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CANINE SEMEN CRYOPRESERVATION AFTER SEMINAL PLASMA REMOVAL IN DOGS WITH BENIGN PROSTATIC HYPERPLASIA

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ABSTRACT

Benign prostatic hyperplasia (BPH) can alter the composition of canine seminal plasma (SP) and specific prostatic proteins may impair sperm cryotolerance. The present study evaluated a large-volume cryopreservation protocol with SP removal in dogs with BPH. Whole ejaculates were collected from 10 healthy dogs and 10 dogs with BPH. SP was removed by brief low-speed centrifugation. Samples were dispensed into cryovials in 500 µL aliquots and subjected to equilibration. They were then frozen using an accelerated ultralow temperature profile and subsequently thawed for computer-assisted sperm analysis. Post-thaw progressive motility declined in both groups when compared to fresh samples ($p < 0.001$), while linearity, straightness, and wobble remained stable within groups and were comparable between groups. Curvilinear, straight-line, and average path velocities decreased after freezing compared to fresh controls ($p < 0.05$) in both groups. The amplitude of lateral head displacement and beat-cross frequency exhibited significantly greater post-thaw reductions in BPH dogs than in healthy controls ($p < 0.05$). These findings indicate that the developed cryopreservation protocol enables effective long-term storage of canine semen from patients with BPH, while maintaining key trajectory parameters despite cryoinjury.

Key words: *Canis familiaris*, Spermatozoa, Cryotolerance, Computer-assisted sperm analysis, Sperm kinematics.

Introduction

Technologies for the long-term storage of genetic material at ultralow temperatures began to develop in the mid-20th century. They have proven their advantages in *in vitro* procedures used to enrich and improve gene pools in animals (Estudillo *et al.* 2021; Hiemstra *et al.* 2006; Mara *et al.* 2013). Nonetheless, cryopreservation can compromise the structure and function of spermatozoa, thereby reducing their viability and fertility (Lemma 2011; Muiño-Blanco *et al.* 2008). Furthermore, age-related susceptibility to cryodamage has been reported in dogs (de la Fuente-Lara *et al.* 2019; Hesser *et al.* 2017) by comparing the quality parameters of spermatozoa from fresh and frozen-thawed semen from dogs of different ages. Numerous studies on the role of different freezing rates and protective media in canine semen cryopreservation have been conducted, and it has been shown that the spermatozoa of this species are more sensitive to low temperatures, similar to other species such as boars and horses (Knox 2015; Oldenhof *et al.* 2012; Schäfer-Somi *et al.* 2022; Yeste 2016).

With the rise in the population of domestic dogs, there has been a corresponding increase in reports of patients developing benign prostatic hyperplasia (BPH). Proteins specific to the seminal plasma (SP) of dogs with BPH that are not present in healthy individuals have been previously documented (Genov *et al.* 2020; Genov et Ivanova 2021; Gradinarska *et al.* 2019). Prostatic fluid

(PF), which forms the liquid phase of the ejaculate, has shown context-dependent effects on canine spermatozoa during cryopreservation. Some prostate components may have different positive or negative effects on sperm cell functionality following low-temperature storage or cryopreservation (Sirivaidyapong *et al.* 2001; Strzeżek et Fraser 2009). A positive effect on sperm motility and viability has been reported when PF is added to canine epididymal spermatozoa (Hori *et al.* 2005). However, such positive effects were not observed after semen freezing and thawing. Korochkina *et al.* (2014) suggested that some of the specific proteins present in PF are likely "detrimental" to sperm cell survival during low-temperature storage or cryopreservation. Thus, the role of SP in canine sperm cryotolerance remains unclear, especially in dogs with BPH.

Despite extensive research on cryopreservation protocol optimisation, it remains unclear whether removing SP before freezing improves post-thaw function, specifically in semen from dogs with BPH, where SP composition is altered. To address this gap, we evaluated whether a large-volume cryopreservation protocol with SP removal preserves post-thaw motility and kinematic parameters in dogs with BPH compared with healthy controls.

Materials and Methods

Ejaculate collection and primary analysis

Whole ejaculates were manually collected through digital stimulation from 20 dogs of various breeds aged between 4 and 7 years (10 healthy dogs and 10 dogs with BPH). BPH diagnosis was based on clinical evaluation, digital rectal palpation, laboratory tests (bloodwork and urinalysis), and diagnostic imaging (abdominal ultrasonography, radiography, and, when necessary, computed tomography) to confirm prostatomegaly and parenchymal changes. In cases where clinical signs and imaging findings were inconclusive, a prostatic biopsy was conducted to rule out prostatitis, abscessation, or neoplasia. Dogs that met these criteria and showed no signs of infection or neoplasia were categorised into the BPH group, whereas healthy dogs served as controls. All regulations and requirements regarding humane treatment and animal well-being were strictly followed.

Primary semen analysis was performed using computer-assisted sperm analysis (CASA). The ejaculates from healthy (n=10) and BPH dogs (n=10) to undergo SP removal and freezing were selected to ensure comparable pre-freeze motility, specifically similar total and progressive motility.

Semen extenders and dilution

After the initial semen assessment, samples from both groups were centrifuged at 300 × g for 20 s to remove SP. After centrifugation of the ejaculates, the SP was discarded and sperm cell dilution was performed with cryoprotective medium (TRIS 3 g, citric acid 1.7 g, fructose 1.3 g, distilled water up to 100 ml, glycerol 8 ml, egg yolk 20% v/v). This dilution resulted in a final sperm concentration of 200-300×106/ml to ensure adequate sperm numbers post-thaw within dosing ranges reported for artificial insemination with frozen–thawed canine semen (Farstad 2009; Pesch et Hoffmann 2007; Silva *et al.* 2005).

Freezing of canine semen samples

The subsequent equilibration phase in sample processing was initiated by aliquoting 500 µL of each sample into a cryovial. This phase serves as the adaptation period for spermatozoa to acclimate to low temperatures in a protective medium containing cryoprotective agents. Each cryovial containing diluted semen was stored at 4°C for 180 min.

After the equilibration period, cryovials were subjected to accelerated freezing at ultralow temperatures in a biofreezer at -150°C. A temperature probe was used to monitor freezing rate consistency. Accelerated freezing started from +4°C (the temperature of the samples after equilibration) and reached -130°C for 29 min at an average rate of temperature decrease over the entire period of - 4.6°C/min (Gradinarska 2019). The samples were then stored at -150°C.

Thawing of cryovials with canine semen samples

Each semen sample was thawed in a water bath at 65°C for 60–80 sec, with the cryovials being gently agitated until the solid phase was completely dissolved. The samples were then incubated at 37°C in a temperature-controlled incubator and assessed using CASA.

Computer-assisted sperm analysis

Thawed samples were assessed by CASA for motility, progression, velocity, and kinematic parameters of frozen-thawed spermatozoa. The Sperm Class Analyser® CASA System (Microptic, Spain) and "Motility and Concentration" software module were used in the present study. The CASA software was configured for canine semen analysis using a Nikon BM 10× objective, with coverslip dimensions of 18 × 18 mm and a droplet volume of 8 µL. For each sample, a minimum of 1000 spermatozoa were analysed across at least five captured fields.

The basic parameters measured were concentration, motility, progression, velocity, and head area. Kinematic parameters were evaluated for the entire sample and within specific sperm subpopulations classified as static, slow, medium, or rapid. These included the curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP). Additional kinematic parameters included linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF).

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). A two-tailed analysis was performed. Differences between the healthy and BPH groups (separately for fresh and frozen-thawed samples) were assessed using the non-parametric Mann–Whitney U test. Within-group changes (fresh vs. post-thaw) were assessed using the Wilcoxon signed-rank test. Differences were considered statistically significant at $p < 0.05$. All analyses were performed using Statistica v.6.0 (StatSoft Inc.).

Results and Discussion

In the present study, the evaluated cryopreservation protocol used pre-freeze SP removal via gentle 300 × g for 20 s separation. Low g-force and brief duration were chosen to minimise mechanical stress and potential structural damage to spermatozoa, particularly in BPH cases (Koderle *et al.*, 2009; Lazov *et al.* 2019; Tsvetkov et Daskalova 2023).

Accelerated freezing results in a rapid transition of cells from partial to complete anabiosis, thus shortening the time for the main damaging factors to cause effects during cryopreservation (Eilts 2005; Kim *et al.* 2012). Subsequent rapid thawing at higher temperatures also minimises the duration of ice crystal growth and recrystallisation during the warming phase, which can cause severe mechanical and osmotic damage to spermatozoa (Mazur 1984; Watson 2000). In this protocol, the combination of high-temperature thawing and precise timing ensures that gametes pass quickly through the critical temperature zone between −50°C and −5°C, thereby improving the preservation of structural integrity and post-thaw functionality of the sperm.

Comparative CASA results for motility, progression, and velocity of spermatozoa in healthy dogs and dogs with BPH, before and after freezing, are presented in Table 1.

Ejaculates from healthy and BPH dogs to undergo this study were selected to ensure comparable baseline motility. Prior to freezing, the two groups showed no significant differences in the main CASA parameters used for post-thaw interpretation (e.g. total and progressive motility). Exception to this was a larger rapid subpopulation in healthy dogs before freezing (15.73±3.16 % vs 8.91±2.11 % in BPH group), indicating greater energetic potential of the spermatozoa (Table 1).

Table 1: CASA of motility, progression, and velocity of fresh and frozen spermatozoa in dogs with BPH and healthy dogs.

Group	State	Static (%)	Non-progressive (%)	Progressive (%)	Rapid (%)	Medium (%)	Slow (%)
BPH	Fresh	35.03±7.79§	52.87±4.69	12.10±3.47§	8.91±2.11§	10.09±3.29	45.96±3.85
	Frozen	72.87±8.42$^{\#}$	25.77±8.01	1.36±0.54$^{\#}$	1.58±1.13	2.20±1.48	23.35±6.13
Healthy	Fresh	31.38±8.19§	57.84±7.34	10.77±3.32§	15.73±3.16$^{\#}$	11.61±4.46	41.27±6.65
	Frozen	60.45±7.21$^{\#}$	37.68±6.76	1.87±0.62$^{\#}$	0.70±0.25	2.18±0.65	36.67±6.49

Note: Data are presented as mean ± SEM (n=10 per group). Symbols (§, #) were used to highlight specific differences. Within columns, values with different superscript symbols differ significantly ($p < 0.05$).

After thawing, both groups showed the expected decline in total and progressive motility. The cryopreservation process results in the deterioration of the general biological parameters of spermatozoa and an increase in static spermatozoa (Korochkina *et al.* 2014), which is the reason for the significant decrease in motility after thawing in both groups compared to fresh spermatozoa ($p < 0.001$). Post-thaw progressive motility did not differ between BPH and healthy dogs (Table 1).

The head area of spermatozoa in fresh ejaculates was larger in healthy dogs than in dogs with BPH. Post-thaw, between-group differences in head area were not observed (healthy: 22.19 ± 2.16 μm²; BPH: 20.04 ± 0.96 μm²). These results indicate that the freezing protocol preserved head morphometry to comparable levels in both groups. Prior observations of BPH-related changes in SP proteins (Gradinarska *et al.* 2019) and of prostatic or SP components which can modulate post-thaw sperm quality (Korochkina *et al.*, 2014; Neagu *et al.*, 2011) suggest a plausible role of SP composition in the preservation of structural and functional integrity of spermatozoa during cryopreservation that should be tested in future targeted studies.

The results from the CASA of sperm kinematics from healthy dogs and dogs with BPH before and after freezing are presented in Table 2.

Both groups demonstrated similar velocity kinematics before cryopreservation. After thawing, a significant decrease in the values of VCL, VSL, and VAP of sperm cells from healthy dogs and dogs with BPH was observed compared to the pre-freeze values ($p < 0.05$) (Table 2). These results are natural consequences of the detrimental effects of low temperatures during freezing. No significant differences were observed between BPH and healthy spermatozoa in the post-thaw velocity parameters (Table 2).

Table 2: CASA of kinematic parameters of fresh and frozen spermatozoa in dogs with BPH and healthy dogs.

Group	State	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
BPH	Fresh	42.07±6.32§	21.39±3.31§	28.69±4.65§	50.41±2.25	74.90±1.56	67.22±2.4	3.21±0.13	10.84±0.25
	Frozen	22.30±1.85$^{\#}$	9.79±1.57$^{\#}$	13.85±1.67$^{\#}$	43.42±4.93	68.73±4.19	61.42±4.09	1.53±0.42§	5.97±1.86§
Healthy	Fresh	48.92±7.52§	25.05±4.31§	33.00±4.74§	53.19±6.49	74.69±4.65	68.84±4.47	2.49±0.46	9.62±1.25
	Frozen	25.80±1.19$^{\#}$	11.30±0.80$^{\#}$	16.72±1.01$^{\#}$	43.49±1.67	67.33±1.54	64.46±1.35	2.82±0.17$^{\#}$	9.51±0.73$^{\#}$

Note: Data are presented as mean ± SEM (n=10 per group). Symbols (§, #) were used to highlight specific differences. Within columns, values with different superscript symbols differ significantly ($p < 0.05$).

Post-thaw trajectory parameters demonstrated that movement quality was largely maintained across the groups (Table 2). Both STR and progressive motility in dogs with BPH remained comparable to those of healthy controls after freezing, despite the expected overall decline in both groups. LIN, STR, and WOB remained stable in the healthy and BPH groups at both the pre-freeze and post-thaw assessments, showing no within-group change and no between-group differences. A notable post-thaw divergence was that ALH and BCF exhibited a greater decrease in BPH than in healthy dogs ($p < 0.05$), indicating greater susceptibility of the flagellar beat pattern to cryoinjury in this group.

These findings indicate that cryopreservation after gentle SP removal preserved the biological potential of spermatozoa from dogs with BPH, with post-thaw motility and kinematic profiles comparable to those of healthy controls. Thus, the evaluated SP-removal cryopreservation protocol is a viable approach for long-term gamete storage in dogs with BPH.

Conclusion

Under the evaluated freezing protocol which involved gentle SP removal prior to accelerated freezing, post-thaw progressive motility, LIN, STR, and WOB remained similar between BPH and healthy dogs. Progressive motility and velocity parameters (VCL, VSL, and VAP) decreased in both groups, with ALH and BCF showing a greater reduction in BPH than in healthy controls. These data indicate that the protocol is effective in maintaining key trajectory parameters across both healthy and BPH dogs, while also revealing a selective vulnerability in the flagellar beat dynamics of the BPH group following the freeze-thaw process.

References

1. De la Fuente–Lara A., Hesser A., Christensen B., Gonzales K., Meyers S. (2019). *Effects from aging on semen quality of fresh and cryopreserved semen in Labrador Retrievers*. Theriogenology 132: 164–171.
2. Eilts BE. (2005). *Theoretical aspects of canine semen cryopreservation*. Theriogenology 64(3): 692–697.
3. Estudillo E., Jiménez A., Bustamante–Nieves PE., Palacios–Reyes C., Velasco I., López–Ornelas A. (2021). *Cryopreservation of gametes and embryos and their molecular changes*. International Journal of Molecular Sciences 22(19): 10864.
4. Farstad W. (2009). *Cryopreservation of canine semen – new challenges.* Reproduction in Domestic Animals 44(Suppl 2): 336–341.
5. Genov M., Ivanova M. (2021). *Comparative diagnosis of benign prostatic hyperplasia in dogs by ultrasound, X–ray, and computed tomography*. Bulgarian Journal of Veterinary Medicine 24(2): 219.

6. Genov M., Ivanova M., Gradinarska D. (2020). *Ultrasonographic and radiographic studies of healthy dogs and dogs with benign prostatic hyperplasia in relation to the biological potential of spermatozoa*. Tradition and Modernity in Veterinary Medicine 5(1): 20–24.
7. Gradinarska D. (2019). *Seminal plasma proteins – role in the reproductive process and low–temperature storage of canine spermatozoa*. PhD Thesis. Institute of Biology and Immunology of Reproduction "Acad. K. Bratanov"– Bulgarian Academy of Sciences, Sofia, Bulgaria.
8. Gradinarska D., Ivanova M., Genov M., Tsvetkov T., Daskalova D. (2019). *Comparative assay of seminal–plasma proteins in healthy dogs and dogs with benign prostatic hyperplasia*. Comptes Rendus de l'Academie Bulgare des Sciences 72(8): 1137–1143.
9. Hesser A., Darr C., Gonzales K., Power H., Scanlan T., Thompson J., Love C., Christensen B., Meyers S. (2017). *Semen evaluation and fertility assessment in a purebred dog breeding facility*. Theriogenology 87: 115–123.
10. Hiemstra SJ., van der Lende T., Woelders H. (2006). *The potential of cryopreservation and reproductive technologies for animal genetic resources conservation strategies*. In: The role of biotechnology in exploring and protecting agricultural genetic resources. Eds. Ruane J, Sonnino A. FAO. 45–60.
11. Hori T., Hagiuda K., Endo S., Hayama A., Kawakami E., Tsutsui T. (2005). *Unilateral intrauterine insemination with cryopreserved caudal epididymal sperm recovered from refrigerated canine epididymides*. Journal of Veterinary Medical Science 67: 1141–1147.
12. Kim S., Lee Y., Yang H., Kim YJ. (2012). *Rapid freezing without cooling equilibration in canine sperm*. Animal Reproduction Science 130(1–2): 111–118.
13. Knox RV. (2015) *The fertility of frozen boar sperm when used for artificial insemination*. Reproduction in Domestic Animals 50(Suppl 2): 90–97.
14. Koderle M., Aurich C., Schäfer–Somi S. (2009). *The influence of cryopreservation and seminal plasma on the chromatin structure of dog spermatozoa*. Theriogenology 72(9): 1215–1220.
15. Korochkina E., Johannisson A., Goodla L., Morrell JM., Axner E. (2014). *Effect of prostatic fluid on the quality of fresh and frozen–thawed canine epididymal spermatozoa*. Theriogenology 82: 1206–1211.
16. Lazov K., Georgiev B., Taushanova P., Gradinarska D. (2019). *Comparative analysis of cryopreservation of male gametes with and without seminal plasma from Canis Lupus Familiaris*. Bulgarian Journal of Veterinary Medicine 22(1): 129–134.
17. Lemma A. (2011). *Effect of cryopreservation on sperm quality and fertility*. In: Artificial Insemination in Farm Animals 191–216. InTech. doi: 10.5772/16563
18. Mara L., Casu S., Carta A., Dattena M. (2013). *Cryobanking of farm animal gametes and embryos as a means of conserving livestock genetics*. Animal Reproduction Science 138(1–2): 25–38.
19. Mazur P. (1984). *Freezing of living cells: mechanisms and implications*. American Journal of Physiology–Cell Physiology 247(3): C125–C142.
20. Muiño–Blanco T., Pérez–Pé R., Cebrián–Pérez J. (2008). *Seminal plasma proteins and sperm resistance to stress*. Reproduction in Domestic Animals 43: 18–31.
21. Oldenhof H., Friedel K., Akhoondi M., Gojowsky M., Wolkers WF., Sieme H. (2012). *Membrane phase behavior during cooling of stallion sperm and its correlation with freezability*. Molecular Membrane Biology 29(3–4): 95–106.
22. Pesch S., Hoffmann B. (2007). *Cryopreservation of spermatozoa in veterinary medicine*. Journal of Reproductive Medicine and Endocrinology 4(2): 101–105.
23. Schäfer–Somi S., Colombo M., Luvoni GC. (2022). C*anine spermatozoa – predictability of cryotolerance*. Animals 12(6): 733.

24. Silva AR., Cardoso RCS., Silva LDM. (2005). *Comparison between different dilution rates on canine semen freezing using Tris–buffer with the addition of egg–yolk and glycerol*. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 57: 764–771.
25. 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*. Journal of Reproduction and Fertility (Supplement 57): 383–386.
26. Strzeżek R., Fraser L. (2009). *Characteristics of spermatozoa of whole ejaculate and sperm–rich fraction of dog semen following exposure to media varying in osmolality*. Reproductive Biology 9(2): 113–126.
27. Tsvetkov TS., Daskalova DB. (2023). *Effect of seminal plasma protein fractions on cooled dog semen kinetics*. Macedonian Veterinary Review 46(2): 177–183.
28. Watson PF. (2000). *The causes of reduced fertility with cryopreserved semen*. Animal Reproduction Science 60: 481–492.
29. Yeste M. (2016). *Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs*. Theriogenology 85(1): 47–64.