC, Rothblat GH. Importance of different pathways of cellular cholesterol efflux. Arterioscler Thromb Vasc Biol 2003;23(5):712–9. https://doi.org/10.1161/01.ATV.0000057572.97137.DD.
[15] Palme N, Becher AC, Merkl M, Glösmann M, Aurich C, Schäfer-Somi S. Immunolocalization of the cholesterol transporters ABCA1 and ABCG1 in canine reproductive tract tissues and spermatozoa. Reprod Domest Anim 2014;49(3):441–7. https://doi.org/10.1111/rda.12294.
[16] Favari E, Zanotti I, Zimetti F, Ronda N, Bernini F, Rothblat GH. Probucol inhibits ABCA1-mediated cellular lipid efflux. Arterioscler Thromb Vasc Biol 2004;24(12):2345–50. https://doi.org/10.1161/01.ATV.0000148706.15947.8a.
[17] Tsujita M, Yokoyama S. Selective inhibition of free apolipoprotein-mediated cellular lipid efflux by probucol. Biochemistry 1996;35(40):13011–20. https://doi.org/10.1021/bi960734h.
[18] Sakr SW, Williams DL, Stoudt GW, Phillips MC, Rothblat GH. Induction of cellular cholesterol efflux to lipid-free apolipoprotein A-I by cAMP. Biochim Biophys Acta Mol Cell Biol Lipids 1999;1438(1):85–98. https://doi.org/10.1016/S1388-1981(99)00041-4.
[19] Schäfer-Somi S, Budik S. The ABCA1 blocking agent probucol decreases capacitation in ejaculated dog spermatozoa. Acta Vet Scand 2020;62(1):2. https://doi.org/10.1186/s13028-019-0500-2.
[20] Bernecic NC, Zhang M, Gadella BM, Brouwers JFHM, Jansen JWA, Arkesteijn GJA, et al. BODIPY-cholesterol can be reliably used to monitor cholesterol efflux from capacitating mammalian spermatozoa. Sci Rep 2019;9(1):9804. https://doi.org/10.1038/s41598-019-45831-7.
[21] Hewitt DA. Oocyte maturation and fertilization in the bitch. The use of in vitro culture. Ph.D. Thesis. UK: University of London; 1997. 1997
[22] Witte TS, Schäfer-Somi S, Kuchar A, Möstl E, Iben C, Aurich C. Effect of hen's egg yolk on capacitation and acrosome reaction of diluted canine spermatozoa. Anim Reprod Sci 2009;110(3–4):293–305. https://doi.org/10.1016/j.anireprosci.2008.01.022.
[23] Petrunkina AM, Simon K, Günzel-Apel A-R, Töpfer-Petersen E. Kinetics of protein tyrosine phosphorylation in sperm selected by binding to homologous and heterologous oviductal explants: how specific is the regulation by the oviduct? Theriogenology 2004;61(9):1617–34. https://doi.org/10.1016/j.theriogenology.2003.09.011.
[24] Petrunkina AM, Simon K, Günzel-Apel A-R, Töpfer-Petersen E. Specific order in the appearance of protein tyrosine phosphorylation patterns is functionally coordinated with dog sperm hyperactivation and capacitation. J Androl 2003;24(3):423–37. https://doi.org/10.1002/j.1939-4640.2003.tb02691.x.
[25] Divar MR, Azari M, Mogheiseh A, Ghahramani S. Supplementation of melatonin to cooling and freezing extenders improves canine spermatozoa quality measures. BMC Vet Res 2022;18(1):86. https://doi.org/10.1186/s12917-022-03186-8.
[26] Farshad A, Khalili B, Fazeli P. The effect of different concentrations of glycerol and DMSO on viability of Markhoz goat spermatozoa during different freezing temperatures steps. Pakistan J Biol Sci 2009;12(3):239–45. https://doi.org/10.3923/pjbs.2009.239.245.