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INFLUENCE OF DIFFERENT BUFFERS ON THE VIABILITY CHARACTERISTICS OF CANINE SPERMATOZOA DURING STORAGE AT 4–6 °C

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Background. Maintaining high fertilization capacity of spermatozoa is a key factor for successful artificial insemination in dogs. Given that the use of chilled semen provides higher pregnancy rates and larger litter sizes compared with frozen semen, the demand for this method of gamete preservation is increasing. Preservation of sperm fertilizing ability in an in vitro system directly depends on the storage medium; however, analysis of current literature indicates that most studies focus on the effects of antioxidants, energy substrates, or membrane protectants, whereas the impact of buffer system parameters on gamete homeostasis during cooling remains insufficiently studied. Therefore, the aim of this study was to determine the influence of different buffers on the characteristics of chilled canine semen at 4–6 °C.

Materials and Methods. Extenders differing in their buffer system were added to the second fraction of canine semen: T-BSA (TRIS, pH 6.2), hT-BSA (TRIS, pH 7.3), and HEPES-BSA (HEPES, pH 6.2). Sperm motility, membrane integrity (HOS test), and the level of DNA fragmentation (SCD method, HALOMAX test system) were evaluated every 24 hours over a period of 10 days.

Results and Discussion. It was established that the T-BSA extender (pH 6.2) most effectively ensures long-term preservation of the functional parameters of canine spermatozoa during chilled storage at 4–6 °C. At the same time, DNA integrity remained

a stable parameter in all groups, even under conditions of complete loss of sperm motility. Thus, optimization of the buffer parameters of the extender is a key factor in prolonging the shelf life of chilled canine semen in clinical practice.

Conclusion. The T-BSA extender (pH 6.2) was found to be the most effective for preserving the functional parameters of canine spermatozoa during prolonged storage at 4–6 °C. DNA integrity remained a stable parameter in all groups, even in the case of complete loss of motility. Therefore, optimization of the buffer parameters of the extender is a key factor for extending the shelf life of chilled canine semen in clinical practice.

Keywords: semen preservation, TRIS-citrate buffer, HEPES, reproductive biotechnology, fertility

Registration number and the Funding source: none

INTRODUCTION

Artificial insemination (AI) in dogs is a fundamental tool of modern clinical veterinary practice (Mason, 2018), enabling the overcoming of logistical barriers (Hori *et al*., 2014), control of the genetic pool (Donner *et al*., 2018), and prevention of infectious diseases (Quartuccio *et al*., 2020). However, the effectiveness of AI critically depends on the quality of gametes, which, due to the absence of self-repair mechanisms, require the use of specialized extenders to maintain homeostasis and motility (Martínez-Barbitta & Rivera Salinas, 2022; Sinagra *et al*., 2024).

A key factor in sperm survival in an in vitro system is the stability of the medium pH (Contri *et al*., 2013). Despite the wide tolerance range of canine spermatozoa to fluctuations in acidity (pH 5–10), their maximum functional activity is observed at pH values of 7.0–8.5 (Wales & White, 1958). It should be noted that the pH of the second fraction of the native ejaculate is 6.1–6.2 (Martínez-Barbitta & Rivera Salinas, 2022; Litvinchuk *et al*., 2025). The situation is further complicated by the variability of the pH of the female reproductive tract (6.2–7.6), which requires synchronization of these parameters to ensure successful fertilization (Antonov *et al*., 2014).

While buffer systems have been widely investigated in various domestic animals, the extrapolation of these results to canine reproduction is limited by significant species-specific differences in sperm physiology and seminal plasma biochemistry (Dziekońska & Partyka, 2023). Specifically, the native pH of the canine sperm-rich fraction (6.1–6.2) differs from that of other domestic animals, such as bovine (6.8–6.9) (Lavanya *et al*., 2021) or boars (7.1) (Rivas *et al*., 2022), necessitating the development of specialized extenders tailored to the unique homeostatic requirements of dog spermatozoa.

In view of the above, the development of protocols for chilling canine semen requires careful selection of the buffer system and its acidity. Therefore, the aim of the study was to evaluate the influence of different buffers on the functional characteristics of canine spermatozoa in an in vitro system in order to improve the effectiveness of artificial insemination in dogs in clinical practice.

MATERIALS AND METHODS

The study was conducted during 2024–2025. Experimental investigations were carried out on a group of five German Shepherd dogs (*Canis lupus familiaris*), kept

at the Kennel of the Department of the State Protection of Ukraine (Kyiv). All animals involved were clinically healthy, had a reproductive history, and had produced offspring within the previous year.

Semen collection and processing. Work with the biomaterial was carried out at the Educational and Scientific Laboratory "Center for Animal Reproductive Biology with a Semen and Embryo Bank" of the National University of Life and Environmental Sciences of Ukraine (Kyiv). Semen was collected by masturbation, three times from each of five dogs with a 14-day sexual rest period between collections. The ejaculate was obtained fractionally and collected into separate sterile containers (Biosigma, Italy). This approach, involving repeated sampling from the same animals, was implemented to minimize intra-individual variability and ensure a sufficient volume of biological material (total n = 15 ejaculates), thereby achieving statistical power and reliability of the results. For further work, the second (sperm-rich) fraction was used, allowing minimal presence of the first and third fractions. Transportation to the laboratory was carried out within 30–60 min at a temperature of 20–24 °C.

After dilution of the native semen, its motility, concentration, proportion of abnormal forms, membrane status, level of sperm DNA fragmentation, and pH were analyzed. The samples were then aliquoted into tubes (Sarstedt, Germany) at a dose of 150 million gametes per sample and centrifuged for 20 min (200 g). After removal of the supernatant, 5 cm³ of one of three experimental extenders (composition provided in **Table**) was added to the pellet.

Composition and pH of canine semen extenders

Components	Extender		
	T-BSA	hT-BSA	Hepes-BSA
TRIS, mg	2400	3000	-
Citric acid, mg	1400	1400	-
HEPES, mg	-	-	450
Glucose, mg	4900	4900	4900
BSA, mg	300	300	300
Gentamicin, mg	200	200	200
Deionized water, cm³	100	100	100
pH	6.2	7.3	6.2

For the preparation of media based on deionized water (Diowater, Ukraine), reagents from Sigma (USA) were used: bovine serum albumin (BSA), citric acid, gentamicin sulfate, D-(+)-glucose, HEPES, as well as Tris(hydroxymethyl)aminomethane (TRIS) from Merck (Germany).

The obtained samples were stored in a refrigerator at a temperature of 4–6 °C. Evaluation of sperm parameters was performed every 24 hours. Aliquots of 0.2 cm³ were incubated in a thermostat for 30 minutes at 37 °C, after which sperm motility, membrane integrity, DNA preservation, and pH were assessed.

Semen quality assessment. Sperm concentration was determined by counting in a Goryaev chamber. Sperm motility was evaluated using the "crushed drop" method at 120× magnification under a microscope (Litvinchuk *et al*., 2025). Measurement of hydrogen ion concentration (pH) was performed using a calibrated EcoTestr pH 1 device (Thermo Scientific, USA).

Membrane integrity (hypo-osmotic swelling test, HOS test). The functional integrity of sperm plasma membranes in all experimental groups was assessed using the hypo-osmotic swelling test (HOS test), according to the manufacturer's protocol.

DNA fragmentation. DNA integrity was analyzed using the species-specific test system "HALOMAX" (Halotech DNA, Spain), developed for *Canis familiaris*, according to the manufacturer's protocol. A comprehensive step-by-step description of the procedure can be found in Litvinchuk *et al*. (2025).

Statistical analysis. Experimental data were processed by methods of variation statistics using Microsoft Excel and XLSTAT software. The obtained results were processed by calculating the mean value and its standard error ($x \pm SE$). A two-way analysis of variance (ANOVA) was employed to evaluate the effects of the extender, storage duration, and their interaction. Post-hoc multiple comparisons were conducted using the Bonferroni test to assess changes within each extender over time, while Tukey's HSD test was applied to identify significant differences between different extenders at specific time points. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The functional status of spermatozoa is directly dependent on the pH of the medium (Mishra *et al*., 2018); however, the influence of acid–base balance on male gametes is described in the literature only fragmentarily (De Pauw *et al*., 2003; Dai *et al*., 2024). Therefore, within the current stage of the study, we investigated the effect of different buffers on the functional parameters of spermatozoa by selectively altering the buffer component in the T-BSA extender, which had previously demonstrated high efficacy (Litvinchuk *et al*., 2025).

Thus, over the 10-day experimental period, a statistically significant decrease in total sperm motility was recorded in all studied groups (**Fig. 1*A***).

Up to day 3, no significant differences were observed among the studied samples (total motility 88.0–91.6 %), whereas from day 4 a significant advantage of the T-BSA extender was noted ($P < 0.05$), where motility remained at 87.7 ± 2.8 % compared with 77.4 ± 3.1 % for hT-BSA. However, by day 8, the differences between the parameters became statistically non-significant ($P > 0.05$), despite the continued advantage of T-BSA. The lowest efficiency was demonstrated by the HEPES-BSA extender: on day 6, total sperm motility was only 31.8 ± 4.0 %, which was 2.4 times lower than in the T-BSA group ($P < 0.001$), and by day 8, a complete loss of motility was observed.

The obtained data indicate that for canine spermatozoa, not only the pH level (which in T-BSA and HEPES-BSA was identical – 6.2) is decisive, but also the chemical nature of the buffer. The TRIS-citrate buffer proved more effective compared with HEPES, which is consistent with the findings of J. L. Yániz *et al*. (2012). The low efficiency of HEPES may be explained by inhibition of ion channels and mitochondrial functions (Mendola *et al*., 2024). Blockade of Ca^{2+} CatSper channels leads to flagellar

paralysis (Lishko *et al*., 2012), while an increase in intracellular pH (pHi) stimulates oxidative stress and ATP depletion (Koppers *et al*., 2008).

Along with total motility, the quality of sperm movement is a sensitive marker of functional competence (**Fig. 1*B***). Thus, when using the HEPES-BSA and T-BSA extenders, a proportional decrease in the percentage of motile spermatozoa and cells exhibiting progressive linear motility was observed. In contrast, analysis of progressive linear motility in the hT-BSA group (pH 7.3) revealed a sharp decline in this parameter: by day 6 of the experiment, it was 2.3 times lower than in T-BSA group ($P < 0.001$), and from day 8 onward, only spermatozoa with an oscillatory type of movement were recorded, whereas in T-BSA group, the studied parameter was maintained at the level of 31.2 ± 3.3 % up to day 10 ($P < 0.001$). The loss of progressive linear motility while preserving total motility indicates profound functional impairment of spermatozoa. The likely causes of this phenomenon include premature activation of spermatozoa under alkaline pH conditions (Suarez, 2008) and a decrease in mitochondrial membrane potential (Contri *et al*., 2013).

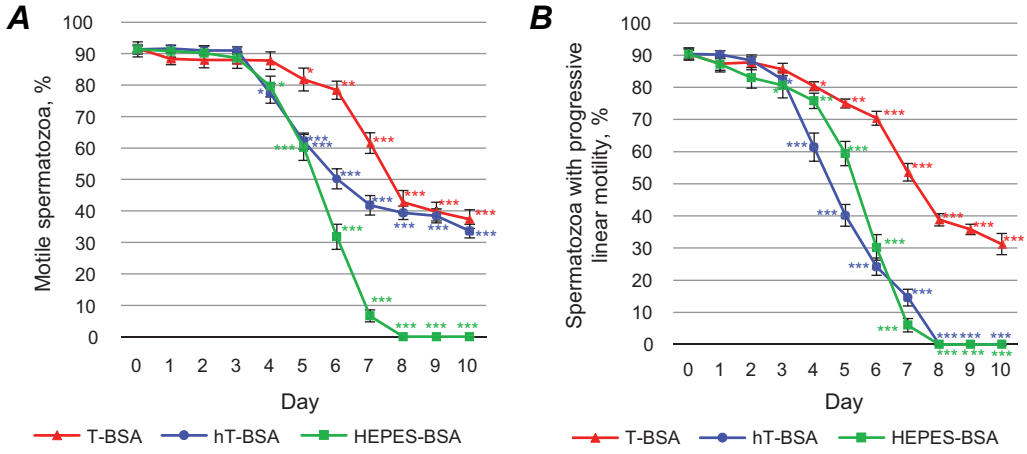

Fig. 1. Dynamics of canine sperm motility parameters during storage using semen extenders with different buffering systems: ***A*** – total motility; ***B*** – progressive linear motility. * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$ compared with the control (day 0) and between groups within a row (x ± SE, n = 5)

The results of the HOS test (**Fig. 2*A***) confirmed the destructive effect of alkaline pH when using the hT-BSA extender. Thus, by day 4, membrane integrity sharply decreased to 68 ± 4.1 % ($P < 0.01$), which temporally correlates with the onset of a rapid loss of the spermatozoa's ability for progressive linear motility. In contrast, semen diluted with media at pH 6.2 maintained more than 80 % HOS-positive spermatozoa until the end of the experiment. This indicates the initiation of oxidative stress and disruption of membrane permeability under alkaline pH conditions (Aitken *et al*., 2014).

At the same time, the level of DNA fragmentation remained consistently low in all groups (**Fig. 2*B***), without statistically significant differences from the control, which is consistent with the findings of D. Bencharif *et al*. (2013) and I. K. Puja *et al*. (2018). This confirms the hierarchical model of sperm degradation: coordinated motility is lost first, followed by membrane integrity, while the genetic material remains stable for the longest period (Zduńczyk *et al*., 2025).

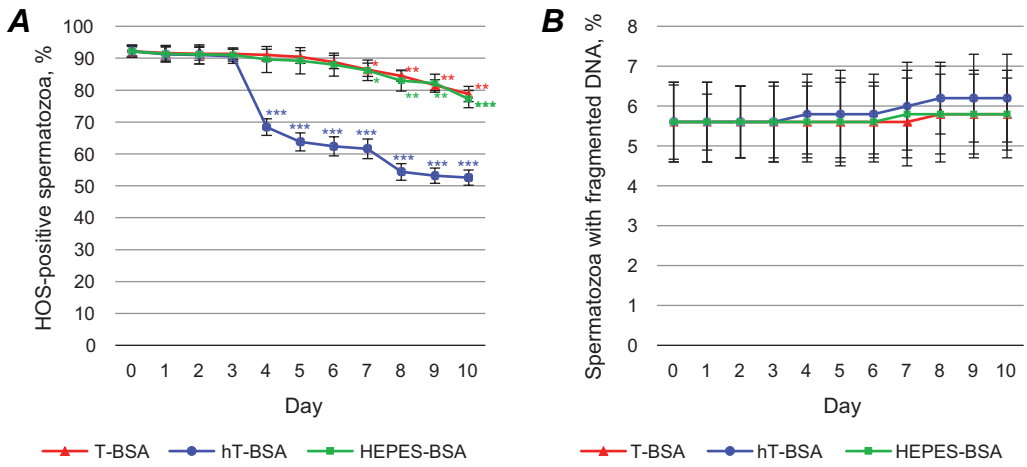

Fig. 2. Indicators of functional competence of canine spermatozoa when using extenders with different buffering properties during storage: ***A*** – plasma membrane integrity; ***B*** – DNA fragmentation. * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$ compared with the control (day 0) and between groups within a row ($x \pm SE$, $n = 5$)

CONCLUSION

It was established that the T-BSA extender (TRIS-citrate buffer, pH 6.2) is optimal for long-term storage of canine semen at 4–6 °C, allowing preservation of progressive linear motility in 31.2 ± 3.3 % of spermatozoa up to day 10. The use of the TRIS-citrate buffer with an increased pH of 7.3 proved to be ineffective due to premature desensitization of plasma membranes and metabolic exhaustion of gametes. Although the pH when using HEPES was also 6.2, this extender was ineffective, likely due to inhibition of ion channel activity and mitochondrial functions, confirmed by the absence of motile spermatozoa in the samples on day 8 of the experiment. At the same time, the genetic material of spermatozoa demonstrates high stability: the level of DNA fragmentation remains stable even when spermatozoa lose motility.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: the authors declare that they have no commercial or financial relationships that could be construed as a potential conflict of interest.

Animal rights compliance: the experimental procedures involving animals were conducted in accordance with the principles of humanity and bioethics and in strict adherence to the Law of Ukraine “On the Protection of Animals from Cruelty” (No. 3447-IV), as well as the General Ethical Principles of Experiments on Animals adopted by the First National Congress of Bioethics (Kyiv, 2001). The study protocols were fully aligned with

the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986). The experimental part of the study was officially approved by the Local Bioethics Commission of the National University of Life and Environmental Sciences of Ukraine (Protocol No. 006/2024 dated 29 February 2024).

Human rights compliance: this article does not contain descriptions of any studies involving humans as research subjects.

AUTHOR CONTRIBUTIONS

Conceptualization, [Yu.V.; V.V.; O.S.]; methodology, [Yu.V.; V.V.; O.S.]; validation, [Yu.V.]; formal analysis, [Yu.V.].; investigation, [Yu.V.; O.S.]; resources, [V.V.; S.S]; data curation, [Yu.V.; V.V.; O.S.] ; writing – original draft preparation, [Yu.V.; V.V.; O.S.]; writing – review and editing, [Yu.V.; V.V.; O.S.; S.S; O.A; Yu.V.]; visualization, [Yu.V.; O.S.] supervision, [V.V.; S.S]; project administration, [V.V.; S.S; O.A.]; funding acquisition, [V.V.; S.S; O.A.].

All authors have read and agreed to the published version of the manuscript.

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ВПЛИВ РІЗНИХ БУФЕРІВ НА ХАРАКТЕРИСТИКИ ЖИТТЄЗДАТНОСТІ СПЕРМАТОЗОЇДІВ ПСІВ ПІД ЧАС ЗБЕРІГАННЯ ЗА ТЕМПЕРАТУРИ 4–6 °C

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Обґрунтування. Збереження високих показників запліднення сперматозоїдів є важливою умовою успішного штучного осіменіння собак. Зважаючи на те, що охолоджена сперма забезпечує вищі показники вагітності та більший розмір приплоду у гнізді, порівняно із замороженою, зростає попит на цей метод збереження гамет. Збереження фертильності сперматозоїдів у системі *in vitro* напряму залежить від середовища зберігання, проте аналіз сучасних літературних джерел свідчить, що більшість наукових пошуків зосереджена на вивченні впливу антиоксидантів, енергетичних субстратів чи мембранопротекторів, тоді як вплив параметрів буферних систем на гомеостаз гамет під час охолодження залишається малодослідженим. Тому метою нашої роботи було встановити вплив різних буферів на характеристики охолодженої сперми псів за температури 4–6 °C.

Матеріали та методи. До другої фракції сперми псів додавали розріджувачі, що відрізнялися буферною системою: T-BSA (TRIS, pH 6.2), hT-BSA (TRIS, pH 7.3) та HEPES-BSA (HEPES, pH 6.2). Рухливість сперматозоїдів, цілісність їхньої мембрани (HOS-тест) та рівень фрагментації ДНК (метод SCD, тест-система “HALOMAX”) оцінювали кожні 24 год упродовж 10 діб.

Результати. Встановлено, що розріджувач T-BSA (pH 6.2) найефективніше забезпечує тривале збереження функціональних параметрів сперматозоїдів собак

під час зберігання методом охолодження за температури 4–6 °C. Водночас цілісність ДНК залишалася стабільним параметром у всіх групах, навіть за умови повної втрати рухливості сперматозоїдів. З огляду на це, підбір оптимальних буферних характеристик розріджувача відіграє вирішальну роль у пролонгації зберігання охолодженої сперми псів.

Висновки. Встановлено, що розріджувач T-BSA (pH 6.2) є найбільш ефективним для збереження функціональних показників сперматозоїдів псів під час тривалого зберігання за температури 4–6 °C. Тоді як цілісність ДНК виявилася стабільним показником у всіх групах, навіть за умови повної втрати рухливості. Отже, оптимізація буферних параметрів розріджувача є ключовим фактором для продовження терміну придатності охолодженої сперми псів у клінічній ветеринарній практиці.

Ключові слова: збереження сперми, TRIS-цитратний буфер, HEPES, репродуктивна біотехнологія, фертильність

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