Polish Journal of Veterinary Sciences Vol. 21, No. 1 (2018), 133–138

DOI 10.24425/119031

Original article

Effects of seminal plasma concentration on sperm motility and plasma and acrosome membrane integrity in chilled canine spermatozoa

C. Pan, Y. Wu, Q. Yang, J. Ye

Nanchang Police Dog Base of the Ministry of Public Security,
No.518 Xingguo Road, Nanchang Xinjian District, China

Abstract

Depending on the mammal species, the use of seminal plasma during semen processing for cryopreservation has been found to have both beneficial and detrimental effects. This study was designed to determine the effects of seminal plasma concentration on the motility, sperm movement characteristics, and plasma and acrosome membrane integrity of chilled canine spermatozoa. After pooling the semen from seven dogs, samples for each assay were preserved at 4°C for 96h in extenders containing different seminal plasma concentrations (0, 25, 50, 75 and 100% (v/v) seminal plasma). After 96h cold storage, group 25% (v/v) seminal plasma showed significantly higher percentages of sperm cells with motility [46.4 ± 1.65% ($p<0.05$)], intact plasma membrane [46.5 ± 3.11% ($p<0.05$)] and intact acrosome[58.5 ± 1.86 % ($p<0.05$)] than other groups. In conclusion, supplementing semen extender with an appropriate seminal plasma concentration (25% (v/v) seminal plasma) is able to adequately preserve the sperm motility, integrity of the plasma and acrosome membrane in canine spermatozoa chilled at 4°C.

Key words: seminal plasma, chill, canine, sperm

Introduction

Artificial insemination (AI) allows rapid dissemination of genetic material from a small number of superior sires to a large number of females (Vishwanath and Shannon 1997). The first successful use of AI in dogs dates back to 1954 (Harrop 1954). Semen used for this purpose can be stored either for short-term at 4°C in the liquid form or for long-term in cryopreserved state in liquid nitrogen (Maxwell and Watson 1996). Cryopreservation of semen is practiced worldwide for many reasons, but the use of chilled semen for AI has its own advantages. The main benefit of liquid semen is that a comparatively higher conception rate is achieved with relatively lower number of spermatozoa (Michael et al. 2010). Furthermore, it is easier to transport than frozen semen, which requires liquid nitrogen, and easy to inseminate (intravaginal vs intrauterine insemination) (Rota et al. 1995).

Correspondence to: J. Ye, e-mail: pcx19921219@163.com

For preservation by cooling to be successful, semen needs to be in contact with an appropriate extender that is able to create an environment which is metabolically and physiologically favourable to the survival of sperm cells and protecting them from cold shock. In the processing of semen, both beneficial and detrimental effects of seminal plasma on sperm function have been detected and such effects are much affected by species (Morrell and Rodriguez-Martinez 2009).

In the dog, seminal fluid has been found to be deleterious for the *in vitro* preservation of sperm quality (Rota et al. 1995). Other studies designed to clarify the effect of prostatic fluid on preserved canine semen indicate that its presence before semen cryopreservation is also detrimental to sperm quality after thawing (Sirivaidvapong et al. 2001, Niżański 2006); The addition of heterologous prostatic fluid has shown no beneficial effects on motility variable and a clear negative trend and lower motility have been observed compared to semen diluted with control medium (Koderle et al. 2009, Milani et al. 2010). However, studies also have shown that the presence of canine seminal plasma fluid in the extender protects the sperm acrosome during cold storage (Manosalva et al. 2005, Treulen et al. 2012).

Seminal plasma is beneficial or detrimental for semen preservation in dog, but inconsistence results have been given in studies. Most of studies discussed the influence of seminal plasma on canine semen quality under the process of freezing and thawing, and their experimental design either with or without seminal plasma. However, no study has examined the effects of different concentration of seminal plasma on semen preservation at refrigeration temperatures. The aim of this study was to examine the effects of seminal plasma concentration on the motility, sperm movement characteristics and plasma and acrosome membrane integrity of chilled canine spermatozoa.

Materials and Methods

Chemical agents

Unless otherwise stated, all chemicals used were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

Semen collection and evaluation

A total of 7 male healthy dogs (two Dobermans, five Rottweilers) were included in the study, weighting between 20 kg and 30 kg and ranged from 2 to 5 years of ages. The dogs were housed in individual pens and fed with commercial canine food. Water was available ad libitum. The owners of the dogs included in the study provided written consent. All experimental procedures were carried out in compliance with the ethical committee of Jiangxi Province Key Lab for Police Dog Breeding Reproduction and Behavior Science. The animal's health status was periodically checked by a veterinarian. The dogs were trained for semen collection before the study and were used routinely as semen donors. Six replicate trials were conducted using semen collected once a week from each dog by manual manipulation aided with pre-warmed graduated glass funnels. The second and third fractions of the ejaculates were collected in this study.

Sperm concentration and motility was estimated. The percentage of motile sperm was estimated by phase contrast microscopy as described later (see Sperm evaluation section). Sperm concentration was determined using a calibrated spectrophotometer. Only samples showing motility of ≥ 80% and sperm concentration of ≥ 1.5 x 10^8 cell/ml were included in the study. In each experimental trial (n = 6), aliquots of samples from the seven dogs showing similar spermatozoa concentrations were pooled to eliminate variability between the different samples and ensure any differences observed were attributable to the effects of time and/or extenders (Verstegen et al. 2005).

Semen processing

The EYT-based extender (Verstegen et al. 2005) with slight modifications was freshly prepared before each trial using the components: Tris (hydroxymethyl)-aminomethane (0.2 M), sodium citrate monohydrate (0.06 M), glucose (0.06 M), benzylpenicillin 1653 UI/ml, dihydrostreptomycin sulphate 750 UI/ml, fresh egg yolk 20% (v/v) and distilled water (100 ml). The osmolarity of the solution was adjusted to 326 Osm/l and pH 6.8 (Shahiduzzaman and Linde- Forsberg 2007).

Ejaculates from the seven dogs were centrifuged at 12000x g for 6 min at 4°C (Barrios et al. 2000), and the seminal plasma (SP) was collected in sterile tubes, aliquoted and frozen (-80°C) until use. The extenders containing different seminal plasma concentration (0, 25, 50, 75 and 100% (v/v) seminal plasma) were then prepared. To prepare 1 ml of five extenders with different seminal plasma concentration in brief, five amounts of seminal plasma (0, 0.25, 0.5, 0.75 and 1 ml) and EYT (1, 0.75, 0.5, 0.25 and 0 ml) were mixed, respectively.

The semen pool was centrifuged at 720x g for 3 min (Rijesselaere et al. 2002) at room temperature. The supernatant was then removed, and the sperm pellet was first resuspended in TRIS buffer (TRIS buffer containing glucose: Pena et al. 2003) to obtain a final concentration 1.0 x 10^8 spermatozoa/ml. This sperm suspension was divided into five equal aliquots and placed in five sterile tubes, which were centrifuge at 720x g for 3min and the supernatants removed. The resulting pellets were diluted respectively with 1 ml extenders containing different seminal plasma concentration (0, 25, 50, 75 and 100% (v/v) seminal plasma) as follows: 0% SP, 25% SP, 50% SP, 75% SP and 100% SP group. After preparing all dilutions, the final sperm concentration was 5.0 x 10^7 spermatozoa/ml. To avoid cooling too rapidly and to minimize the effects of cold shock, the extended semen samples were placed in a room temperature cooler to reach a temperature of 4° in approximately 45 min; the cooling rate in the cooler was -0.5°C/min (Ponglowhapan et al. 2004). Subsequently, all samples were stored at 4°C in a refrigerator for 96 h.

Sperm evaluation

The samples were analysed during cold storage at 24, 48, 72 and 96 h. At each of these time points, aliquots were taken from each sperm sample and washed (720x g for 5 min) with TRIS buffer. The sperm suspensions of each of the groups were adjusted to 5.0 x 10^6 cells / ml in TRIS buffer and rewarmed to 37°C for 6 min. Subsequently, aliquots for the different sperm evaluation techniques were prepared for each group.

Motion analysis

Motion analysis of the chilled semen was additionally performed using the computer-assisted semen analysis (CASA, Hamilton-Thorne Biosciences IVOS, Version 12.3, Bevery, MA, USA). the analysis captured 100 frames of images each time and the scan rate was 60 frames per second. 5 µl of semen sample was put on a glass slide and covered by a glass cover slide and estimated at 37°C under a phase contrast microscope (400x). A minimum of 300 spermatozoa in at least five different microscopy fields was observed. The mean value calculated from these five analyzed fields was used for statistical analysis.

Progressive motility (PMOT%): The spermatozoa moved fast and progressively (as a percentage).

VCL: curvilinear line velocity (expressed in µm/s).

VSL: straight-line velocity (expressed in µm/s).

VAP: average path velocity (expressed in µm/s).

ALH: lateral head displacement (amplitude of the lateral movements of the head expressed in µm).

Plasma membrane integrity

The plasma membrane integrity was evaluated using the hypoosmotic swelling test (HOST). The assay was performed by mixing 50 µl of semen with 1ml of 150 mOsm/kg hypo-osmotic solution (England & Plummer. 1993). After incubation for 60 min at 37°C, sperm swelling was assessed by placing 15 µl well-mixed samples on a warm slide (37°C) under a phase-contrast microscope at 400x magnification. The spermatozoa were classified as positive or negative based on the presence or the absence of coiled tail. The percentages of sperm with swollen and curled tails were then recorded in at least 300 sperm cells per slide. Each sample was measured five times and the average was taken for statistical analysis.

Acrosome membrane integrity

Acrosome integrity was assessed using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) staining, in a procedure slightly modified from the method described by Petrunkina et al (2004). Briefly, the 30 µl semen sample was used to prepare smears on microscope slides. After air-drying, sperm smears were fixed with absolute methanol for 10 min at 20-22°C and allowed to dry. Then, approximately 30 µl FITC-labelled peanut agglutinin (FITC-PNA) solution (100 µg/ml) in PBS was spread over each slide. Subsequently, the slides were incubated in a dark and moist chamber for 30 min at 37°C. After incubation, the slides were rinsed with PBS, air-dried and mounted with 10 µl of Antifade Solution to preserve fluorescence. The slide smear was coverslipped and sealed with colourless nail polish. The acrosome status of the sperm was examined using an epifluorescence microscope. The whole acrosome was visualized with strong green fluorescence under a fluorescence microscope and was scored as acrosome-intact sperm cells. The percentage of fluorescent acrosome-intact spermatozoa was counted in at least 300 sperm cells per slide. Each sample was measured five times and the average was taken for statistical analysis.

Statistical analysis

All analyses were performed using Statistical Product and Service Solutions (SPSS 20.0 for Win-

Table 1. Means of PMOT, VAP, VSL, VCL and ALH in extenders with different seminal plasma concentration at 24, 48, 72 and 96h of storage at 4°C.

Parameter		PMOT (%)	VAP(μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)
24h	0% SP	$76.5^{a} \pm 2.21$	$49.4^{a} \pm 1.88$	$33.0^{a} \pm 0.28$	$78.6^{a} \pm 3.11$	$2.9^{a} \pm 0.06$
	25% SP	$78.5^{a} \pm 1.25$	$49.2^{a} \pm 1.39$	$33.6^{a} \pm 0.78$	$83.1^{a} \pm 3.29$	$3.0^{a} \pm 0.10$
	50% SP	$63.6^{b} \pm 2.28$	$42.8^{b} \pm 1.62$	$25.8^{b} \pm 1.01$	$75.5^{a} \pm 2.81$	$2.8^{ab} \pm 0.09$
	75% SP	$53.3^{c} \pm 2.05$	$38.7^{b} \pm 0.97$	$23.0^{b} \pm 0.23$	$65.6^{b} \pm 2.33$	$2.6^{b} \pm 0.13$
	100% SP	$52.3^{c} \pm 1.41$	$37.1^{b} \pm 1.10$	$25.8^{b} \pm 0.77$	$52.8^{c} \pm 2.01$	$2.5^{b} \pm 0.07$
48h	0% SP	$61.1^{a} \pm 1.33$	$37.9^{a} \pm 2.11$	$24.1^{a} \pm 0.12$	$71.3^{a} \pm 2.39$	$2.5^{a} \pm 0.11$
	25% SP	$65.3^{a} \pm 2.95$	$38.3^{a} \pm 1.38$	$26.9^{a} \pm 0.92$	$74.9^{a} \pm 3.04$	$2.7^{a} \pm 0.09$
	50% SP	$49.5^{b} \pm 2.36$	$30.5^{b} \pm 1.11$	$18.0^{b} \pm 0.09$	$57.4^{b} \pm 2.99$	$2.6^{a} \pm 0.13$
	75% SP	$24.9^{c} \pm 1.46$	$27.5^{b} \pm 0.62$	$15.8^{bc} \pm 0.51$	$47.6^{c} \pm 1.85$	$1.5^{b} \pm 0.03$
	100% SP	$20.6^{c} \pm 1.59$	$27.3^{b} \pm 1.01$	$14.1^{c} \pm 0.09$	$32.7^{d} \pm 1.92$	$1.4^{b} \pm 0.03$
72h	0% SP	$46.2^{b} \pm 2.49$	$30.3^{a} \pm 0.48$	$19.8^{a} \pm 0.34$	$60.6^{a} \pm 2.16$	$2.0^{ab} \pm 0.09$
	25% SP	$54.0^{a} \pm 1.77$	$33.8^{a} \pm 0.82$	$22.6^{a} \pm 0.85$	$63.6^{a} \pm 2.77$	$2.2^{a} \pm 0.09$
	50% SP	$35.2^{c} \pm 0.54$	$22.9^{b} \pm 0.66$	$15.4^{b} \pm 0.16$	$51.4^{b} \pm 3.12$	$1.8^{b} \pm 0.10$
	75% SP	$12.2^{d} \pm 2.06$	$14.0^{c} \pm 0.12$	$11.8^{c} \pm 0.08$	$32.4^{c} \pm 1.28$	$0.7^{c} \pm 0.07$
	100% SP	$0.0^{e} \pm 0.00$	$0.0^{d} \pm 0.00$	$0.0^{d} \pm 0.00$	$0.0^{d} \pm 0.00$	$0.0^{d} \pm 0.00$
96h	0% SP	$36.5^{b} \pm 1.01$	$25.3^{a} \pm 0.27$	$13.5^{b} \pm 0.22$	$51.1^{b} \pm 2.16$	$1.5^{a} \pm 0.09$
	25% SP	$46.4^{a} \pm 1.65$	$28.1^{a} \pm 1.18$	$18.9^{a} \pm 0.31$	$59.6^{a} \pm 2.75$	$1.6^{a} \pm 0.11$
	50% SP	$18.8^{c} \pm 1.56$	$20.2^{b} \pm 0.97$	$13.2^{b} \pm 0.09$	$40.5^{c} \pm 1.94$	$1.3^{b} \pm 0.03$
	75% SP	$7.3^{d} \pm 0.85$	$10.4^{c} \pm 0.737$	$5.6^{c} \pm 0.05$	$28.2^{d} \pm 0.99$	$0.5^{c} \pm 0.02$
	100% SP	$0.0^{e} \pm 0.00$	$0.0^{d} \pm 0.00$	$0.0^{d} \pm 0.00$	$0.0^{e} \pm 0.00$	$0.0^{d} \pm 0.00$

In the same column, values with different letter superscripts mean significant difference ($p<0.05$) and with the same letter superscripts mean no significant difference ($p>0.05$). The same as below.

dows; SPSS, Chicago, IL, USA). All results were expressed as mean values ± SD. The mean values of the percentages of motile sperm, acrosome-intact, plasma membrane-intact sperm were compared using One-Way ANOVA. The results were considered statistically significant when the p-value was less than 0.05.

Results

Sperm mobility characteristics

The mean baseline progressive sperm motility was 91.2 ± 1.77%. After 48 h of chilling, no differences ($p>0.05$) were observed in progressive motility between 0% SP and 25% SP. However, in this time point, sperm sample diluted in 75% SP and 100% SP decreased significantly in sperm motility to 24.9 ± 1.46% and 20.6 ± 1.59%, respectively. At 96 h, the parameter of 25% SP still obtained 46.4 ± 1.65%, higher than other groups significantly ($p<0.05$) (Table 1).

The mean baselines of VAP, VSL, VCL and ALH were 53.5 ± 1.19 μm/s, 36.8 ± 2.12 μm/s, 88.2 ± 2.63 μm/s and 3.2 ± 0.06 μm, respectively. After 72 h of chilling, there was no significant differences ($p>0.05$) between 0% SP and 25% SP in parameters of VAP, VSL, VCL and ALH. However, values of 25% SP were higher than those of 0% SP at all time points in the four parameters. After 96 h, statistically significant differences ($p<0.05$) were observed between 25% SP, 50% SP, 75% SP and 100% SP for parameters VAP, VSL, VCL and ALH (Table 1).

Plasma membrane integrity

The mean baseline percentage of sperm with an intact plasma membrane was 90.2 ± 1.94%. After 24 h of chilling, no differences ($p>0.05$) were observed in plasma membrane integrity between the samples stored in the five extenders. However, after 48 h, sperm sample diluted in 100% SP decreased significantly in the percentage of intact plasma membrane to 20.3 ± 2.22%. At 72 h, sperm plasma membrane integrity in 75% SP declined to 12.8 ± 1.78% dramatically, while there was no significant difference between 0% SP and 50% SP. After 96 h, the parameter of 25% SP still obtained 46.5 ± 3.11%, higher than other groups significantly ($p<0.05$) (Table 2).

Acrosome integrity

The mean baseline percentage of sperm with an intact acrosome membrane was 87.2 ± 1.24%. The results shown in Table 2 indicate no significant

Table 2. Percentage of spermatozoa with intact plasma membrane and intact acrosome in extenders with different seminal plasma concentration at 24, 48, 72 and 96h of storage at 4°C.

Parameter	24h	48h	72h	96h
Intact plasma membrane (%)				
0% SP	$83.6^{a} \pm 2.45$	$58.0^{b} \pm 1.82$	$35.3^{b} \pm 0.66$	$32.2^{b} \pm 1.04$
25% SP	$84.8^{a} \pm 1.76$	$68.6^{a} \pm 3.001$	$52.2^{a} \pm 1.06$	$46.5^{a} \pm 3.11$
50% SP	$83.0^{a} \pm 3.93$	$61.2^{b} \pm 2.08$	$30.2^{bc} \pm 1.97$	$24.2^{c} \pm 1.03$
75% SP	$82.1^{a} \pm 2.72$	$50.6^{c} \pm 2.08$	$12.8^{d} \pm 1.78$	$8.7^{d} \pm 0.41$
100% SP	$81.4^{a} \pm 1.54$	$20.3^{d} \pm 2.22$	$9.7^{d} \pm 0.33$	$7.8^{d} \pm 0.41$
Intact Acrosome (%)				
0% SP	$82.2^{a} \pm 1.32$	$67.6^{b} \pm 0.77$	$51.8^{b} \pm 1.05$	$44.3^{b} \pm 0.66$
25% SP	$83.0^{a} \pm 0.54$	$77.2^{a} \pm 2.77$	$68.1^{a} \pm 2.01$	$58.5^{a} \pm 1.86$
50% SP	$81.2^{a} \pm 0.29$	$65.9^{b} \pm 2.15$	$52.8^{b} \pm 1.36$	$43.2^{b} \pm 1.62$
75% SP	$79.3^{a} \pm 2.20$	$63.2^{b} \pm 1.92$	$49.3^{b} \pm 0.55$	$41.7^{b} \pm 1.08$
100% SP	$79.1^{a} \pm 2.10$	$34.4^{c} \pm 1.22$	$18.0^{c} \pm 0.29$	$16.0^{c} \pm 0.85$

differences in this variable between the five groups up to 24 h of chilling ($p>0.05$). After 48 h, sperm sample diluted in 100% SP decreased significantly in the percentage of intact acrosome to $34.4 \pm 1.22\%$. At 96 h, no differences ($p>0.05$) in the number of sperm with intact acrosome membrane were observed between 0% SP, 50% SP and 75% SP groups ($44.3 \pm 0.66\%$, 43.2 ± 1.62 and $41.7 \pm 1.08\%$, respectively). At the same time point, this parameter in 25% SP group was higher than in other groups significantly ($58.5 \pm 1.86\%$) ($p<0.05$) (Table 2).

Discussion

Seminal plasma is known to have beneficial as well as detrimental effects during semen processing. However, no study has well examined the effects of different concentration of seminal plasma on canine semen preservation during cold storage. In our research, extender with 25% SP showed the best preservation effect in motility of canine spermatozoa, and 0% SP was the next-best. Results also show that extender with high seminal plasma concentration is detrimental to sperm quality, which is consistent with other researcher's results (Rota et al. 1995, Milani et al. 2010). If the sperm plasma membrane is not functional, the sperm is considered deteriorated and is not capable to fertilize *in vivo* (Silva and Gadella 2006). Our results suggest that supplementation of the cooling extender with 25% (v/v) seminal plasma improves the protective effect of the cooling extender on the sperm plasma membrane during storage. Besides, among other damage induced by cryopreservation of dog sperm, acrosomal abnormalities are observed with high frequency. In the present study, after 96 h of cold storage, a ideal percentage of intact acrosome was gained in 25% SP. Similar studies have shown a protective action of seminal plasma fluids on the acrosome membrane (Treulen et al. 2012).

Those results are probably because of those complex components in seminal plasma (Heriberto et al. 2011). For instance, Mogielnicka-Brzozowska et al. (2014) observed that high molecular weight protein complexes of canine seminal plasma possess the ability to bind zinc ions and the positive effect of this phenomenon on the motility of spermatozoa stored at low temperatures. Besides, super-oxide dismutase (SOD) in seminal plasma is an important component of the enzyme antioxidant system that protects canine spermatozoa against the deleterious effects of ROS, which also plays a protective role in canine semen during storage (Cassani et al. 2005). However, when seminal plasma concentration reaches a certain threshold, contents of nutritional components and cryoprotectant in semen extender decrease, which is also significant to the survival of sperm cells and protect them from cold shock.

In conclusion, our findings indicate that extender with a appropriate seminal plasma concentration (25% SP) is able to adequately preserve the sperm motility, integrity of the plasma and acrosome membrane in canine spermatozoa chilled at 4°C.

Acknowledgements

Funding has been provided by The National Key Research and Development Program of China: No. 2016YFD0501008 and No. 2016YFC0800310, and Jiangxi Province Key Lab for Police Dog Breeding Reproduction and Behavior Science.

References

Barrios B, Perez R, Gallego M, Tato A, Osada J, Muiño T, Cebrian J (**2000**) Seminal plasma proteins revert the cold shock damage on ram sperm membrane. Biol Reprod 63: 1531-1537.

Cassani P, Beconi M, O'Flaherty C (**2005**) Relationship between total superoxide dismutase activity with lipid peroxidation, dynamics and morphological parameters in canine semen. Anim Reprod Sci 86: 163-173.

England GC, Plummer JM (**1993**) Hypo-osmotic swelling of dog spermatozoa. J Reprod Fertil Suppl 47: 261-270.

Harrop AE (**1954**) Artificial insemination of a bitch with preserved semen. Br Vet J 110: 424-425.

Koderle M, Aurich C, Schäfer-Somi S (**2009**) The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa. Theriogenology 72: 1215-1220.

Maxwell WM, Watson PF (**1996**) Recent progress in the preservation of ram semen. Anim Reprod Sci 42: 55-65.

Michael AJ, Alexopoulos C, Pontiki EA, Hadjipavlou-Litina DJ, Saratsi P, Ververidis HN, Boscos CM (**2010**) Effect of N-acetyl-L-cysteine supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. Reprod Domest Anim 45: 201-207.

Milani C, Fontbonne A, Sellem E, Stelletta C, Gerard O, Romagnoli S (**2010**) Effect of post-thaw dilution with caffeine, pentoxifylline, 2'-deoxyadenosine and prostatic fluid on motility of frozen-thawed dog semen. Theriogenology 74: 153-164.

Mogielnicka-Brzozowska M, Dziekońska A, Strzeżek R, Zalecki M, MAjewska A (**2014**) Effect of seminal plasma zinc-binding proteins on motility and membrane integrity of canine spermatozoa stored at 5ºC. Bull Vet Inst Pulawy 58:163-168.

Mogielnicka-Brzozowska M, Strzezek R, Wasilewska K, Kordan W (**2015**) Prostasomes of canine seminal plasma-zinc-binding ability and effecs on motility characteristics and plasma membrane integrity of spermatozoa. Reprod Domest Anim 50: 484-491.

Niżański W (**2006**) Intravaginal insemination of bitches with fresh and frozen-thawed semen with addition of prostatic fluid: use of an infusion pipette and the Osiris catheter. Theriogenology 66: 470-483.

Pena A, Johannisson A, Linde-Forsberg C (**1999**) Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. Theriogenology 52: 965-980.

Petrunkina AM, Gropper B, Gunzel-Apel AR, Topfer-Petersen E (**2004**) Functional significance of the cell volume for detecting sperm membrane changes and predicting freezability in dog semen. Reproduction 128: 829-842.

Ponglowhapan S, Essén-Gustavsson B, Linde Forsberg C (**2004**) Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. Theriogenology 62: 1498-1517.

Rijesselaere T, Soom AV, Maes D, Kruif AD (**2002**) Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. Theriogenology 57: 1669-1681.

Rodriguez-Martinez H, Kvist U, Ernerudh J, Sanz L, Calvete JJ (**2011**) Seminal plasma proteins: what role do they play? Am J Reprod Immunol 66(suppl 1): 11-22.

Rota A, Strom B, Lindeforsberg C (**1995**) Effects of seminal plasma and three extenders on canine semen stored at 4 degrees C. Theriogenology 44: 885-900.

Shahiduzzaman AK, Linde-Forsberg C (**2007**) Induced immotility during long-term storage at +5 degrees C does not prolong survival of dog spermatozoa. Theriogenology 68: 920-933.

Silva PF, Gadella BM (**2006**) Detection of damage in mammalian sperm cells. Theriogenology 65: 958 – 978.

Sirivaidyapong S, Ursem P, Bevers MM, Colenbrander B (**2001**) Effect of prostatic fluid on motility, viability and acrosome integrity of chilled and frozen-thawed dog spermatozoa. J Reprod Fertil Suppl 57: 383-386.

Treulen F, Sanchez R, Risopatron J (**2012**) Effects of Seminal Fluid Fractions on Plasma and Acrosome Membrane Integrity and Mitochondrial Membrane Potential Determined by Flow Cytometry in Chilled Canine Spermatozoa. Reprod Domest Anim 47: 1043-1048.

Verstegen JP, Onclin K, Iguer-Ouada M (**2005**) Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris-glucose extender: in vitro and in vivo studies. Theriogenology 64: 720-733.

Vishwanath R. Shannon P (**1997**) Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. Reprod Ferti & Develop 9: 321-332.

Vitro RM (**2009**) Biomimetic techniques for improving sperm quality in animal breeding: a Review. Open Andrology J 1: 1-9.