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Original article

Evaluation of the efficacy of different semen extenders for chilled storage of Aseel rooster sperm

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Abstract

Semen extenders are pivotal in maintaining the integrity and quality of avian sperm throughout the storage period. In this study, we evaluated the effectiveness of three commonly used extenders, Lake (LAKE), Beltsville Poultry Semen Extender (BPSE), and EK extender, in the storage of Aseel rooster semen at 5°C for 48 hours. The control group was provided with Lactated Ringer's solution. Spermatological analyses were conducted at 0, 24, and 48 hours to evaluate the time-dependent effects of these extenders on sperm motility, viability, morphological changes, mitochondrial function, membrane functionality, and both acrosome and plasma membrane integrity in sperm cells. The results indicated that the BPSE and EK extenders exhibited higher total and progressive motility rates compared to the control and LAKE extenders at 24 and 48 hours. Additionally, morphological damage rates were lower in the EK and BPSE extenders than in the LAKE extender at these time points. Mitochondrial activity was higher in the EK extender than in the LAKE extender at 24 hours. Acrosome integrity and plasma membrane integrity rates declined over time; however, they remained similar among all groups at corresponding time points. In conclusion, BPSE and EK extenders, characterized by higher fructose content and greater osmolality, were found to be more effective in preserving the sperm quality of Aseel rooster semen during cold storage compared to the LAKE extender.

Keywords: poultry sperm, semen extenders, semen storage

Introduction

Reproductive biotechnology has become increasingly important in the modern era of agriculture and food production, particularly in meeting the ever-growing demand for meat worldwide. Along with increasing productivity, there is a need to preserve the numbers of wild avian species or endangered local species, and short- or long-term storage of semen is carried out for this purpose. While many advances have been made in the field of semen preservation, poultry semen remains more challenging to preserve compared to semen from other farm animals such as bulls. This has led researchers to focus on developing different extenders and additives to protect rooster semen during storage and transportation (Ciftci and Aygün 2018).

During the cooling process, the low temperature has an adverse effect on the structural, biochemical, and functional properties of spermatozoa, leading to decreased motility, vitality and morphological integrity (Ak et al. 2010). Therefore, diluents containing appropriate cryoprotectants, osmotic pressure, and pH values must be used to protect rooster sperm from cold shock during cooling. Semen extenders are specifically formulated to augment semen volume to an ideal level, ensuring the prolonged preservation of spermatozoa vitality. These extenders must contain essential chemicals required for the intracellular functions of spermatozoa while also eliminating any metabolites that may be formed in the environment (Alkan et al. 1997).

Rooster semen is denser, more viscous, and lower in volume than semen from other farm animals, and it is therefore necessary to dilute the semen promptly after collection to avoid damage due to external factors (Lake and Stewart 1978). Successful and widespread artificial insemination practices in poultry depend on the development of species-specific extenders and semen storage methods (Mian et al. 1990). Semen extenders are essential in preserving the viability of spermatozoa throughout the processes of semen storage and transportation. These extenders are typically buffered salt solutions designed to increase semen volume, which enables the insemination of a larger number of females and reduces the cost of male maintenance for breeders. Diluting the semen also ensures homogeneous distribution of spermatozoa (Mian et al. 1990, Vasícek et al. 2015).

To achieve optimal results, semen extenders should imitate the concentrations of electrolytes, energizers, and other substances present in the semen of the target species. Glutamic acid, recognized as the primary anionic constituent in poultry seminal plasma, has been established as a standard ingredient in semen extenders (Lake and Mc Indoe 1959, Lake 1995). Furthermore, a range of additives are incorporated into semen extenders to preserve spermatozoa motility, fertilizing ability, and membrane integrity (Sarlos et al. 2002, Riha et al. 2006). Therefore, the design and formulation of semen extenders are critical for the success and spread of artificial insemination practices in poultry.

Short-term storage of poultry semen (up to 4 hours) can be conducted at moderate temperatures (25°C) without significant deterioration in sperm quality. However, for long-term storage (up to 24 hours), particularly for use in artificial insemination programs and to maintain sperm physiology, storage at 5°C is recommended (Blank et al. 2021). It has been reported that the fertilization potential of rooster sperm can be maintained in vitro at 5°C for 24 to 48 hours. (Partyka and Niżański 2022). However, due to time-dependent losses in sperm membrane lipid composition (Zaniboni and Cerolini 2009), sperm viability declines, leading to reduced reproductive performance after 6 hours (Sexton and Fewlass 1978).

One of the popular ornamental chicken breeds is the Aseel, which is famous for its fighting structure, well-developed muscular body formations, and endurance. The Aseel rooster was brought to Anatolia and adapted to the region during the Ottoman Empire period, making it an important part of Turkey's cultural heritage. Therefore, it is crucial to reveal and preserve the spermatological characteristics of this species for the ornamental poultry sector (Günay et al. 2023).

In the current literature, poultry sperm storage studies have primarily focused on a limited number of variables, such as sperm motility, fertility or morphology (Blank et al. 2021). In this study, an objective investigation was conducted to evaluate the effects of different extender compositions (LAKE, BPSE, and EK) and cold storage durations (0, 24, and 48 hours) on multiple characteristics of chicken spermatozoa, including sperm motility, acrosome and membrane integrity, viability, mitochondrial activity and morphology. This study aims to advance the development of efficacious semen preservation techniques for Aseel roosters, potentially influencing semen storage methods for other ornamental chicken breeds.

Materials and Methods

Ethics statement

The Permission for the study was approved by the Istanbul University-Cerrahpaşa Animal Experiments Local Ethics Committee (2022/41).

Animal subjects and semen collection

The study was conducted at the Department of Reproduction and Artificial Insemination, Istanbul University-Cerrahpaşa Faculty of Veterinary Medicine. The experimental subjects consisted of ten Aseel breed roosters, each two years old, with body weights ranging from 2.1 to 3.4 kg, displaying the characteristic phenotypic traits of the breed. Each rooster was housed individually in a separate cage (1 m length × 75 cm width × 75 cm height) and provided ad libitum access to commercial poultry feed.

Semen collection was performed using the abdominal massage technique, allowing the roosters to acclimate to the procedure. Semen collection was carried out during the autumn season (September–October) when the ambient temperature ranged between 20-25°C. The procedures were conducted twice a week at 9 a.m. over a five-week period (n=10). Each semen sample was collected in 1.5 mL microcentrifuge tubes and transported to the laboratory within 5 minutes for further analysis.

Extender composition

The chemicals used in this study were obtained from Sigma Chemical Co. (Saint Louis, MO, USA).

In this study, Lactated Ringer's solution was used as the control diluent. The other diluents used were as follows:

LAKE extender composition:

The LAKE extender (Lake 1960) comprised 100 mL of ultrapure water, 1.35 g sodium glutamate (Cat. No: G5889), 0.128 g potassium citrate × H_2O (Cat. No: C3029), 0.51 g sodium acetate (Cat. No: S8750), 0.08 g magnesium acetate × $4H_2O$ (Cat. No: M0631), and 0.8 g glucose (Cat. No: G6152). The solution's pH was calibrated to 7.20, with an osmolality of 310 mOsm/kg.

EK extender composition:

The EK extender formulation (Lukaszewicz 2002) included 100 mL of ultrapure water, 1.4 g sodium glutamate (Cat. No: G5889), 0.14 g potassium citrate × H_2O (Cat. No: C3029), 0.7 g glucose (Cat. No: G6152), 0.2 g D-fructose (Cat. No: F3510), 0.7 g inositol (Cat. No: I7508), 0.02 g protamine sulfate (Cat. No: P3369), 0.1 g polyvinylpyrrolidone (Cat. No: P0930), 0.98 g anhydrous sodium hydrogen phosphate (Cat. No: 1.06559), and 0.21 g anhydrous sodium dihydrogen phosphate (Cat. No: 5.43840). The pH of the solution was adjusted to 7.30, and its osmolality was measured at 390 mOsm/kg.

BPSE extender composition:

The BPSE extender (Sexton 1977) consisted of 100 mL ultrapure water, 0.867 g sodium glutamate (Cat. No: G5889), 0.064 g potassium citrate × $3H_2O$ (Cat. No: C3029), 0.50 g D-fructose (Cat. No: F3510), 0.195 g TES (Cat. No: 1.10695), 1.270 g potassium diphosphate × $3H_2O$ (Cat. No: P5504), 0.065 g potassium monophosphate (Cat. No: P5655), 0.430 g anhydrous sodium acetate (Cat. No: S8750) and 0.034 g magnesium chloride (Cat. No: M2670). The pH of this solution was adjusted to 7.50, with an osmolality of 333 mOsm/kg.

Fresh sperm evaluation, dilution and cooling

Each rooster's ejaculate was individually analyzed for spermatological parameters. Only samples with a total motility rate of at least 80%, a concentration of 2–3 × 10^9, and a mass activity of at least 3 out of 5 were selected for pooling. The pooled sperm was divided into four equal volumes and assigned to groups according to the extenders used (1:5, v/v): EK, LAKE, BPSE extenders, and lactated Ringer's solution. Subsequently, the diluted semen samples were cooled in a water bath inside a refrigerator set at +5°C for approximately 2 hours.

Sperm Quality Assessments

Spermatological analysis was performed at 0 (immediately after cooling), 24, and 48 hours following the incubation period.

Computer-assisted semen analysis (CASA)

The CASA (SCA®, Sperm Class Analyser, Microptic SL, Barcelona) analysis, including total and progressive motility, was conducted at three time points: 0, 24, and 48 hours of cold storage. In brief, 5 µL of each semen sample was placed on a prewarmed glass slide, covered with a coverslip, and examined under standard settings (Nguyen et al. 2015), with 600–800 spermatozoa analyzed per sample. The percentage of motile sperm was defined as the proportion of spermatozoa exhibiting an average path velocity (VAP) > 5 µm/s, while progressive cells were classified as those with a VAP > 50 µm/s and straightness index (STR) > 75%. The CASA system settings were as follows: Area (µm²) (min): 5, Area (µm²) (max): 170, VAP points (pixels): 5, and Connectivity (pixels): 18.

Assessment of sperm viability and morphology

To determine the proportions of viable, dead, and abnormal spermatozoa, eosin-nigrosine staining was

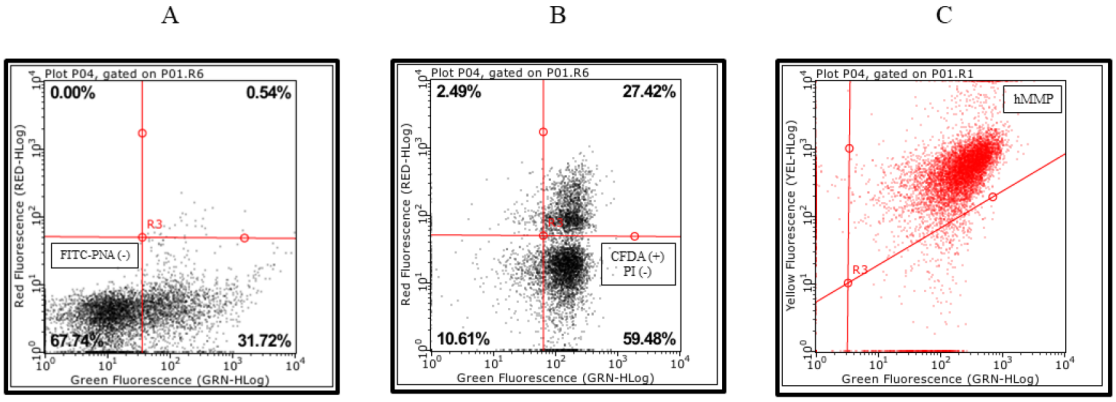

Fig. 1. Sample data plot graphs of flow cytometry analyses: **(A)** acrosome integrity, spermatozoa that did not exhibit green fluorescence [FITC-PNA (-)] were classified as having intact acrosomes; **(B)** membrane integrity, spermatozoa exhibiting green fluorescence without red fluorescence stimulation [CFDA (+), PI (-)] were classified as viable and possessing an intact plasma membrane; and **(C)** mitochondrial activity, spermatozoa exhibiting orange fluorescence were classified as having high mitochondrial activity.

used (Lukaszewicz 1988). A drop of semen was placed on a prewarmed (37°C), clean slide and mixed with a blend of one drop of 5% eosin and two drops of 10% nigrosine stain. The slides were prepared, air-dried, and then examined under a microscope (Eclipse Ni-U, Nikon, Tokyo, Japan) at 400× magnification. In each sample, 100 spermatozoa were analyzed. Spermatozoa that were either fully or partially stained were classified as dead. The proportion of spermatozoa with morphological abnormalities was calculated by counting a total of 100 spermatozoa and identifying visible deformities in their head, neck, midpiece, and tail regions.

Hypo-osmotic swelling test

The functionality of spermatozoa membranes was assessed using the hypo-osmotic swelling test (HOST), following the methodology described by Zhang et al. (2022). In this procedure, 25 µL of the semen sample was mixed with 975 µL of a HOST solution (100 mOsm/kg) containing 57.6 mM fructose and 19.2 mM sodium citrate. The mixture was then incubated at 37°C for 30 minutes. After incubation, 5 µL of the solution was placed onto a preheated (37°C) slide and covered with a coverslip. A total of 200 spermatozoa were examined under a phase-contrast microscope at 100× magnification using immersion oil. Spermatozoa displaying a curved tail, swollen head, or spiral-like form were classified as positive in the HOST.

Spermatological parameters examined by flow cytometry

Evaluation of acrosome integrity

Acrosome integrity was assessed using fluorescein isothiocyanate-conjugated to Arachis hypogaea (peanut) lectin (FITC-PNA, Cat. No: L7381). Initially, the semen samples were diluted to a concentration of 50×10^6 sperm/mL using the EK extender. Then, 1 µL of FITC-PNA (100 µg/mL) and 200 µl of the EK extender were added to 50 µl of the semen sample. The samples were incubated for 10 minutes in the dark. After incubation, they were analyzed using flow cytometry (Guava easyCyte™, Guava® Technologies, Hayward, CA, USA), with 10,000 cells counted per sample. Spermatozoa that did not exhibit green fluorescence (emission range: 519–590 nm) were classified as having intact acrosomes (Marco-Jiménez et al. 2005).

Assessment of plasma membrane integrity

The protocol was modified based on the staining method described by Câmara et al. (2011). The sperm sample was diluted with an EK solution to achieve a concentration of 50×10^6 sperm/mL. Then, 0.5 µL of carboxyfluorescein diacetate (CFDA, Cat. No: C5041; 0.46 mg/mL stock), 0.5 µL of propidium iodide (PI, Cat. No: 81845; 0.5 mg/mL stock), and 200 µL of an EK solution were sequentially added to 100 µL of diluted semen in the dark.

Flow cytometric analysis was performed by counting 10,000 cells, with excitation wavelengths ranging from 519 to 630 nm. Spermatozoa exhibiting green fluorescence without red fluorescence stimulation ($CFDA^+$, PI^-) were classified as viable and possessing an intact plasma membrane.

Spermatozoa mitochondrial activity

Sperm mitochondrial activity was assessed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, Cat. No: T4069). Initially, semen samples were diluted to a concentration of 50×10^6 sperm/mL using the EK extender. To these samples, 200 µL of EK extender and 0.5 µL of a 3 mM

Table 1. Total and progressive motility data of the Aseel rooster groups detected in cold incubated sperm (n=10).

Group	Total motility (%)			Progressive motility (%)		
	0 h	24 h	48 h	0 h	24 h	48 h
Control	88.33 ± 3.89^{A}	58.47 ± 7.08^{bB}	33.23 ± 5.25^{bC}	46.42 ± 3.86^{abA}	19.99 ± 2.60^{bB}	9.84 ± 1.48^{bC}
LAKE	81.37 ± 6.27^{A}	44.43 ± 8.77^{bB}	21.07 ± 7.03^{bC}	43.78 ± 5.50^{bA}	15.74 ± 3.86^{bB}	4.02 ± 1.13^{bC}
EK	95.42 ± 1.54^{A}	89.15 ± 3.05^{aB}	75.01 ± 4.66^{aC}	55.41 ± 3.80^{abA}	43.39 ± 3.91^{aA}	32.19 ± 5.63^{aB}
BPSE	96.94 ± 1.29^{A}	87.18 ± 2.44^{aB}	68.64 ± 7.05^{aC}	58.65 ± 4.02^{aA}	44.71 ± 3.57^{aA}	27.22 ± 4.56^{aB}

^{ab} Different letters in the same column describe statistical difference ($p<0.05$).
^{ABC} Different letters in the same line indicate that the time-dependent difference is statistically significant ($p<0.05$).

JC-1 stock solution (3 mM JC-1 in DMSO) were added. The samples were then incubated at 38°C in a water bath for 40 minutes (Gillan et al. 2005).

After incubation, flow cytometric analysis was performed, with 10000 cells counted per sample. In the analysis, the sperm population was classified as having high and low mitochondrial membrane potential (MMP) (Dariush et al. 2019). Spermatozoa exhibiting orange fluorescence were categorized as having high mitochondrial membrane potential (hMMP).

Data graphs for all flow cytometry analyses are presented in Fig. 1.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). For comparisons of normally distributed data across groups, one-way analysis of variance (ANOVA) was employed, with Duncan's test used for post hoc analysis. The Kruskal-Wallis test was applied for non-normally distributed data, followed by Tamhane's T2 test for post hoc comparisons.

For statistical evaluations across three different time points, repeated measures ANOVA was used for normally distributed data, whereas the Friedman--Wilcoxon test was applied for non-normally distributed data. The analysis data, classified based on normal and non-normal distribution, are presented below:

Normally distributed data: Progressive motility, viability, membrane integrity, mitochondrial activity, and morphological defect data.

Non-normally distributed data: Total motility, HOST and acrosome integrity.

Results are expressed as mean ± standard error (SE). Differences with p-values <0.05 were considered statistically significant.

Results

The percentages of total motility were evaluated in both the control and experimental groups at three distinct time points: immediately after cooling (0 hour), and at 24 and 48 hours post-cooling. At 0 hour, the analysis revealed no significant difference in total motility between the control and experimental groups ($p>0.05$). However, at 24 and 48 hours, the EK and BPSE extenders exhibited significantly higher total motility compared to the control and LAKE extenders ($p<0.05$). Moreover, total motility values decreased over time in all extenders ($p<0.05$).

When the progressive motility data at 0 hour were evaluated, it was observed that the BPSE extender had the highest success rate, with 58.65 ± 4.02%. The progressive motility rate in the BPSE extender was significantly higher than in the LAKE extender ($p<0.05$), but it was similar to that observed in both the control and EK extenders ($p>0.05$).

At 24 and 48 hours of cold storage, the EK and BPSE extenders were found to better preserve the progressive motility of spermatozoa compared to the control and LAKE extender ($p<0.05$). In the control and LAKE extenders, progressive motility percentages declined sharply over time ($p<0.05$). However, the progressive motility percentages in the EK and BPSE extenders at 24 hours showed no significant change compared to 0 hour ($p>0.05$) (Table 1).

Throughout the various time points of cold storage, no significant differences were observed between the groups in terms of HOST-positive findings ($p>0.05$) (Table 2). However, the percentage of HOST-positive spermatozoa decreased over time in all extenders except for the EK extender ($p<0.05$). Notably, the EK extender maintained a stable HOST-positive rate between 24 and 48 hours ($p>0.05$), whereas the other extenders exhibited a significant decline over time.

Spermatozoa viability data are presented in Table 2. The analysis revealed that the percentage of viable spermatozoa in the LAKE extender was significantly lower than in the other extenders following the cooling process ($p<0.05$). At the 24-hour point, viability rates were similar across all extenders ($p>0.05$). However, by the 48-hour point, the control group exhibited the lowest viability rate ($p<0.05$). Over time, the viability rate in the control, EK, and BPSE extenders showed a declining trend ($p<0.05$). In contrast, the viability rate in the

Table 2. Membrane functionality (HOST+) and sperm viability percentages of the Aseel rooster groups at different cold incubation times (n=10).

Group	HOST+ (%)			Viability (%)		
	0 h	24 h	48 h	0 h	24 h	48 h
Control	93.85±0.82^{A}	89.15±2.29^{B}	81.35±4.91^{C}	71.60±3.70^{abA}	56.10±4.51^{B}	39.70±5.03^{bC}
LAKE	91.70±1.07^{A}	88.45±1.67^{B}	82.95±1.99^{C}	62.30±7.29^{b}	57.40±5.42	48.60±4.90^{ab}
EK	93.20±1.10^{A}	89.70±0.98^{B}	87.75±2.31^{B}	82.60±2.95^{aA}	68.00±3.51^{B}	52.20±4.67^{abC}
BPSE	93.60±1.01^{A}	91.35±0.78^{B}	86.70±2.17^{C}	76.30±2.83^{aA}	65.50±3.35^{B}	57.00±3.72^{aC}

^{ab} Different letters in the same column describe statistical difference ($p<0.05$).
^{ABC} Different letters in the same line indicate that the time-dependent difference is statistically significant ($p<0.05$).

Table 3. Time-dependent regional and total morphological defects of Aseel spermatozoa during chilling (n=10).

Group	Acrosome (%)			Head (%)			Mid-piece (%)			Tail (%)			Total (%)		
	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h
Control	22.50±5.56^{a}	19.70±4.58	17.50±2.91^{ab}	3.30±0.44^{B}	11.70±2.05^{A}	6.20±1.59^{AB}	20.60±3.14^{aB}	37.90±5.72^{aA}	40.80±5.34^{aA}	7.80±2.96^{ab}	11.60±3.23^{ab}	15.40±3.65^{a}	54.20±5.65^{aB}	80.90±6.16^{aA}	79.90±6.12^{aA}
LAKE	17.40±4.47^{ab}	19.60±5.88	22.50±4.54^{a}	3.60±0.58	6.30±1.06	7.60±1.56	17.90±2.65^{aB}	33.30±4.56^{aA}	34.80±3.41^{aA}	8.80±1.71^{ab}	14.70±2.44^{a}	9.70±1.77^{ab}	47.70±3.34^{abB}	73.90±2.87^{aA}	74.60±2.74^{aA}
EK	5.10±1.60^{b}	9.00±2.54	8.00±1.46^{b}	3.30±0.49^{B}	13.90±6.61^{AB}	7.80±1.00^{A}	16.30±3.32^{a}	17.60±2.95^{b}	22.20±2.70^{b}	11.30±2.54^{a}	10.50±3.05^{ab}	14.60±3.43^{a}	36.00±3.49^{bc}	51.00±6.14^{b}	52.60±4.24^{b}
BPSE	15.20±3.98^{ab}	15.60±4.25	11.80±3.06^{b}	4.90±1.26	6.40±0.73	7.50±1.46	7.80±1.73^{bB}	15.80±4.09^{bAB}	18.60±2.56^{bA}	4.10±1.50^{b}	5.00±1.93^{b}	4.80±1.25^{b}	32.00±4.84^{c}	42.80±5.40^{b}	42.70±2.86^{b}

^{abc} Different letters in the same column describe statistical difference ($p<0.05$).
^{AB} Different letters in the same line indicate that the time-dependent difference is statistically significant ($p<0.05$).

Table 4. Acrosome integrity, plasma membrane integrity and high mitochondrial activity data analyzed by flow cytometry at different cold incubation times (n=10).

Group	Acrosome integrity (%)			Plasma membrane integrity (%)			High mitochondrial activity (%)		
	0 h	24 h	48 h	0 h	24 h	48 h	0 h	24 h	48 h
Control	89.05±0.80^{A}	88.12±0.47^{A}	84.89±1.74^{B}	51.85±4.60^{A}	47.41±4.10^{A}	40.38±4.55^{B}	72.38±2.06^{B}	76.11±2.28^{abAB}	80.59±1.92^{A}
LAKE	88.91±0.76^{A}	86.22±0.63^{B}	83.42±1.48^{C}	51.25±3.36^{A}	47.94±2.86^{B}	44.56±2.89^{C}	70.05±1.65^{C}	73.95±1.27^{bB}	80.95±2.61^{A}
EK	88.83±0.88^{A}	86.58±0.76^{B}	83.54±1.21^{C}	51.65±2.93^{A}	46.83±2.91^{B}	45.10±3.07^{C}	73.91±2.98^{C}	80.98±2.14^{aB}	86.24±1.66^{A}
BPSE	88.31±0.73^{A}	85.87±1.68^{B}	82.37±2.60^{C}	46.93±3.07^{A}	42.75±2.89^{B}	36.36±1.82^{C}	73.17±1.78^{C}	78.51±2.20^{abB}	86.65±2.32^{A}

^{ab} Different letters in the same column describe statistical difference ($p<0.05$).
^{ABC} Different letters in the same line indicate that the time-dependent difference is statistically significant ($p<0.05$).

LAKE extender remained stable throughout the study period, showing no significant change ($p>0.05$).

The total morphological damage percentage and the distribution of morphological damage according to localization are shown in Table 3. The percentage of total morphological damage in spermatozoa was significantly lower in the EK and BPSE extenders compared to the control ($p<0.05$). Additionally, no significant variation was observed over time in the rate of total morphological damage within these extenders ($p>0.05$).

The data from flow cytometric analyses are presented in Table 4. Upon completion of the 48-hour cold storage period at 5°C, a significant decline in both acrosome and plasma membrane integrity was observed across all extenders ($p<0.05$). However, no significant differences were detected between extenders in terms of acrosome and membrane integrity ratios ($p>0.05$). Additionally, mitochondrial activity rates significantly increased over time in all extenders ($p<0.05$). Notably, by the 24-hour point, the proportion of spermatozoa exhibiting high mitochondrial activity was significantly greater in semen diluted with EK compared to the LAKE extender ($p<0.05$).

Discussion

Short- and long-term semen preservation can induce detrimental structural and biochemical alterations in spermatozoa. Therefore, the use of appropriate extenders is crucial to establishing a protective environment for spermatozoa during storage (Sharafi et al. 2015). Extenders play a key role in regulating pH and osmolarity levels, which are essential for sperm viability and functionality (Partyka and Nizanski 2022).

In the field of poultry semen preservation, extenders such as LAKE (Lake and Ravie 1984), Beltsville Poultry Semen Extender (BPSE) (Sexton and Fewlass 1978), and EK (Siudzinska and Lukaszewicz 2008)

have received considerable attention. The present study aims to evaluate the effects of these extenders on the cooled storage of semen from Aseel roosters.

This study revealed that total motility, progressive motility and sperm viability decreased in all diluents as the storage time was extended. These findings are consistent with previous research indicating that prolonged semen storage leads to a decline in sperm motility and viability (Lukaszewicz 1988, Blesbois et al. 1999, Antalan et al. 2015, Das et al. 2016, Keerthy et al. 2016, Blank et al. 2021, Burilo and Kashoma 2023).

Notably, reduced sperm motility significantly compromises the fertilization potential of semen, as it directly affects the sperm's ability to navigate through the female reproductive tract and reach the site of fertilization. Moreover, in agreement with Burilo and Kashoma (2023), adverse morphological alterations in spermatozoa were observed over time, likely due to the detrimental effects of storage conditions on sperm structure and functionality.

The measurement of sperm motility is a critical indicator of semen quality, with higher motility rates generally correlating with better fertility outcomes. The results revealed that the BPSE and EK diluents were superior in maintaining higher total and progressive motility rates compared to the other diluents used in the study. Notably, the BPSE diluent exhibited the highest progressive motility at 0 hours of storage, while the EK diluent maintained the highest progressive motility after 48 hours. Conversely, the LAKE diluent showed a significant reduction in motility rates as storage time increased. These findings are consistent with a study investigating the effects of BPSE and LAKE extenders, as well as cold storage duration, on the semen quality of Horasi roosters (Burilo and Kashoma 2023). The composition of the extender, particularly its pH, is a critical factor influencing sperm motility (Donoghue and Wishart 2000) and, in this study, the lower pH of the LAKE diluent may have contributed to the observed decline in motility rates.

Our findings indicated that the LAKE extender exhibited the lowest sperm viability compared to the EK and BPSE extenders, particularly at the 0-hour point. However, viability rates were similar across all extenders at both 24 and 48 hours. Notably, sperm viability in the LAKE extender remained relatively stable over time when compared to the other extenders. A similar trend was observed in Horasi roosters, where BPSE was found to be relatively more effective than LAKE (Burilo and Kashoma 2023).

Numerous studies have established a critical relationship between HOST-positivity and key aspects of sperm functionality, particularly fertilization and cryopreservation potential (Takahashi et al. 1990, Jeyendran et al. 1992, Neild et al. 2000, Perez-Llano et al. 2001, Padrik et al. 2012). In this study, the proportion of HOST-positive spermatozoa decreased over time for all diluents, except for EK. In the case of the EK diluent, HOST-positive rates remained consistent at both 24 and 48 hours. Although no significant difference was observed between the extenders, this finding may potentially be associated with the higher osmolality of the EK extender.

Morphological damage plays a crucial role in determining sperm fertility (Donoghue and Wishart 2000). Our study found that both EK and BPSE extenders were more effective in preventing morphological damage during semen storage compared to the LAKE extender. Notably, no significant difference was observed between the EK and BPSE diluents regarding their ability to prevent morphological damage to spermatozoa. This suggests that both EK and BPSE extenders may be equally suitable for preserving the morphological integrity of spermatozoa during long-term storage.

Sperm motility and fertility are closely linked to the availability of adenosine triphosphate (ATP) and the condition of the mitochondria, underscoring the importance of these factors in reproductive functions (Słowińska et al. 2018). Moreover, mitochondrial activity has been shown to be associated with capacitation in several studies (Medrano et al. 2005, Ramio-Lluch et al. 2011, 2012, 2014, Davila et al. 2016). Blank et al. (2021) reported a trend toward a sharp decrease in the mitochondrial activity index (DAB assay) within 24 hours in rooster sperm diluted with the LAKE extender. However, in the JC-1 examination, time-dependent mitochondrial activation remained unchanged. Conversely, a decrease in mitochondrial membrane potential was observed in turkey sperm within the first 24 hours (Słowińska et al. 2018). In contrast, the present study found an increase in mitochondrial activity over time across all extender groups. This is in contrast to the motility data obtained. Sangani et al. (2017) reported that breed variations in roosters influence sperm mitochondrial ATP levels, mitochondrial ROS production, and mitochondrial electron transport chain complex activity. Therefore, the discrepancies between studies may be attributed to differences in dilution rates and/or breed variations.

In both chickens and turkeys, increases in acrosome reaction values have been linked to the duration of liquid storage, likely due to elevated amidase activity (De las Heras et al. 1996). This finding was corroborated by Lemoine et al. (2011) and Słowińska et al. (2012), who reported that sperm's capacity to undergo the acrosome reaction was significantly higher after 24 hours of liquid storage, even at 4°C. Similarly, Blank et al. (2021) observed a time-dependent decrease in

acrosome integrity in LAKE-diluted semen stored at 37°C, while no significant differences were noted at lower temperatures (5°C and 25°C). In the present study, acrosome and membrane integrity rates declined proportionally with the duration of cold incubation across all diluent groups, although no significant differences were observed among the groups.

In conclusion, this study examined the effects of various extenders on the quality of Aseel rooster semen during storage. Our findings revealed that sperm motility and viability rates were significantly higher in BPSE and EK extenders compared to the LAKE extender. Morphological integrity and mitochondrial activity were also better preserved in BPSE and EK extenders. Based on these results, it can be concluded that high osmolality and energy availability positively influence the spermatological characteristics of Aseel rooster semen during storage. Therefore, BPSE and EK extenders are recommended for the cooling and storage of Aseel rooster semen. Despite these promising findings, further research is needed to assess the impact of these extenders on fertility rates in practical field applications.

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