https://doi.org/10.52973/rcfcv-e362793 Revista Científica, FCV-LUZ / Vol. XXXVI

Effects of different extenders on the short-term storage of Southern Karaman rams semen

Efectos de diferentes diluyentes en el almacenamiento a corto plazo del semen de carneros de la raza Karaman del Sur

Tutku Can Acısu[1]*, Ülkü Gülcihan Şimşek[2], Tülay Canatan Yılmaz[3], Aslıhan Çakır Cihangiroğlu[4], Nida Badıllı[1], Emre Kaya[5], Yasin Baykalır[6]

[1]Fırat University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination. 23119, Elazığ, Türkiye.
[2]Fırat University, Faculty of Veterinary Medicine, Department of Animal Science. 23119, Elazığ, Türkiye.
[3]Bahri Dağdaş International Agricultural Research Institute. 42290, Konya, Türkiye.
[4]Siirt University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination. 56500, Siirt, Türkiye.
[5]Fırat University, Faculty of Veterinary Medicine, Department of Biochemistry. 23119, Elazığ, Türkiye.
[6]Balıkesir University, Faculty of Veterinary Medicine, Department of Biostatistics. 10145, Balıkesir, Türkiye.
**Corresponding author: tcacisu@firat.edu.tr*

ABSTRACT

The conservation of native sheep breeds, the preservation of genetic diversity and the provision of sustainable livestock production are of great importance. The aim of this study was to determine the short-term storage viability of semen collected from Southern Karaman rams, one of Türkiye native sheep breeds at risk of extinction, using two different extenders. A total of six rams were used. Since the animals could not be trained to use an artificial vagina, semen was collected using an electro-ejaculator during the breeding season. The semen samples collected were diluted with an extender containing Tris-egg yolk and a liposome-based extender (OptiXcell®, IMV Technologies, France) and stored at 4°C. Spermatological parameters such as motility, viability and morphological characteristics were evaluated at 0 and 96 hours (h). In addition, some biochemical parameters were examined, primarily changes in malondialdehyde levels, which are considered an important indicator of oxidative stress and antioxidant enzyme levels. The results showed that the type of extender had no statistically significant effect on spermatological parameters. However, significant changes in these parameters were observed during the storage period. The interaction between types of extenders and storage time was not found to be statistically significant. At 96 h, malondialdehyde levels in semen were significantly higher in both extenders compared to 0 h values, while reduced glutathione levels and catalase activity showed a significant decrease. These findings suggest that both extenders can be used for the short-term storage of ram semen without significant differences in spermatological parameters, although storage duration remains a critical factor affecting sperm quality and oxidative stability.

Key words: Extenders; oxidative stress; ram semen; short term storage; Southern Karaman breed

RESUMEN

La conservación de razas ovinas autóctonas, la preservación de la diversidad genética y la producción ganadera sostenible revisten una gran importancia. El objetivo de este estudio fue determinar la viabilidad del almacenamiento a corto plazo del semen colectado de carneros de Karaman del Sur, una de las razas ovinas autóctonas de Turquía en peligro de extinción, utilizando dos diluyentes diferentes. Se utilizaron un total de seis carneros. Dado que no fue posible entrenar a los ejemplares para utilizar una vagina artificial, el semen se colecto utilizando un electroeyaculador durante la temporada de cría. Las muestras de semen colectadas se diluyeron con un diluyente que contenía Tris-yema de huevo y un diluyente basado en liposomas (OptiXcell®, IMV Technologies, Francia) y se almacenaron a 4°C. Se evaluaron parámetros espermatológicos como la motilidad, la viabilidad y las características morfológicas a las 0 y 96 horas (h). Además, se examinaron algunos parámetros bioquímicos, principalmente los cambios en los niveles de malondialdehído, que se consideran un indicador importante del estrés oxidativo, y los niveles de enzimas antioxidantes. Los resultados mostraron que el tipo de diluyente no tuvo un efecto estadísticamente significativo sobre los parámetros espermáticos. Sin embargo, se observaron cambios significativos en estos parámetros durante el período de almacenamiento. No se encontró una interacción estadísticamente significativa entre los tipos de diluyentes y el tiempo de almacenamiento. A las 96 h, los niveles de malondialdehído en el semen fueron significativamente más altos en ambos diluyentes en comparación con los valores de 0 h, mientras que los niveles de glutatión reducido y la actividad de la catalasa mostraron una disminución significativa. Estos hallazgos sugieren que ambos diluyentes pueden utilizarse para el almacenamiento a corto plazo de semen de carnero sin diferencias significativas en los parámetros espermáticos, aunque la duración del almacenamiento sigue siendo un factor crítico que afecta a la calidad del esperma y a la estabilidad oxidativa.

Palabras clave: Diluyentes; estrés oxidativo; semen de carnero; almacenamiento a corto plazo; raza Karaman del Sur

Received: 11/09/2025 Accepted: 07/02/2026 Published: 18/02/2026

INTRODUCTION

The conservation of native sheep (*Ovis aries*) breeds is a fundamental component in preserving genetic diversity and ensuring the long-term sustainability of livestock production systems [1].

Türkiye sheep population comprises diverse local breeds adapted to various ecological zones. Among these, the Southern Karaman breed, which is widespread in the Toros Mountains, represents a distinct genetic group. This breed emerged as a result of the crossbreeding of Karagül rams, brought by Turkmen migrants from Turkistan during the Ottoman period, with the local Akkaraman and Dağlıç sheep in the mountainous regions of Antalya, Mersin, and Adana. The Southern Karaman breed is highly valued not only for its superior meat and milk production but also for its exceptional resistance to environmental stress factors, particularly cold climates. This breed is primarily raised in the Toros Mountains at high altitudes between 2000 and 2500 meters. As a native and resilient breed, Southern Karaman represents an important element of biological diversity [2, 3].

However; the widespread use of commercial breeds and inadequate conservation programs have led to genetic erosion in this breed. Therefore, the development of biotechnological methods is essential for the conservation of the breed [4].

The artificial insemination (AI) of domestic animals is one of the most commonly used assisted reproductive techniques for genetic improvement and development [5]. In sheep breeding, semen must be readily available to inseminate a large number of animals in a short period of time. For this purpose, two methods have been developed for semen preservation. The first method involves the short-term storage of diluted semen in liquid by gradually lowering its temperature to slow down sperm cells' metabolic activity. The second method consists of the long-term storage of semen through cryopreservation at -196°C [6].

In practical breeding applications, the use of cooled ram semen for short-term storage is more prevalent than frozen-thawed semen. Due to the reduced fertility rates associated with cervical insemination using frozen-thawed sperm, research efforts have increasingly focused on optimizing short-term semen preservation techniques [7, 8].

Semen extenders are essential for maintaining both the viability and functional integrity of ram semen during storage. These formulations act by stabilizing the plasma membranes of sperm cells and mitigating the mechanical and chemical stresses encountered during handling and preservation. Among them, Tris-egg yolk-based extenders represent a conventional and widely accepted approach, owing to their lipid and protein constituents that support sperm cell energy metabolism and contribute to membrane protection [9, 10].

In recent years, liposome-based semen extenders such as OptiXcell® have emerged as chemically defined, egg yolk-free alternatives designed to mitigate the risk of microbial contamination and enhance procedural standardization. These extenders have demonstrated encouraging outcomes in species including bulls (*Bos taurus*), buffalo (*Bubalis bubalus*) and ram [11, 12].

However, comparative evaluations in rams have produced variable results. Notably, some studies suggest that conventional Tris-egg yolk extenders may still confer superior preservation of sperm motility and plasma membrane integrity during hypothermic storage, relative to liposome-based formulations such as OptiXcell® [13, 14].

Oxidative stress is one of the main factors causing a decline in sperm quality during both short-term and long-term storage of semen. The unbalance between the formation of reactive oxygen species and antioxidant defense mechanisms leads to lipid peroxidation of the sperm plasma membrane, resulting in decreased motility and viability, increased morphological abnormalities, and reduced fertilization capacity [8, 15].

Among the biomarkers of oxidative damage, malondialdehyde (MDA) is widely used as a reliable indicator of lipid peroxidation levels. Additionally, several antioxidant enzymes such as reduced glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) are critical components of the antioxidant defense mechanisms of sperm cells against oxidative stress and serve as important markers for assessing the oxidative stability of semen [16].

Due to limited data on the oxidative stability of semen from endangered native rams breeds such as the Southern Karaman, the present study aimed to evaluate the preservation potential of two extenders, Tris egg yolk based and liposome based, during both short-term storage of semen by assessing conventional spermatological parameters as well as oxidative stress-related biochemical markers. These findings were expected to contribute to the development of more effective semen preservation strategies for native and endangered breeds.

MATERIAL AND METHODS

Animals and ethical approval

In this study, six clinically healthy Southern Karaman rams, aged 1.5 years, with known fertility and no pathological findings upon genital examination, were used as animal material (FIG. 1).

FIGURE 1. Representative rams of the Southern Karaman breed used in the study

All procedures were carried out according to animal welfare principles. The animals were housed at the Bahri Dağdaş International Agricultural Research Institute, located in Konya, Türkiye, where they were provided with high-quality roughage and concentrate feed. Fresh drinking water was available *ad libitum*.

The approval certificate (30.05.2025/193) was taken from the Local Ethics Committee of the Ministry of Agriculture and Forestry, Bahri Dağdaş International Agricultural Research Institute Directorate.

Extenders

In this study, two different semen extenders were utilized to evaluate the short-term preservation potential of ram semen. The first was a conventional Tris-based formulation enriched with egg yolk, widely recognized for its membrane-protective properties and nutritional support to sperm cells. The second was a commercially available, chemically defined liposome-based extender, developed to minimize the risk of microbial contamination and enhance procedural standardization. The comparative use of these two extenders enabled a comprehensive assessment of their effects on sperm quality under low-temperature storage conditions.

In the present study, the components of the Tris-egg yolk extender consisted of 3.63 grams (g) Tris (tris(hydroxymethyl)aminomethane), 1.99 g citric acid, and 0.5 g glucose dissolved in 100 mL of distilled water. Into this solution, 15 mL of fresh egg yolk, 100,000 IU of Penicillin G and 100 mg of Streptomycin were then added.

The extender was prepared under sterile conditions and used directly for semen dilution. All chemicals used in the preparation of the Tris-egg yolk extender, such as Tris (hydroxymethyl) aminomethane, citric acid, and glucose, were bought from SigmaAldrich (USA).

OptiXcell® (IMV Technologies, France), a commercial liposome-based extender, was used as the second extender in the research. OptiXcell® is an egg yolk-free product with a balanced composition developed to reduce microbial contamination risks and increase repeatability in semen preservation protocols. It contains a composition of phospholipids, antioxidants, and energy substrates encapsulated within liposomes to protect sperm membranes during low-temperature storage [17]. The extender was prepared and used according to the producer's guidelines.

Semen collection and assessment of spermatological characteristics

The research was carried out during the breeding season. Since the rams could not get used to the artificial vagina, semen collection procedure was performed using an electro-ejaculator (Beltron Instruments, USA) in accordance with the described technique. During the procedure, the rams were kept in a designated area under physical restraint, and all animal welfare regulations were strictly observed. In the present study, semen samples were collected using an electro-ejaculator twice per week at two-day intervals over a period of three weeks, and the ejaculates obtained from each individual were subsequently pooled (FIG. 2).

The collected ejaculates were equally divided into two portions. One half was diluted at a 1:1 ratio with the Tris-egg yolk extender, while the other half was diluted with OptiXcell®, a commercially

FIGURE 2. A semen sample collected from a Southern Karaman ram

available semen extender. For short-term storage, routine spermatological evaluations, including volume, concentration, motility, viability, and morphological assessments, were performed. Among these parameters, motility, viability (live-to-dead sperm ratio), and morphological characteristics were reassessed at 96 hours (h) to evaluate changes during storage.

Volume: The volume of the collected semen was recorded in "mL" by reading the graduations on a graduated semen collection tube. This analysis was conducted prior to dilution with semen extenders.

Concentration: Semen concentration was determined using the hemocytometric method (Marienfeld Superior, Germany) [18].

Motility: A clean microscope slide was first placed on a warming stage adjusted to 37°C and allowed to equilibrate. Then, 0.5 mL of either Tris-egg yolk extender or the commercial semen extender OptiXcell® was placed on the slide, followed by 3 µL of semen. The semen was mixed with the extender on the slide and covered with a coverslip. Motility was evaluated under a microscope (Celestron, USA) at 400× magnification. Assessment was performed by observing at least three different microscopic fields, and the average motility was calculated. Results were recorded as percentages ranging from 0 to 100% [18, 19].

Morphological examination (abnormal sperm ratio): Smears were prepared using Indian ink (Schmincke Indian Ink, Germany), a suspension of fine carbon black particles in a water-based binder, to determine the proportion of abnormal sperm cells in the semen samples. The percentage of morphologically abnormal sperm cells was calculated and expressed as a percentage [18, 19].

Viability assessment (live-dead sperm ratio): Semen smears were prepared and stained using Eosin-Nigrosin stain [18]. Sperm cells with heads stained pink were considered non-viable (dead), whereas unstained sperm cells were considered viable (live). The ratio of live to dead sperm cells was then calculated.

Oxidative stress analyzes

Semen samples previously collected from six rams and pooled were used for oxidative stress analyses conducted at 0 and 96 h. The samples were stored at -20°C in a deep freezer (Arçelik, Türkiye) until the analyses were performed. When the time of analysis arrived, the frozen samples were removed from storage and thawed at room temperature.

The thawed ram semen samples were centrifuged (Nüve, Türkiye) to separate the semen extender, which had been added to increase semen volume, maintain sperm viability for a longer period, protect sperm cells from sudden temperature changes, and prevent microbial contamination. Centrifugation was performed at 600 g for 10 min at 4°C. The resulting cellular pellet was washed three times with distilled water. After each wash, centrifugation was repeated at 600 g for 10 min at 4°C, and the pellet was preserved. The final pellet was separated, weighed with precision scales (Sartorius, Germany), and diluted at a ratio of 1:10 (weight/volume) with distilled water. The diluted samples were homogenized using a Bullet Blender Tissue Homogenizer (Next Advance, USA), at speed 10 for 4 min. Stainless steel beads with a diameter of 0.9 to 2.0 mm (SSB14B) were used, in a volume equal to the sample volume.

The homogenate was centrifuged at 3000 g for 15 min at 4°C using a Hettich Mikro 200R centrifuge (Tuttlingen, Germany) for the determination of MDA, GSH, CAT, GST, and SOD levels. For the measurement of GSH-Px activity, the samples were centrifuged at 10000 g for 55 min at 4°C. The resulting supernatants were used in all biochemical analyses.

Malondialdehyde level was measured using the method described by Placer *et al.* [20] which is based on the reaction of thiobarbituric acid with MDA, a lipid peroxidation product. GSH level was determined using the method of Ellman *et al.* [21] a spectrophotometric assay (Thermo Scientific, USA) based on the formation of a yellow-colored product when 5,5'-dithiobis-2-nitrobenzoic acid reacts with sulfhydryl groups. CAT activity was determined by the method of Aebi [22], by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm.

Glutathione peroxidase activity was evaluated using the method of Beutler [23]. In this assay, GSH is oxidized to glutathione disulfide (GSSG) by hydrogen peroxide, and the rate of GSSG formation is measured via the glutathione reductase reaction. GST activity was determined by the method of Habig *et al.* [24] which measures the conjugation of GSH with 1-chloro-2,4-dinitrobenzene at 340 nm at 37°C. SOD activity was assessed based on the method described by Sun *et al.* [25] by quantifying the generation of superoxide anion ($O_2^{\cdot-}$) through the reaction of xanthine and xanthine oxidase with nitroblue tetrazolium. Protein concentration was measured using the Lowry *et al.* method [26].

Statistical analysis

For the analysis of spermatological parameters, data were analyzed using SPSS version 22.0 (IBM Corp., USA). A two-way analysis of variance (ANOVA) was performed to examine the interactive effects of extender and time on various parameters. Effect sizes were calculated for each factor to determine their relative influence on motility, abnormal sperm rate, and sperm viability. Statistical significance was defined as $P<0.05$.

Results were expressed as means ± standard error of the mean (SEM). Descriptive statistics, including minimum and maximum values within 95% confidence intervals, were used to summarize body weight, semen volume, and semen concentration of the rams [27]. For the oxidative stress parameters measured in semen, all statistical analyses were conducted using two-way repeated measures ANOVA in SPSS version 22.

This analysis was performed to evaluate both the between-subjects effects of the diluents and the within-subjects effects of time. Prior to the ANOVA, Mauchly's Test of Sphericity was conducted to assess the assumption of sphericity, and the Epsilon value was calculated. Since the Epsilon value was set to 1.000, the Huynh-Feldt correction was applied to adjust the degrees of freedom and acquire the correct P values. Statistical significance was set at $P<0.05$. Data were presented as mean±standard error of the mean (SEM) [28].

RESULTS AND DISCUSSION

In the present study, the body weights, semen volume, and semen concentration of Southern Karaman rams were determined and shown in TABLE I.

The average body weight was 43.60 kg, and this value varied between 35.89 kg and 51.30 kg. The average semen volume was 0.51 mL, varying from 0.16 mL to 0.87 mL. The mean semen concentration was 1593.33 × 10^6 sperm·mL^{-1}, with observed values ranging between 976.15 and 2210.51 × 10^6 sperm·mL^{-1}. These findings are consistent with previous studies on native sheep breeds [29, 30]. Al-Kawmani *et al.* [31] reported semen volumes ranging from 0.8 to 2.5 mL per ejaculation in rams, with semen concentrations averaging between 2900 × 10^6 and 4010 × 10^6 sperm per mL. Similarly, Gündoğan observed that the semen volume of Akkaraman rams varied from 0.8 to 1.7 mL, with an average of 1.3 mL, and semen concentrations ranging from 2,800 to 3000 × 10^6 sperm·mL^{-1} [32].

In a study conducted using semen from Southern Karaman rams, the reported values for semen volume and concentration were found to be similar to those obtained in the present study. However, the average body weights of the rams used in the current study were observed to be lower than those reported in the previous study [2].

TABLE I
Average values and variability (mean ± SD, 95% CI) of body weight, semen volume and semen concentration in Southern Karaman rams used in the study

Parameters	Mean ± SD	95% CI* Lower – Upper
Body weight (kg)	43.60 ± 7.34	35.89 – 51.30
Semen volume (mL)	0.51 ± 0.33	0.16 – 0.87
Concentration (× 10^6 sperm·mL^{-1})	1593.33 ± 588.10	976.15 – 2210.51

CI: Confidence Interval, SD: Standard deviation, *: 95% CI. The upper and lower range values in the 95% confidence interval are shown

The results of spermatological parameters, such as sperm motility, morphological abnormalities, and sperm viability, obtained in the study are comprehensively presented in TABLE II.

TABLE II
Effects of Tris-egg yolk and OptiXcell® semen extenders and short-term storage (up to 96 hours) on sperm motility, abnormal sperm rate and viability in Southern Karaman rams

Extender \ Parameters	Total Motility (%)	Abnormal Sperm Rate (%)	Sperm Viability (%)
Tris-egg yolk	64.16	5.66	72.41
OptiXcell®	58.33	7.66	64.33
SEM	4.89	0.75	4.24
Hour			
0 h	73.33[a]	4.91[a]	78.25[a]
96 h	49.16[b]	8.41[b]	58.50[b]
SEM	4.89	0.75	4.24
Extender × Hour			
Tris-egg yolk – 0 h	73.33	4.50	78.50
Tris-egg yolk – 96 h	55.00	6.83	66.33
OptiXcell® – 0 h	73.33	5.33	78.00
OptiXcell® – 96 h	43.33	10.00	50.66
SEM	6.92	1.06	5.99
P values			
Extender	0.409	0.075	0.193
Hour	0.002	0.004	0.004
Extender × Hour	0.409	0.286	0.221
Effect size			
Extender	0.034	0.150	0.083
Hour	0.379	0.351	0.352
Extender × Hour	0.034	0.057	0.074

[a, b]: Different letters in the same column indicate differences between groups. SEM: Standard Error Mean, h: hour

The effects of different extenders on short-term semen storage were evaluated. No statistically significant effect of extender type was observed on sperm motility, abnormal sperm percentage, and viability ($P>0.05$). However, all these parameters exhibited significant changes over the storage duration ($P<0.01$), independently of the extender used. Furthermore, no significant interaction was detected between extender type and storage time ($P>0.05$).

Notably, an increase in abnormal sperm percentage was observed concomitant with the progression of storage time, while both sperm motility and viability showed significant declines. Effect size analysis revealed that storage duration exerted the most pronounced influence on all evaluated parameters, whereas the effect of extender type was comparatively limited.

The results of spermatological parameters, such as sperm motility, abnormal sperm morphology, and sperm viability obtained from the present study, are consistent with those reported in a similar investigation where OptiXcell® and egg-yolk-based extenders were used for short-term semen preservation [14]. Another study conducted with ram semen indicated that, although several factors affect semen quality during preservation, storage duration has a more decisive impact than the type of extender used, emphasizing the importance of storage time in maintaining sperm functionality and integrity [33]. In a study conducted by Gündoğan [34], various extenders were evaluated for the short-term storage of ram semen, and the best results were obtained using a Tris-egg-yolk-based extender.

In contrast, the present study did not reveal any statistically significant differences between the extenders used. Moreover, in the same study, sperm motility in all groups declined to below 50% starting from the fifth day of storage. Therefore, in the present study, the short-term preservation of semen obtained from Southern Karaman rams was likewise conducted for a duration of up to 96 h.

Beyond the assessment of semen quality indicators, oxidative stress parameters were further examined to investigate the biochemical effects of the extenders, storage time, and their interaction. The effects of Tris-egg yolk and OptiXcell® extenders and short-term storage for a duration of up to 96 h on oxidative stress parameters in semen samples from Southern Karaman rams are shown in TABLE III.

No statistically significant differences were observed between the two extenders at any of the evaluated time points, specifically at 0 h and 96 h, in terms of MDA and GSH levels, as well as CAT, GSH-Px, GST, and SOD activities ($P>0.05$). At 96 h, MDA levels in semen samples were significantly higher compared to those measured at 0 h in both extenders ($P<0.001$), whereas GSH levels ($P<0.011$) and CAT activities ($P<0.002$) were significantly

TABLE III
Effects of Tris-egg yolk and OptiXcell® semen extenders, and short-term storage (up to 96 hours), on oxidative stress parameters in Southern Karaman ram semen samples

Parameters	Extender	Hour		*P* value	
		0 h	96 h	between-subjects	within-subjects
MDA (nmol·g^{-1})	Tris-egg yolk	0.49 ± 0.01	0.59 ± 0.01	0.725	**< 0.001**
	OptiXcell®	0.50 ± 0.01	0.58 ± 0.01		
GSH (µmol·mL^{-1})	Tris-egg yolk	5.50 ± 0.13	5.27 ± 0.10	0.616	**0.011**
	OptiXcell®	5.44 ± 0.14	5.18 ± 0.04		
CAT (k·g^{-1} protein)	Tris-egg yolk	13.62 ± 0.90	11.45 ± 0.74	0.784	**0.002**
	OptiXcell®	14.18 ± 0.48	11.30 ± 0.54		
GSH-Px (U·g^{-1} protein)	Tris-egg yolk	77.16 ± 3.27	75.56 ± 2.27	0.282	0.145
	OptiXcell®	78.99 ± 2.27	70.61 ± 1.68		
GST (U·mg^{-1} protein)	Tris-egg yolk	20.63 ± 0.77	20.02 ± 0.15	0.498	0.223
	OptiXcell®	20.37 ± 0.36	19.56 ± 0.59		
SOD (U·mg^{-1} protein)	Tris-egg yolk	3.30 ± 0.11	3.44 ± 0.08	0.384	0.604
	OptiXcell®	3.23 ± 0.23	3.28 ± 0.13		

between subjects: Comparisons between extenders, within-subjects: Comparisons between 0 and 96 hours, MDA: malondialdehyde, GSH: reduced glutathione, CAT: catalase, GSH-Px: glutathione peroxidase, GST: glutathione S-transferase, SOD: superoxide dismutase

lower. No significant differences were found in GSH-Px, GST, and SOD activities at either 0 h or 96 h in samples treated with either extender ($P>0.05$).

The absence of statistically significant differences in oxidative stress parameters such as MDA and GSH levels, as well as CAT, GSH-Px, GST, and SOD enzyme activities between the two extenders, suggests that both formulations provide a similar degree of biochemical protection during short-term semen storage at 4°C. MDA is a highly reactive compound formed as an enol and is a byproduct of polyunsaturated fatty acid peroxidation in cells.

The increase in semen MDA levels observed at 96 h in both extenders is considered an indicator of oxidative stress resulting from the oxidative degradation of lipids in the cell membrane. GSH is a tripeptide that protects cells from oxidative stress by neutralizing free radicals and peroxides, particularly by detoxifying harmful molecules such as hydrogen peroxide (H_2O_2). CAT is an antioxidant enzyme that converts H_2O_2 into harmless components, namely water (H_2O) and oxygen (O_2) [35, 36].

The decreases observed at 96 h compared to 0 h in both extenders are thought to have resulted from the consumption of both GSH and CAT in response to oxidative agents. These findings were in agreement with previous studies reporting that, regardless of the extender used, prolonged semen storage leads to oxidative deterioration [37, 38]. This observation is consistent with the findings of Bucak *et al*. and Çoyan *et al*. [39, 40] who demonstrated that even extenders supplemented with antioxidant agents could not fully prevent oxidative stress over extended storage durations.

Similarly, Kulaksız *et al.* [41] found that the addition of myo-inositol to extenders did not significantly alter MDA, CAT, SOD, GSH or GSH-Px levels after long term storage, indicating limited protective efficacy under oxidative conditions. Pasciu *et al.* [42] showed that during 7 h of storage at 15°C, a skim milk-based extender provided a better oxidative balance, with lower reactive oxygen species and higher antioxidant activity, compared to a commercial extender. Moreover, the lack of significant differences in GSH-Px, GST, and SOD activities in the present study may be attributed to their relatively stable expression or delayed responsiveness to oxidative conditions under refrigeration. Collectively, these results underline that while the type of extender may not significantly influence oxidative stress markers during short-term cold storage, the duration of storage is a decisive factor affecting the oxidative stability and overall quality of ram semen [41, 42].

This highlights the need to limit storage duration and further optimize extender compositions, possibly through targeted antioxidant supplementation, to better preserve semen viability, especially in the context of conserving genetic resources such as the Southern Karaman breed.

CONCLUSIONS

The findings showed that the type of extender did not significantly affect spermatological parameters such as motility, abnormal sperm rate, and viability. However, storage duration had a significant negative impact on these parameters, as motility and viability decreased while abnormal sperm rate increased over time. The effect size analysis confirmed that storage time had a significantly greater influence on these parameters than the type of extender used, underscoring the more crucial role of storage duration in determining semen quality.

Regarding oxidative stress markers, no significant differences were found between the two extenders at either 0 h or 96 h in terms of MDA and GSH levels, or CAT, GSH-Px, GST, and SOD activities. Nevertheless, semen samples stored for 96 h exhibited higher MDA levels and lower GSH levels and CAT activity compared to those stored for 0 h, indicating that oxidative stress increased with storage time, regardless of the extender applied.

These results suggest that reducing storage duration is more critical than extender selection for preserving semen quality and limiting oxidative damage under liquid storage conditions. For producers working with Southern Karaman or similar indigenous breeds, using semen within 24 to 48 h of collection is recommended to maintain fertility potential. Additionally, this study may serve as a foundation for future research on the long-term cryopreservation of Southern Karaman ram semen, which remains an underexplored area requiring further investigation.

ACKNOWLEDGEMENTS

We would like to thank Bahri Dağdaş International Agricultural Research Institute Directorate for their valuable contributions to this study.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this article.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable requests.

Author Contributions

Tutku Can ACISU: Conceptualization, supervision, methodology, writing-original draft preparation, review, and editing. Tülay CANATAN YILMAZ, Aslıhan ÇAKIR CİHANGİROĞLU and Nida BADILLI: Methodology, data curation. Ülkü Gülcihan ŞİMŞEK, Emre KAYA and Yasin BAYKALIR: Methodology, conceptualization, and validation.

BIBLIOGRAPHIC REFERENCES

[1] Food and Agriculture Organization of the United Nations (FAO). The Second Report on the State of the World's Animal Genetic Resources for Food and Agriculture [Internet]. Rome (Italy): FAO Commission on Genetic Resources for Food and Agriculture Assessments; 2015 [cited Sep 3, 2025]; 606 p. Available in: https://goo.su/lWDVDmu

[2] Acısu TC, Yuksel E, Canatan-Yılmaz T, Çakır-Cihangiroğlu A, Badıllı N, Baykalır Y, Kaya E, Şimşek UG. Determination of andrological and spermatological parameters in Southern Karaman breed rams under threat of extinction. Open Vet. J. [Internet]. 2025; 15(8):3711–3718. doi: https://doi.org/qrc3

[3] Tüney-Bebek D, Keskin M. Some reproductive, fattening and milk yield traits of South Karaman sheep. MKU. Tar. Bil. Derg. [Internet]. 2021; 26(1):1–7. doi: https://doi.org/qrc5

[4] Aydin KB, Bi Y, Brito LF, Ulutaş Z, Morota G. Review of sheep breeding and genetic research in Türkiye. Front. Genet. [Internet]. 2024; 15:1308113. doi: https://doi.org/pcsx

[5] Kukovics S, Gyökér E, Németh T, Gergatz E. Artificial insemination of sheep – possibilities, realities and techniques at the farm level. In: Manafi M, editor. Artificial insemination in farm animals [Internet] Rijeka (Croatia): IntechOpen; 2011. doi: https://doi.org/qrc6

[6] Gibbons AE, Fernandez J, Bruno-Galarraga MM, Spinelli MV, Cueto MI. Technical recommendations for artificial insemination in sheep. Anim. Reprod. [Internet]. 2019; 16(4):803–809. doi: https://doi.org/qrc7

[7] O'Hara L, Hanrahan JP, Richardson L, Donovan A, Fair S, Evans ACO, Lonergan P. Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. Theriogenology [Internet]. 2010; 73(4):541–549. doi: https://doi.org/ddkrpf

[8] Rizkallah N, Chambers CG, De Graaf SP, Rickard JP. Factors affecting the survival of ram spermatozoa during liquid storage and options for improvement. Animals [Internet]. 2022; 12(3):244. doi: https://doi.org/g7p4mq

[9] Kulaksız R, Çebi Ç, Akçay E. The effect of different extenders on the motility and morphology of ram sperm frozen or stored at 4°C. Turk. J. Vet. Anim. Sci.[Internet]. 2012; 36(2):177–182. doi: https://doi.org/qrc8

[10] Hameed N, Zubair M, Ahmad N, Durrani AZ, Khan MIUR. Effect of cooling rate, extender type, and storage temperature on chilled sperm quality and pregnancy rate in Kail sheep. Small Rumin. Res. [Internet]. 2024; 231:107202. doi: https://doi.org/g56433

[11] Ansari MS, Rakha BA, Akhter S, Ashiq M. OPTIXcell improves the postthaw quality and fertility of buffalo bull sperm. Theriogenology [Internet]. 2016; 85(3):528–532. doi: https://doi.org/qrdc

[12] Luna-Orozco JR, González-Ramos MA, Calderón-Leyva G, Gaytán-Alemán LR, Arellano-Rodríguez F, Ángel-García O, Véliz-Deras FG. Comparison of different diluents based on liposomes and egg yolk for ram semen cooling and cryopreservation. Iran J. Vet. Res. [Internet]. 2019; 20(2):126–130. doi: https://doi.org/qrdd

[13] Snoeck PPDN, Câmara DR, Moura LCDO, Silva MCD, Machado-Neves M, Teixeira-Neto MR, Henry M. Post-thaw quality of ram sperm frozen with different concentrations of low-density lipoproteins associated with non-enzymatic antioxidants. Anim. Reprod. [Internet]. 2023; 20(1):e20220068. doi: https://doi.org/qrdg

[14] Almeida-Filho RAD, Silva LSD, de Lima LGF, Lourencetti FA, Freitas-Dell´ Aqua C, Ferreira JCP, Oba E. Egg-yolk-and a liposome-based extenders: refrigeration time and effects on ram semen quality. Anim. Reprod. [Internet]. 2025; 22(2):e20240052. doi: https://doi.org/qrdh

[15] Younus AM, Yamanaka T, Shimada M. The protective effects of antioxidants against endogenous and exogenous oxidative stress on bull sperm. *In Vitro* Cell. Dev. Biol. – Animal [Internet]. 2024; 60:969–982. doi: https://doi.org/qrdj

[16] Salavoura A. Chemical environmental pollutants and their effect on health [Internet]. Cham (Switzerland): Springer; 2025. Chapter 3, Oxidative Stress; p. 37–53. doi: https://doi.org/qrdk

[17] Wojtusik J, Stoops MA, Roth TL. Animal protein-free OptiXcell and shortened equilibration periods can replace egg yolk-based extender and slow cooling for rhinoceros semen cryopreservation. Cryobiology [Internet]. 2019; 89:21–25. doi: https://doi.org/qrdm

[18] Bearden HJ, Fuquay JW. Applied animal reproduction. 3rd ed. Englewood Cliffs (NJ, USA): Prentice Hall; 1992.

[19] Hafez ESE. Reproduction in farm animals. 5th ed. Philadelphia (PA, USA): Lea and Febiger; 1987.

[20] Placer ZA, Cushman LL, Johnson BC. Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. Anal. Biochem. [Internet]. 1966; 16(2):359–364. doi: https://doi.org/b96rpj

[21] Ellman GL, Courtney KD, Andres jr V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. [Internet]. 1961; 7(2):88–95. doi: https://doi.org/fwdkkz

[22] Aebi H. Catalase *in vitro*. Methods Enzymol. [Internet]. 1984; 105:121–126. doi: https://doi.org/dnf7v9

[23] Beutler E. Red cell metabolism: A manual of biochemical methods. 3rd ed. New York (NY, USA): Grune & Starton; 1984.

[24] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. J. Biol. Chem. [Internet]. 1974; 249(22):7130–7139. doi: https://doi.org/gjjzqq

[25] Sun YI, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin. Chem. [Internet]. 1988; 34(3):497–500. doi: https://doi.org/gj74fn

[26] Lowry O, Rosebrough N, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. [Internet]. 1951; 193(1):265–275. doi: https://doi.org/ghv6nr

[27] Kim HY. Statistical notes for clinical researchers: Two-way analysis of variance (ANOVA)-exploring possible interaction between factors. Restor. Dent. Endod. [Internet]. 2014; 39(2):143–147. doi: https://doi.org/gf2vpg

[28] Blanca MJ, Arnau J, García-Castro FJ, Alarcón R, Bono R. Repeated measures ANOVA and adjusted F-tests when sphericity is violated: which procedure is best? Front. Psychol. [Internet]. 2023; 14:1192453. doi: https://doi.org/g89wmv

[29] Aksu EH. Current semen extenders for bulls. Vet. J. KU. [Internet]. 2023; 2(2):34–40. doi: https://doi.org/qrdn

[30] Fernandez-Novo A, Santos-Lopez S, Barrajon-Masa C, Mozas P, de Mercado E, Caceres E, Garrafa A, Gonzalez-Martin JV, Perez-Villalobos N, Oliet A, Astiz S, Perez-Garnelo SS. Effect of extender, storage time and temperature on kinetic parameters (CASA) on bull semen samples. Biology [Internet]. 2021; 10(8):806. doi: https://doi.org/g69qzq

[31] Al-Kawmani AA, Farah MA, Nahdi S, Harrath AH, Al-Jawdah, H. A comparative study of semen characteristics and testosterone concentration in ram breeds of Saudi Arabia. Indian J. Anim. Res. [Internet]. 2020; 54(12):1476–1480. doi: https://doi.org/qrdp

[32] Gündoğan M. Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi rams. Turk. J. Vet. Anim. Sci.[Internet] 2006 [cited Sep 3, 2025]; 30(1):95–100. Available in: https://goo.su/6rCtU

[33] Mata-Campuzano M, Álvarez-Rodríguez M, Álvarez M, Tamayo-Canul J, Anel L, de Paz P, Martínez-Pastor F. Post-thawing quality and incubation resilience of cryopreserved ram spermatozoa are affected by antioxidant supplementation and choice of extender. Theriogenology [Internet]. 2015; 83(4):520–528. doi: https://doi.org/f3rvtq

[34] Gündogan M. Short term preservation of ram semen with different extenders. Kafkas Univ. Vet. Fak. Derg. [Internet]. 2009; 15(3):429–435. doi: https://doi.org/qrdq

[35] Budai C, Egerszegi I, Olah J, Javor A, Kovacs A. The protective effect of antioxidants on liquid and frozen stored ram semen-review. Sci. Pap. Anim. Sci. Biotechnol. [Internet]. 2014 [cited Sep 12, 2025] 47(1):46–52. Available in: https://goo.su/sTGE

[36] Sies H, Berndt C, Jones DP. Oxidative stress. Annu. Rev. Biochem. [Internet]. 2017; 86(1):715–748. doi: https://doi.org/b6gx

[37] Falchi L, Galleri G, Zedda MT, Pau S, Bogliolo L, Ariu F, Ledda S. Liquid storage of ram semen for 96 h: Effects on kinematic parameters, membranes and DNA integrity, and ROS production. Livest. Sci. [Internet]. 2018; 207:1–6. doi: https://doi.org/gczcc6

[38] Ben-Moula A, Hamidallah N, Badi A, El Fadili M, El Amiri B. Adjusting ram semen preservation: Exploring the impact of oxygen exposure during liquid storage. Reprod. Domest. Anim. [Internet]. 2024; 59(5):e14618. doi: https://doi.org/qrdr

[39] Bucak MN, Ateşşahin A, Yüce A. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. Small Rumin. Res. [Internet]. 2008; 75(2–3):128–134. doi: https://doi.org/cbzgkf

[40] Çoyan K, Başpınar N, Bucak MN, Akalın PP, Ataman MB, Ömür AD, Güngör Ş, Küçükgünay S, Özkalp B, Sarıözkan, S. Influence of methionine and dithioerythritol on sperm motility, lipid peroxidation and antioxidant capacities during liquid storage of ram semen. Res. Vet. Sci. [Internet]. 2010; 89(3):426–431. doi: https://doi.org/ckdq4f

[41] Kulaksız R, Bucak MN, Akçay E, Sakin F, Daşkın A, Ateşşahin A. The effects of different extenders and myo-inositol on post-thaw quality of ram semen. Kafkas Univ. Vet. Fak. Derg. [Internet]. 2011; 17(2):217–222. doi: https://doi.org/qrds

[42] Pasciu V, Nassif C, Dattena M, Succu S, Sotgiu FD, Cannas A, Cossu I, Baralla E, Chessa F, Berlinguer F, Mara L. Effect of different extenders on the oxidative status and fertility of Sarda ram liquid semen stored at 15°C. Antioxidants [Internet]. 2025; 14(8):932. doi: https://doi.org/qrdt