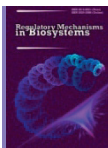

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Effects of extenders on the functional activity of chilled canine spermatozoa during prolonged storage

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Preserving the quality and providing a high fertile capacity of spermatozoa are some of the main objectives of successful fertilization of dogs. Insemination using chilled semen produces higher pregnancy parameters and larger litters, compared with using frozen semen. Therefore, this practice is in high demand. Nevertheless, there is an issue of prolonged storage of chilled semen, especially relevant during transportation to long distances. Considering that high-quality parameters of spermatozoa directly depend on a medium in which they are stored, the objective of this study was determining the effects of different types of extenders on the functional characteristics of chilled canine semen during prolonged storage. We studied the effects of the extenders on the motility, ability to move in a straight line, integrity of the membrane, and DNA fragmentation of canine spermatozoa. The second fraction of ejaculate was collected from five dogs using the masturbation method. The semen was centrifuged to achieve settlement of the spermatozoa and remove the plasma. To the spermatozoa sediment, one of the following extenders was added, which had been heated up to the room temperature: T – TRIS + citric acid + glucose (base content); T-EG – base content + egg yolk; T-BSA – base content + BSA; T-EDTA – base content + EDTA in a calculation of 150×10^6 spermatozoa in 5 cm^3 of extender. After pipetting, the samples were cooled to 4 °C with subsequent daily monitoring until the tenth day of storage. The results of the study revealed that the extender T-BSA provided the highest parameters of the general motility and straight-line movement of the spermatozoa over the storage period. In the descending order, the effectiveness of extenders in preserving the sperm motility at a 50% level was as follows: T-BSA (7 days); T and T-EDTA (6 days); T-EG (5 days). At the same time, according to the percentage of gametes with straight-line movements, the results were as follows: T-BSA (7 days); T and T-EG (5 days); T-EDTA (4 days). The percentage of spermatozoa with damaged membrane over the period of storage increased (by 13.4–16.0%, compared with day zero), although no effect of the extender composition on this parameter was observed. The level of DNA fragmentation in the chilled canine sperm increased insignificantly (up to 0.4% by day 10) over the storage period, without significant effect of the extender on this parameter. In summary, the extender T-BSA demonstrated the highest effectiveness in the preservation of sperm motility at a 4 °C temperature, maintaining the general motility at the level of 37.4 ± 5.9% and the straight-line movement at the level of 31.2 ± 6.6% on day 10 of storage. This allows us to consider T-BSA an optimal composition for long storage of dog semen in a chilled state.

Keywords: BSA; egg yolk; EDTA; TRIS; DNA fragmentation; HOS test; HALOMAX; hypoosmotic swelling test; sperm vitality reproductive biotechnologies; fertility.

Introduction

The first successful artificial insemination of dogs was performed by Lazzaro Spallanzani in 1780 (Bozzini et al., 2016). However, only in recent decades has interest in the reproduction of companion animals increased (Martínez-Barbitta & Rivera Salinas, 2022). One of the reasons for this is that while dog breeders aim to preserve valuable pedigrees and genetic diversity by mating animals from different regions, transportation of females and males is stressful to animals and reduces the effectiveness of fertilization, while at the same time increasing the risk of complications during pregnancy and labor. (Hori et al., 2014). A new alternative is the transportation of semen – fresh, chilled, or cryoconserved – with the subsequent artificial insemination (Michael et al., 2009; Nguyen et al., 2019).

Yet, another problem has arisen, which is the preservation of sperm quality during transportation of semen, maintaining a high fertile capacity of spermatozoa. Freshly obtained semen quickly loses its properties. Thus, during storage, spermatozoa in semen samples lose their activity already after 3–4 hours at a temperature of 37 °C and after 5–6 hours at a room temperature (20–24 °C). To expand the period of viability of spermatozoa, their metabolic activity must be reduced, which is achieved by an appropriate storage temperature or medium. Therefore, freshly obtained dog semen are chilled or cryoconserved, depending on the following periods of use (Thomassen & Farstad, 2009). It has to be noted that cryoconservation of semen requires special equipment and a more complex processing than its chilling (Linde-Forsberg, 1991; Eilts, 2005), while the fertile capacity of cryoconserved spermatozoa is much lower (Nizański, 2006). Furthermore, vaginal insemination of female dogs using chilled semen leads to higher pregnancy rates and larger litters compared with frozen semen, which typically requires intrauterine insemination (Rota et al., 1995; Calabria et al., 2023). Therefore, chilled semen is in a greater demand in programs of artificial insemination (Thomassen & Farstad, 2009).

Chilling of semen reduces the metabolic activity of spermatozoa, thus preserving the quality parameters of the sperm, such as motility and viability, for several days (Kutzler, 2005). Considering that the duration of storage of chilled semen is also quite limited, it requires adequate use of extenders that minimize the damage to spermatozoa caused by temperature decrease, provide energy, maintain pH and osmolarity, reduce oxidation, and preserve the integrity of plasmatic, acrosomal, and mitochondrial membranes, etc. (Rota et al., 1995; Nguyen et al., 2019; Martínez-Barbitta & Rivera Salinas, 2022). Extenders used for chilled semen must provide spermatozoa with nutrients, have a substantial buffer system and optimal concentration of electrolytes, thereby preventing the growth of bacteria and providing a protection from heatstroke during cooling (Rota et al., 1995; Michael et al., 2009; Suzuki et al., 2022).

Despite the existence of various commercial and non-commercial extenders for chilling canine semen, one of the most commonly used is Tris-glucose/fructose buffer with 20% egg yolk (Iguer-Ouada &

Verstegen, 2001; Rodenas et al., 2014). However, using yolk is associated with a number of risks – in particular, microbiological contamination. Another problem is variability of yolk composition, which complicates the standardizing of the medium's components (Bousseau et al., 1998; Hermansson et al., 2021; Yurchuk et al., 2022). This necessitates the search for alternative compounds that are able to provide a similar or better protective effect, with none of the biological disadvantages of yolk.

As of now, most of the recent studies pertaining to canine spermatozoa have focused on composition of extenders for chilled semen, with the objective of prolonging the periods during which spermatozoa survive and retain their fertile capacity (Prete et al., 2018; Del Prete et al., 2022; Calabria et al., 2023). Using extenders for long-term storage of dog semen would improve the management of male reproductive processes, thereby enhancing the effectiveness of artificial insemination in females and maximizing reproductive results. Thus, the objective of our experiment was evaluating the life span of spermatozoa, their motility, functional condition of the membrane (hypo-osmotic swelling test), and the level of DNA fragmentation (using a HALOMAX test system) in the canine semen extenders of different compositions.

Materials and methods

The studies were conducted from March to November, 2024. The experiments were performed using five *Canis lupus familiaris* dogs of the German Shepherd breed, kept at the Kennel of the Administration of State Guard of Ukraine (the city of Kyiv). The experiment involving the animals was carried out with adherence to the requirements of the Law of Ukraine On Protection of Animals From Abuse (Article 230 as of 2006), The General Ethical Principles of Experiments on Animals, adopted by the National Congress of Bioethics and coordinated with the positions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasburg, 1986). Prior to the experiments, we received a permit from the local Committee of Bioethics of the National University of Life and Environmental Sciences of Ukraine of Ukraine for using dogs in the experiment (Protocol №006/2024 as of February 29, 2024).

All the animals involved in the experiment had veterinary passports with a stamp indicating their planned immunization, which was carried out at an interval of no longer than 12 months and no sooner than 30 days before the experiment. The dogs were clinically healthy and had a history of confirmed fertility (litters during the previous year).

The dogs were kept in the regular conditions of a German Shepherd kennel, taking into account their basic, psychological, social, and physiological needs, in compliance with the requirements of the Law of Ukraine on protection of animals from abuse, on animals, on the protection of the environment, on veterinary medicine, on the provision of sanitary and epidemiological wellness of the population, and on the protection of the population from infectious diseases. The animals were fed twice a day (at 8:00 and 22:00) with a commercial dry feed and selected chicken meat, and had free access to fresh water. The animals were cared for by one to two qualified workers, each of whom had reached the age of 18 and had undergone special training at cynology organizations. Each day, the animals received a veterinary examination. Whenever signs of diseases were discovered, the dogs immediately received qualified treatment. During the experiment, we adhered to the principles of humanity: during the procedures, we minimized pain, stress, and suffering, causing no harm to their health.

The work with semen was conducted at the Research-Scientific Laboratory Center of Reproductive Medicine of Animals with the Bank of Sperm and Embryos of the National University of Bioresources and Environmental Sciences of Ukraine (Kyiv).

The semen was collected via manual masturbation after an abstinence period of 14 days, performed three times. The ejaculate (divided into fractions) from each dog was collected in sterile capacities for biological fluids (Biosigma, Italy). In the following studies, we used the second (main/spermatozoa-enriched) fraction of the ejaculate. The spermatozoa-enriched fraction was isolated, although it inevitably contained small amounts of the first and third fractions. In 30–60 minutes after yielding, the biological material was transferred to the laboratory at a temperature of 20–24 °C.

After dilution, the native semen samples were analyzed to determine the activity, concentration, percentage of pathological forms, functional state of the membrane, level of DNA fragmentation of spermatozoa, and pH.

The yielded semen samples were divided among centrifuge test tubes (Sarstedt, Germany) in calculation of 150 million spermatozoa in one dose and centrifuged for 20 min at a centrifugal force of 200 g. The supernatant was selected, and a 5 cm^3 of the studied extender was added to the sediment. In the experiment, we compared 10 variations of extenders for canine semen, the contents of which are presented in Table 1.

In the study we used the following chemical compounds, purchased from international producers: Tris(hydroxymethyl) aminomethane (TRIS) (Merck, Germany), Citric acid (Sigma-Aldrich, USA), bovine serum albumin (BSA) (Sigma-Aldrich, США), ethylenediaminetetraacetic acid (EDTA) (Sigma, USA), gentamicin sulfate (Sigma, USA). All the solutions were prepared using deionized water (Diowater, Ukraine).

Table 1
Composition and pH of the canine semen extenders

Components	Extenders			
	T	T-EG	T-BSA	T-EDTA
TRIS, mg	2400	2400	2400	2400
Citric acid, mg	1400	1400	1400	1400
Glucose, mg	4900	4900	4900	4900
Yolk, %	–	20	–	–
BSA, mg	–	–	300	–
EDTA, mg	–	–	–	40
Gentamicin, mg	200	200	200	200
Deionized water, cm^3	100	100	100	100
pH	6.5	6.2	6.2	6.4

The obtained samples were stored at a temperature of 4–6 °C in a refrigerator. To assess the effects of an extender on the spermatozoa, every 24 h, after pipetting, we collected 0.2 cm^3 from each test tube in micro tubes (Sarstedt, Germany). The obtained samples were heated in a thermostat at 37 °C for 60 min, and then were assessed for pH of the suspension, activity of spermatozoa, degree of their agglutination, integrity of the membrane, and DNA fragmentation.

The concentration of spermatozoa in the semen was determined in a Goryaev's chamber. The percentage of dead spermatozoa was determined microscopically in a semen smear, stained with 5% aqueous solution of eosin (the head of the dead spermatozoa stained pink, while remaining unstained in the live ones), and the percentage of pathological ones was determined after staining with 1% solution of methylene blue, at 600 times magnification (the total number of counted spermatozoa was no less than 500).

The activity of the spermatozoa was determined by counting gametes with straight-line gradual movement in a wet mount preparation at a 120 times magnification on a heating table at 38–40 °C. In addition, the samples were visually analyzed for the degree of agglutination: + – <10% agglutinated spermatozoa, high number of free ones; ++ – 10–50%; +++ – >50%; ++++ – all spermatozoa agglutinated. The measurement of pH in the samples was conducted using a pH meter EcoTestr pH 1 (Thermo Scientific, USA).

The functional state of the spermatozoon membrane (HOS test) was assessed in all the analyzed samples during each time interval. An amount of 0.1 cm^3 of a sperm sample was incubated at a temperature of 37 °C for 30 min (no longer than 120 min) in a 1 cm^3 pre–heated Host-medium (FertiPro, Belgium). After the incubation period, a 0.01 cm^3 of the suspension was placed on a Superfrost microscope slide (Thermo Scientific, USA) and covered with a cover slip. The assessments were carried out using a Leica DMR (Germany) microscope at 400× and 1000× magnification. The cells were classified as positive (intact membrane) or negative (damaged membrane), according to the presence or absence of tucked tails, respectively (Fig. 1).

Fig. 1. Hypoosmotic swelling test (HOS test): *a* – HOS-positive spermatozoa (intact membrane, varying degree of tail swelling); *b* – HOS-negative spermatozoa (damaged membrane, no swelling)

The DNA fragmentation of the spermatozoa in each sample was evaluated using a HALOMAX system for dogs (Halotech DNA, Spain), which is species-specific to *Canis familiaris*, according to the manufacturer's protocol. The principle of identification of the mentioned parameter is based on the SCD method (sperm chromatin dispersion) – identification of dispersion of sperm chromatin (Fernández et al., 2003). It has to be noted that for the study, the sperm samples were diluted in phosphate–buffered saline (PBS) (Sigma, USA) so that the concentration of spermatozoa did not exceed 20 M/cm^3. The spermatozoa had been immobilized in agarose gel on a microscope slide, were treated with an acid solution for DNA denudation, and then buffer was used to induce lysis of the membranes and proteins. After the fixation in ethanol solution, in order to visualize the dispersed DNA loops, the samples were stained with Brightfield Staining Kit (Halotech DNA, Spain), according to the manufacturer's recommendations. The samples were examined using the Leica DMR (Germany) microscope at 400× and 1,000× magnification. The spermatozoa with fragmented DNA had very small dispersion halos or were absent completely, whereas the spermatozoa with low level of fragmentation released DNA loops that formed large halos (Fig. 2).

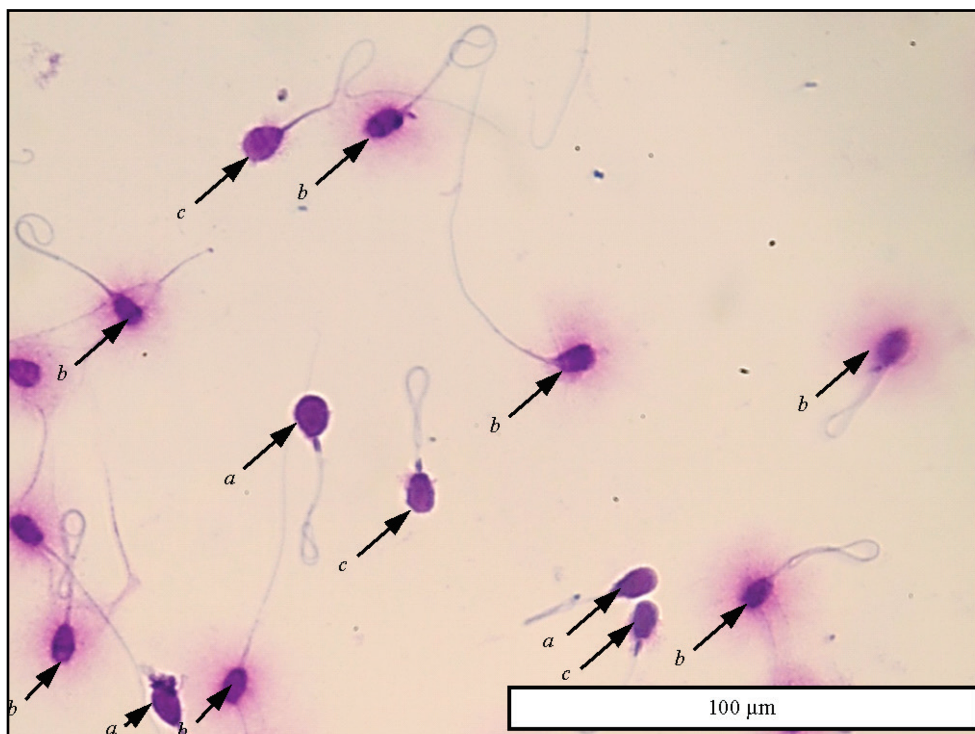

Fig. 2. Fragmentation of DNA of the spermatozoa: *a* – abnormal spermatozoon without halo (DNA fragmentation); *b* – normal spermatozoon with a large halo (no fragmentation); *c* – abnormal spermatozoon with a small halo (DNA fragmentation)

The results are presented as x ± SE (main ± standard error), and $P < 0.05$ were considered significant. To compare the differences in the mean parameters among the different media, we used the Tukey test. The parameters within one medium were compared using one-way ANOVA with the Bonferroni post hoc test.

Results

Prior to the experiment, we had conducted an assessment of the second ejaculate fraction that was later used. The pH of the studied samples ranged 6.1–6.2. The average volume of the main semen fraction from the examined dogs accounted for 3.5 ± 0.9 cm^3, and the mean number of spermatozoa accounted for $248.1 \pm 57.1 * 10^6/cm^3$. The mean percentage of mobile spermatozoa in the fresh semen samples measured 91.5 ± 3.4, while such of the gametes with straight-line movement equaled 90.3 ± 3.7%. The average percentage of live spermatozoa in the studied samples was 92.7 ± 3.9%, whereas such of morphologically normal ones accounted for 90.8 ± 4.0%. According to the results of hypoosmotic swelling test, 92.7 ± 3.1 % of the examined gametes had functionally undamaged membrane. At the same time, according to the results of identification of dispersion of sperm chromatin, the mean percentage of abnormal spermatozoa with fragmented DNA in the studied samples accounted for 5.7 ± 1.7%. No spermatozoid agglutination in the studied samples was observed.

After centrifugation and addition of extenders to the spermatozoa sediment, no changes in the motility of the spermatozoa were noted (day 0 of the study), although, as expected, this parameter significantly decreased in response to 10 day storage (Table 2).

Table 2
Changes in the parameters of the general motility (%) of the canine spermatozoa during storage in the semen extenders of different compositions (x ± SE, n = 5)

Extender	Day										
	0	1	2	3	4	5	6	7	8	9	10
T	91.4 ± 4.8^{a}	89.8 ± 3.4^{a}	87.0 ± 2.8^{a}	86.8 ± 4.1^{a}	$79.2 \pm 4.3^{**a}$	$78.6 \pm 3.3^{**a}$	$58.2 \pm 6.3^{***a}$	$43.6 \pm 5.0^{***a}$	$41.0 \pm 5.5^{***a}$	$33.4 \pm 4.1^{***a}$	$17.2 \pm 4.1^{***a}$
T–EG	91.4 ± 3.0^{a}	87.6 ± 5.2^{a}	86.6 ± 4.8^{a}	86.0 ± 5.1^{a}	84.8 ± 4.7^{a}	$66.6 \pm 7.5^{***ab}$	$40.2 \pm 7.1^{***b}$	$17.6 \pm 3.4^{***c}$	$9.0 \pm 2.4^{***b}$	$0 \pm 0^{***c}$	$0 \pm 0^{***b}$
T–BSA	91.6 ± 1.9^{a}	88.4 ± 3.9^{a}	88.0 ± 5.1^{a}	88.0 ± 5.3^{a}	87.8 ± 5.4^{a}	81.8 ± 7.1^{a}	$78.4 \pm 5.8^{**c}$	$61.6 \pm 6.7^{***b}$	$42.8 \pm 7.2^{***a}$	$39.8 \pm 6.0^{***a}$	$37.4 \pm 5.9^{***c}$
T–EDTA	91.4 ± 4.8^{a}	90.4 ± 3.0^{a}	89.2 ± 3.9^{a}	80.0 ± 5.8^{a}	$58.2 \pm 6.9^{***b}$	$57.6 \pm 4.9^{***b}$	$52.6 \pm 6.7^{***ab}$	$37.8 \pm 6.2^{***a}$	$36.8 \pm 5.5^{***a}$	$19.8 \pm 7.0^{***b}$	$9.2 \pm 4.2^{***b}$

Note: * – $P < 0.05$; ** – $P < 0.01$ *** – $P < 0.001$ compared with the control (day 0) and the groups within a line (ANOVA followed with the Bonferroni post hoc test); letters indicate significant differences among the groups within a column ($P < 0.05$) according to the results of the Tukey Test.

The least effective extender in the preservation of motility of the chilled spermatozoa was the solvent T–EDTA (TRIS + citric acid + glucose + EDTA). As early as on day 4 of the experiment, the total motility decreased by 33.2% ($P < 0.001$), and only 9.2 ± 4.2% of mobile gametes remained on day 10. The decrease occurred gradually throughout the monitoring period.

Compared with T–EDTA, T–EG (TRIS + citric acid + glucose + egg yolk) provided much better parameters, preserving the average motility at a level of 66.6 ± 7.5% on day 5, which was 24.8% lower compared with the initial value (day 0, $P < 0.001$). However, the decrease in the motility in this extender had a pronounced stepwise pattern, with a sharp drop until complete absence of active spermatozoa on day 9. Somewhat better results were demonstrated by the base diluent T (TRIS + citric acid + glucose), in which a reliable decline in the motility started on day 4 (79.2 ± 4.3 %, $P < 0.05$), but happened gradually, which allowed the maintenance of 17.2 ± 4.1% of mobile gametes on day 10 of storage.

The most effective extender was T–BSA (TRIS + citric acid + glucose + serum albumin of bovine cattle). In this formulation, the motility started to significantly decline on day 6 (78.4 ± 5.8%, $P < 0.01$), whereas on day 10, the level of mobile gametes measured 37.4 ± 5.9%, which was significantly higher than in the other examined extenders ($P < 0.01$).

However, when assessing the effects of the extenders on the preservation of spermatozoa, assessment of their general motility is not enough. In order to increase the chances of a successful fertilization during artificial canine insemination, the gametes of a male must be capable of moving in a straight line. Therefore, one of the criteria for evaluation of the extenders was determining the percentage of spermatozoa with straight–line movement (Table 3).

Table 3
Percentage change in the canine spermatozoa with straight–line movement (%) during storage in the semen extenders of different compositions (x ± SE, n = 5)

Extender	Day										
	0	1	2	3	4	5	6	7	8	9	10
T	90.4 ± 2.7^{a}	87.8 ± 2.1^{a}	85.4 ± 3.1^{a}	84.0 ± 3.4^{a}	$75.6 \pm 3.9^{***a}$	$69.4 \pm 6.7^{***a}$	$48.4 \pm 5.8^{***b}$	$37.6 \pm 2.9^{***b}$	$27.0 \pm 5.5^{***b}$	$20.8 \pm 4.6^{***b}$	$9.0 \pm 3.0^{***b}$
T–EG	90.2 ± 4.4^{a}	86.0 ± 4.0^{a}	82.6 ± 2.7^{a}	81.8 ± 3.4^{a}	$78.8 \pm 2.4^{**a}$	$65.8 \pm 6.9^{***a}$	$30.8 \pm 4.5^{***c}$	$9.2 \pm 3.2^{***c}$	$2.6 \pm 1.6^{***d}$	$0 \pm 0^{***d}$	$0 \pm 0^{***c}$
T–BSA	90.4 ± 3.8^{a}	87.4 ± 4.2^{a}	86.8 ± 4.6^{a}	85.6 ± 3.9^{a}	80.4 ± 2.7^{a}	$75.0 \pm 2.8^{***a}$	$70.4 \pm 4.5^{***a}$	$53.6 \pm 5.5^{***a}$	$38.8 \pm 3.9^{***a}$	$35.8 \pm 3.2^{***a}$	$31.2 \pm 6.6^{***a}$
T–EDTA	90.4 ± 2.7^{a}	85.6 ± 7.1^{a}	83.8 ± 6.3^{a}	$78.2 \pm 6.6^{*a}$	$54.2 \pm 5.4^{***b}$	$49.6 \pm 4.2^{***b}$	$34.6 \pm 7.3^{***bc}$	$27.8 \pm 3.2^{***b}$	$16.8 \pm 3.7^{***c}$	$7.8 \pm 5.1^{***c}$	$0 \pm 0^{***c}$

Note: see Table 2.

The analysis of the yielded data revealed a correlation between the percentage of mobile spermatozoa and the percentage of spermatozoa that moved in a straight line. The preservation of ability of the spermatozoa to move in a straight line varied among the extenders of different compositions. Thus, a reliable, below 80%, decrease in the average percentage of gametes with this ability was observed on day 3 of the experiment in T–EDTA, on day 4 in T and T–EG, and on day 5 in T–BSA. As we can see, the addition of T–BSA to the chilled dog semen allowed the preservation of a significantly higher ($P < 0.05$) average percentage of spermatozoa with straight–line movement, compared with the other extenders, until day 6 of storage. At the same time, on day 10 of storage in the BSA extender, the spermatozoa with straight-line movement accounted for 31.2 ± 6.6% of ($P < 0.05$), which was significantly higher compared with the other tested extenders. We should note that starting from day 5 of storage, the spermatozoa diluted in T–EG and T–EDTA were observed to have agglutination sites at a level +, which increased up to a level ++ by day 10. In the T and T–BSA samples, no agglutination was observed throughout the study.

Long-term storage of chilled spermatozoa led to decrease in the density of their plasmatic membrane with time ($P < 0.05$, Table 4). The average percentage of HOS-positive spermatozoa in the native semen accounted for 92.7 ± 3.1%, whereas centrifugation and chilling led to a 0.5% decrease in this parameter in all the studied groups. It has to be noted that over the 10 day storage of the samples, the mean percentage of spermatozoa with a functionally integral membrane decreased by 13.4–16.0% ($P < 0.01$, compared with day 0). Nonetheless, we observed no significant difference in the effects of different extenders on the integrity of the plasmatic membrane of canine spermatozoa.

Table 4
Changes in the integrity of the canine spermatozoon membrane
in the semen extenders of different compositions (HOS-positive spermatozoa (functionally integral membrane), %, x ± SE, n = 5)

Extender	Day										
	0	1	2	3	4	5	6	7	8	9	10
T	92.2 ± 3.7	91.6 ± 4.8	91.4 ± 3.4	91.2 ± 3.4	91.0 ± 3.7	90.2 ± 3.7	88.8 ± 3.4	86.2 ± 4.2	84.0 ± 3.2**	81.0 ± 3.9**	78.0 ± 3.4***
T–EG	92.2 ± 3.0	91.8 ± 3.7	91.4 ± 4.4	91.2 ± 3.8	90.8 ± 3.2	90.2 ± 4.0	87.8 ± 4.5	85.8 ± 3.8*	82.8 ± 3.1**	79.8 ± 3.7***	77.8 ± 4.1***
T–BSA	92.2 ± 3.7	91.6 ± 4.3	91.4 ± 3.9	91.4 ± 3.7	91.0 ± 3.5	90.4 ± 4.0	88.8 ± 4.2	86.4 ± 4.3	84.4 ± 3.2*	81.6 ± 3.4**	78.8 ± 4.7**
T–EDTA	92.2 ± 3.0	91.4 ± 3.0	90.6 ± 2.7	89.4 ± 3.4	88.6 ± 3.4	87.6 ± 4.3	85.2 ± 5.0	83.0 ± 3.1 **	81.0 ± 3.2***	78.4 ± 3.4***	76.2 ± 3.2***

Note: see Table 2.

Table 5
Changes in the mean percentage of the spermatozoa with fragmented DNA (%) over storage
in the semen extenders of different compositions (x ± SE, n = 5)

Extender	Day										
	0	1	2	3	4	5	6	7	8	9	10
T	5.6 ± 2.1	5.6 ± 1.5	5.6 ± 1.3	5.6 ± 2.2	5.6 ± 2.1	5.6 ± 2.4	5.6 ± 1.0	5.8 ± 1.7	5.8 ± 1.3	6.0 ± 2.2	6.0 ± 1.1
T–EG	5.6 ± 1.8	5.6 ± 1.8	5.6 ± 1.2	5.6 ± 2.1	5.6 ± 2.2	5.6 ± 2.2	5.8 ± 2.1	5.8 ± 2.3	6.0 ± 2.6	6.0 ± 2.5	6.0 ± 2.0
T–BSA	5.6 ± 2.1	5.6 ± 2.0	5.6 ± 1.8	5.6 ± 2.1	5.6 ± 2.1	5.6 ± 2.2	5.6 ± 1.9	5.6 ± 2.2	5.8 ± 1.9	5.8 ± 2.1	5.8 ± 2.3
T–EDTA	5.6 ± 1.8	5.6 ± 2.4	5.6 ± 2.5	5.6 ± 2.2	5.6 ± 2.0	5.6 ± 2.1	5.8 ± 2.0	5.8 ± 2.3	6.0 ± 2.0	6.0 ± 2.4	6.0 ± 2.0

Note: see Table 2.

The results of determining the integrity of DNA of the spermatozoa after dilution are presented in Table 5. Diluting and chilling had no effect on the percentage of DNA fragmentation of the canine gametes. It should be noted that the percentage of spermatozoa with damaged DNA did not vary among the extenders. Nonetheless, in the dynamics of 10 day storage, there was observed an insignificant increase in the level of DNA fragmentation (up to 0.4%), although those changes did not achieve statistical significance ($P > 0.05$) and had no practical effect on the general assessment of sperm quality.

Discussion

The objective of chilling semen is storing gametes at low temperatures without reaching the chilling point that causes intracellular changes affecting their vitality and fertilization capacity (Linde–Forsberg, 1991; Martínez-Barbitta & Rivera Salinas, 2022). This method is a good alternative to regular cryoconservation that can be easily adapted for clinical use. In recent decades, the survival of spermatozoa during chilling was expanded from 2–3 to 10–14 days; nevertheless, the main limitation of the technique has been critical deteriorateon of the quality of sperm doses over storage (Martínez–Barbitta & Rivera Salinas, 2022). That is why the development of alternative extenders for canine semen is still important, as confirmed by the myriad studies focused on improvement of the existing or development of new combinations of extenders (Prete et al., 2018; Divar et al., 2022; Suzuki et al., 2022).

Canine spermatozoa are cells that have an ability to survive in the reproductive pathways of a female for over 10 days, which is due to the physiology of the species' reproductive system: a female dog has a long estrous cycle (up to 9 days), while the sexual receptivity is not always synchronous with ovulation (up to 10 days from ejaculation to fertilization) (Bucci et al., 2023). For a successful fertilization, dog spermatozoa must remain live and retain a functional activity over a long time, which differentiates them from male gametes of other mammals (Foutouhi & Meyers, 2022). However, once completely mature, spermatozoa lose the capacity to biosynthesize, recover, grow, divide, with only metabolic processes occurring in them, thus becoming completely dependent on the environment after exit from the epididymis (Contri et al., 2013). That is why they have developed various metabolic strategies: high ability to metabolize glucose (Fernández-Novell et al., 2004), metabolic plasticity (Birkhead & Møller, 1993; Bucci et al., 2010), activation of functional metabolism of glycogen (Ballester et al., 2000) that is necessary to achieve capacitation in the *in vitro* conditions (Albarracín et al., 2004), high ability to intensify their oxidative metabolism, and the ability to provide utilization of hexose in certain way (Foutouhi & Meyers, 2022). However, to support their functions after ejaculation, spermatozoa require exogenous substrates that allow them to maintain intracellular energy reserves, cellular components, and most importantly their motility (Songsasen et al., 2002).

In the experiment and analyses, we used the second fraction of canine ejaculate, parameters of which from all the examined animals remained within the norm (Kustritz, 2007) and were consistent with the data of other researchers (Prinosilova et al., 2012; Hermansson et al., 2021; Teixeira et al., 2022). Taking into account that fertilization of female canines requires no less than 150×106 spermatozoa (Linde-Forsberg, 1991), each studied smear contained this particular concentration of gametes. The pH of the second fraction of ejaculate from the examined animals ranged 6.1–6.2, which correlates with the data of other researchers (Lee et al., 2013; Martínez-Barbitta & Rivera Salinas, 2022). It is worth noting that in cases of non-fraction dog semen, this parameter ranged 6.4 to 6.8 (Kustritz, 2007; Lee et al., 2013). During long storage, semen plasma is replaced with extender. The effects of pH on the characteristics of spermatozoa have been reported (Carr et al., 1985), although no study has described those effects comprehensively. Therefore, in this study, we used the TRIS buffer system, which provided a pH of solvent within 6.2–6.5, which corresponds to the physiological parameters of canine ejaculate.

The functioning of all live cells is an energy-dependent process and sexual gametes are no exception. The main functions of spermatozoa are provided by two metabolic pathways: oxidative phosphorylation and glycosis (du Plessis et al., 2015). The main sources of energy for spermatozoa are considered to be fructose and glucose (Bustani & Baiee, 2021). Besides energy function, sugars support the osmotic pressure and perform cryoprotective action (Yildiz et al., 2000). Their inclusion in extenders helps preserve the motility of spermatozoa, supports their vitality, and preserves the integrity of their acrosome and plasmatic membranes (Yildiz et al., 2000; Rigau et al., 2001). It has to be noted that the reaction of male gametes to different energy substrates or their absence varies depending on species (Serrano et al., 2025). That is why there are conflicting opinions on what sugar is best to use in the content of a canine semen extender (Ponglowhapan et al., 2004, Flores et al., 2010). The most popular extender for chilled canine semen is egg yolk-TRIS-fructose/glucose (Lopes et al., 2009). At the same time, a growing number of studies indicate that for a canine semen extender, it is best to use glucose. Thus, according to the data of Ponglowhapan et al. (2004), consumption of glucose by canine sperm is higher than fructose, in cases of equal presence of both monosaccharids in the medium, which indicates that spermatozoa use glucose rather than fructose. Fernández-Novell et al. (2004) in the study of canine sperm observed that glucose (not fructose) can activete protein kinase B (Akt) in a specific way, directly affecting the regulated Akt pathways, which indicates its action not only as an energy substrate, but also as a direct modulator of the functions. Rahman et al. (2017) reported that extenders that contained glucose decreased the level of active oxygen species more effectively compared with

other monosaccharides. Flores et al. (2010) suggested that glucose provides a better protection of the spermatozoon membrane than fructose. Furthermore, Verstegen et al. (2005) noted a clear advantage of using glucose in an extender, compared with fructose, highlighting the preservation of motility. Despite a high number of publications that declare the necessity of adding monosaccharides to the composition of canine semen extender, there is no unanimous opinion regarding their concentration, which ranges 90 to 2,160 mg/cm^3 (Ponglowhapan et al., 2004; Goericke-Pesch et al., 2012; Prete et al., 2018). Considering the aforementioned, the extenders used in the present study included glucose. Likewise, its concentration was 4,900 g/100 cm^3, which significantly exceeds the amount mentioned by other authors. The reason for this was that our previous studies (unpublished data) have indicated that such a content of sugar provides maximum motility of spermatozoa over a long time, compared with lower concentrations. Thus, the tested base medium T (TRIS + citric acid + glucose) allowed preservation not only of the general motility of the spermatozoa but also their ability to move in a straight line for 10 days, while the permeability of the membrane and the level of DNA fragmentation remained at insufficient levels, which greatly improves the fertilizing value of sperm doses.

Using extenders for chilling canine semen is a direction that is being actively developed and there are no unanimous data regarding the effects of different compounds on the preservation of the functions and the fertilizing ability of gametes over storage. That is why in our study, we chose components that are used for cryoconservation and produce a confirmed positive effect.

As a component of semen extenders, egg yolk has been used since 1939 (Phillips, 1939). To this day, extenders based on TRIS-citric-acid-fructose or glucose and supplemented with 20% egg yolk continue to be the ones most commonly used for storage of chilled canine semen (Nguyen et al., 2019). The use of egg yolk is often justified by the ability to prevent the formation of active oxygen species (Schäfer-Somi et al., 2021) because of a complex interaction among the fraction of low-density lipoproteins, proteins of semen plasma, and the plasmatic membrane of spermatozoon, which prevents the loss of membrane phospholipids and anions (Thérien et al., 1999; Bergeron et al., 2004; Layek et al., 2016). It is also considered a source of long-chain polysaturated fatty acids (Simopoulos & Salem, 1992), lipids, proteins, carbohydrates, and minerals (Raheja et al., 2018).

In the present study, we observed a significant decrease in the sperm motility in the T–EG medium (TRIS+citric acid+glucose+egg yolk) from day 5 of storage until the complete disappearance on day 9, while the straight–line movement disappeared by day 9 starting from day 4, which is consistent with the data yielded by Sumayya et al. (2024). However, Verstegen et al. (2005) indicate that canine spermatozoa are able to retain motility for up to 16 days, and the observed differences can be related to the differences in the chemical composition of egg yolk and its interaction with high levels of glucose. It has to be noted that the T extender was significantly better in preserving those parameters, which can be attributed to the multi-component composition of the studied extenders and a complex effect of the components on the sensitivity of canine spermatozoa (Songsasen et al., 2002). Perhaps, in a medium supplemented with egg yolk, spermatozoa metabolism intensifies, entailing increases in the level of active oxygen species and absorption of energy, decline in pH, and increase in the concentration of lactic acid (Verstegen et al., 2005; Kasimanickam et al., 2012).

Due to a number of disadvantages of egg yolk–based extenders, such as broad composition variability, risks of transmission of diseases or bacteria, and complication of microscopic assessment of sperm quality (Bustani & Baiee, 2021), there is a need to search for alternatives. There are studies suggesting the use of bovine serum albumin (BSA) instead of egg yolk in the composition of extenders for chilled semen, pointing out their advantages. Thus, Sandal et al. (2020), while studying sperm of Saanen buck, observed a positive effect on the motility and survival of spermatozoa from addition of albumin to the extender. Álvarez-Rodríguez et al. (2024) reported that inclusion of bovine serum albumin in the medium for thawing hog semen improved the membrane quality and the acrosome integrity, although it had no effect on the motility of gametes. At the same time, Risopatrón et al. (2002) indicate that BSA – regardless of concentration – presserves the vitality and the function of canine sperm prepared for procedures in the in vitro conditions by short incubation. Positive results, such as decline in the ratio of cholesterol and phospholipids in the external acrosomal membrane and stimulation of hyperactivity of spermatozoa (Uysal et al., 2007), obviously, are evidences of a favorable effect of using BSA as an additive to semen extenders. In addition, it binds with calcium ions, increasing the regulation of entry and exit of Ca^{2+}, which helps prevent harmful accumulation of intracellular Ca^{2+} (Zhang et al., 2015). We should also note the antioxidant activity of BSA, which may be related to its distinctive fast absorption on to the sperm membrane during storage (Blank et al., 1976) through a specific interaction with phospholipids of the plasmatic membrane, which prevents contact with potential oxidative agents and protects sperm from temperature fluctuations (Matsuoka et al., 2006; Zhang et al., 2015). The results of our experimental studies confirmed the protective role of BSA and are in agreement with the observations of the above-mentioned authors. Thus, by adding the base diluent BSA, the motility of spermatozoa and their ability to move in a straight line was preserved for 10 days (37.4 ± 5.9% and 31.2 ± 6.6%, respectively), at a level that was significantly higher compared with the other tested extenders.

Another alternative is EDTA. As is known, processes of chilling and freezing promote changes in the ability of spermatozoa to regulate the influx and outflow of Ca^{2+} (Bailey et al., 2000), which stimulates the capacitation of spermatozoa and causes early acrosomal reaction (Cormier & Bailey, 2003). Ethylenediaminetetraacetic acid is able to decrease the Ca^{2+} level, chelating it (Deco-Souza et al., 2020). Therefore, we may assume that by chelating calcium in the extender, the acrosomal reaction can be decreased (Mahi & Yanagimachi, 1978), thereby prolonging the life span of spermatozoa. However, in this study, the addition of EDTA to the extender did not produce the expected outcomes, since the percentage of mobile spermatozoa and those with straight–line movement began to rapidly plummet starting from day 4 of storage ($P < 0.001$), indicating the negative effect of depletion of extracellular calcium on canine spermatozoa.

In our study, we noted the gradual decline in the mean percentage of HOST-sensitive spermatozoa over the assessment period, regardless of the extender composition. The yielded data correlate with such presented by Yu (2006), who reported that the functional integrity of the canine spermatozoon membrane until day 5 of storage remained close to 85%, and then declined almost linearly, approximately by 2%, down to 65% on day 14. Ponglowhapan et al. (2004) confirmed those data and stated that the integrity of the plasmatic membrane deteriorated over time of storage (mean percentage of spermatozoa with damaged membrane equaled 32.6 ± 5.7 on days 1–3, 40.7 ± 4.1 on days 4–8, 54.2 ± 6.7 on days 10–14, and 76.1 ± 5.7 on days 17–23), while neither type nor concentration of sugar exerted a protective effect on the integrity of the plasmatic membrane.

At the same time, the percentage of spermatozoa with fragmented DNA in our study did not change significantly. The obtained data are consistent with the results of Prinosilova et al. (2012), Bencharif et al. (2013), and Puja et al. (2018), who reported that chilling of canine semen cannot cause significant changes in the DNA integrity. However, Sumayya et al. (2024) reported insignificant denaturation of the sperm DNA over storage, which cannot affect the fertilizing ability of sperm dose.

Conclusion

The composition of the extender significantly affects the preservation of the functional activity of chilled canine spermatozoa. The best results were produced by T–BSA, which provided the preservation of gametes' motility and straight–line movement of over 30% until day 10 of storage at 4 °C. Addition of egg yolk (T–EG) and EDTA (T–EDTA) was observed to be less effective compared with the base medium, indicating their limited applicability in extenders for long storage. The parameters of DNA fragmentation underwent no significant changes across the groups, remaining stable within 10 days. We ob-

served no significant difference in the effects of different extenders on the integrity of the plasmatic membrane of the canine spermatozoa, although over storage this parameter increased. This indicates that storage duration was a much greater factor affecting the integrity of the membranes than the composition of the extender. Thus, T–BSA is an optimal extender for storing chilled canine semen for a period of up to 10 days.

The authors declare no conflict of interest.

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