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Effects of different extenders and additives on liquid storage of Awassi ram semen

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Abstract: The aim of this study was to evaluate the effects of different extenders and additives on ram semen during liquid storage and to demonstrate the role of oxidative stress parameters on this process. In the present study, ejaculates taken by artificial vagina twice a week from 4 rams during the breeding season were used. They were mixed and used if motility and viability were above 70% and there was a 95% intact acrosome. The semen specimens were diluted by Tris-citrate-glucose (TRIS), Tris-TES (TEST), HEPES-buffered Tyrode lactate (TL-HEPES), and Dulbecco's phosphate-buffered saline (PBS) extenders supplemented with different additives [centrifuged egg yolk, Equex-STM, bovine serum albumin (BSA), and ethylenediaminetetraacetic acid (EDTA)]. The specimens were stored at 4 °C for up to 96 h and evaluated for motility, membrane integrity, acrosome integrity, mitochondrial membrane potential (MMP), and oxidative stress parameters. At the end of the 96-h storage, the highest sperm motility was 64.2 ± 3.7% ($P < 0.05$) and significant loss of sperm motility and membrane integrity were not detected in extender TEST-3, but the MMP rate significantly declined. Acrosome integrity was not affected by storage time or extender types. BSA and EDTA decreased lipid peroxidation (LPO) and total oxidant status (TOS), but did not positively affect motility or membrane integrity. As a result, TRIS, TEST, and TL-HEPES-3 were observed to provide better protection for ram semen during liquid storage at 4 °C than other extenders. The role of oxidative stress and MMP are considerable in liquid storage.

Key words: Awassi ram, extenders, liquid storage, supplements, sperm

1. Introduction

Artificial insemination with frozen sperm in cattle has been successfully and widely used. Unlike cows, artificial insemination in sheep using frozen sperm is not common due to the difficulty of the method and low fertility rates (1,2). Therefore, in sheep breeding, instead of frozen sperm, native or liquid preserved sperm has been used and a 60% or higher fertility rate can be achieved (1,3). The greatest difficulty with liquid storage is the 10% to 35% loss of sperm fertility if storage time is over 24 h. Even though sperm can remain motile for up to a week, its fertility capacity can decrease (1,4). Although successful fertility rates have been reported after storage for more than 24 h (3) in some studies, contradictory or low fertility results have also been reported (1). It is necessary to extend the liquid storage time to benefit from artificial insemination techniques on a wider platform. More research on the subject is required to achieve optimum fertility rates in storage periods over 48 h. For this reason, the aim of the present study was to determine how sperm can be stored for longer periods, and the factors that affect the success of liquid storage.

The breeding season is an important part of sheep breeding since it allows early lambing. Regarding the Awassi raised in warm climate zones, lambs born early in the year can develop more quickly as they will not be affected by the hot season, which has more stress factors than the cold and rainy season. There are approximately 30 million sheep in Turkey, and most of them are of domestic races. The Awassi race is found in the Southeastern Anatolia Region, and they number around 4 million (5–7). Awassi sheep are one of the races adapted to the steppe conditions in southeastern Turkey. These are the most commonly bred sheep in the region. In state farms, milk production is 300 to 350 kg per lactation, whereas in conventional farms, it is only 100 to 150 kg per lactation.

In sheep breeding, due to the large scale of the herd to be inseminated and breeding performed over long distances, sperm must be transported without any problems and loss in its fertility capacity. Moreover, to take advantage of the rams for longer periods and in various times of the year, sperm storage technologies should be more advanced. The basic principle of sperm storage is to reduce the spermatozoa metabolism, thus

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extending its life. For this purpose, sperm is stored at low temperatures (4–22 °C, liquid storage) or frozen (−196 °C, long-term storage) (1,8). Short- and long-term storage are used for sperm transport, genetic storage, and medical purposes (9). The success of short- and long-term storage methods is, on the whole, dependent on the storage temperature, cooling rate, chemical composition of the extender, reactive oxygen species (ROS), and seminal plasma composition (9,10). Liquid storage is done at room temperature (18–22 °C) and in cold environments (4 °C). For this purpose, many extenders contain buffers and chemicals such as sugar, egg yolk, and glycine (11,12). The success of extenders depends on their ability to maintain the pH of the environment, to generate the appropriate osmotic pressure, and to provide the necessary energy supply (13). pH buffer chemicals with similar properties such as Tris, N-Tris (hydroxymethyl)-methylaminoethane sulfonic acid (TES), 2 (N-morpholino) ethane sulfonic acid (MES), HEPES, and phosphate are used as extenders (14). Although lactose, TES-Tris, Tris-citrate, and milk-based semen extenders are generally used for liquid storage (1,10), the best chemical composition for the extender depends on the species (15). In the present study, HEPES-buffered Tyrode lactate (TL-HEPES), TRIS, TEST, and PBS were used. The efficiency of TL-HEPES and PBS in sperm processing (16,17) and in short-term storage (18–20) was identified in previous studies. Tris-citrate is a commonly used extender (1), and it is also usually used as the control or main extender in ram sperm-extender studies (21–24). TEST, TL-HEPES, and PBS extenders were found to be effective in protecting the sperm against cold shock when freezing rat sperm and in equilibration at 4 °C (20,25). We hypothesized that the extenders successfully used in rat sperm might be more successful in the liquid storage of ram sperm, because rat sperm is known to have a very sensitive structure compared to the sperm of other species (17,26) and, additionally, ram sperm is more durable than rat sperm. In our previous studies (17,25), we found that rat sperm is more sensitive to cold shock and can be stored better at room temperature. Based on our experience with rat sperm, we found that a combination of TEST, centrifuged egg yolk, and 0.75% Equex paste (EP) was effective in protecting rat sperm against cold shock, and, in addition to being good extenders, TL-HEPES and PBS were successful in the short-term storage of rat sperm although they do not contain any source of energy (20).

To increase the efficiency of the extenders in sperm storage, various chemical substances are used as additives and EP is one of them. EP and Orvus ES paste are the commercial forms of sodium dodecyl sulfate (SDS), which is a water-soluble anionic detergent. It is considered to have protective effects against cold damage and cold shock in sperm, especially when used in combination with egg yolk (27). EP is sold commercially for horse and pig sperm storage and has been reported to make positive contributions to the cryopreservation of rat (26), mouse (28), cat (29), dog (30), pig (31), and ram (32) sperm. EP increases the efficiency of egg yolk by altering the structure of its lipoproteins (33). The centrifuged egg yolk used in the present study can alter its constituents and its efficiency. This alteration in the efficiency of egg yolk also affects the utilization rate and efficiency of EP when used together. The amount of EP added to the extenders for sperm cryopreservation is between 0.5% and 1.5%. In studies on rat sperm, while centrifuged egg yolk and EP did not have an effect on liquid storage at 4 °C, they had a positive effect on sperm incubation (2).

The aim of the present study was to test the efficiency of the extenders Tris-citrate, TES/Tris, TL-HEPES, and PBS and to increase their efficiency using various chemicals (centrifuged egg yolk, ethylenediaminetetraacetic acid (EDTA), BSA, and EP). The chemical composition of the extenders TEST, TL-HEPES, and PBS does not contain sugar. Although ram seminal fluid only contains fructose, spermatozoa can use the glucose and mannose added to the extender as an energy source (1). Previous studies report that 3 to 60 mM glucose is a sufficient source of energy. High amounts of glucose added to the extender can decrease the intracellular pH level of 6.0, can pass through the cell membrane due to its low molecular weight, and does not affect the osmotic pressure of the extender (8,34,35). EDTA forms a chelate with the metal ions, reducing their toxic effects on spermatozoa and delaying the capacitation and induction of the acrosome reaction, and thus 1.25 to 3.7 g/L EDTA is used in commercial extenders (1,8).

BSA is important in liquid storage due to its delaying effect on time-dependent sperm aging (8). The amount of TL-HEPES, which is commonly used in sperm processing in in vitro studies, was added to the working solution at a volume of 3 mg/mL (36). The list of chemicals to be added to the extenders used in the present study was kept shortened. This is because one of the main purposes of this study was to evaluate the efficiency of TL-HEPES and PBS. In addition, a wide range of chemicals are still being tested for liquid storage or cryopreservation, and only a limited number of them (Tris, EDTA, etc.) have been approved.

Antioxidants have been one of the most discussed subjects in recent years and extensive studies on the subject are ongoing. Although several studies have reported that antioxidants positively contribute to the postthaw motility parameters (37) or do not (38), oxidative stress measures suggest that external antioxidants do not significantly reduce the oxidative stress parameters (37,38). In the present study, oxidative stress parameters were determined, and EDTA and BSA were used as an

antioxidant. To increase the TEST, TL-HEPES, and PBS efficiency, glucose (27 mM) as an energy supply, EDTA (2 mg/mL), and BSA (3 mg/mL) as adjuvants to the final versions of the extenders were added.

The aim of the present study was to demonstrate the role of oxidative stress parameters in short-term storage and to test the efficiency of the extenders Tris-citrate, TES/Tris, TL-HEPES, and PBS with different supplementary additives in ram sperm.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (Turkey) unless otherwise stated.

2.1. Animals and semen collection

Four Awassi rams were used in the present study. The rams were housed at Harran University Agriculture-Veterinary Research Farm. From June to October in the breeding season, the semen was collected twice a week using an artificial vagina and was kept in a water bath at 37 °C until use. Native semen was analyzed and used if the following criteria were met: motility and membrane integrity ≥70%, intact acrosome ≥95%, total abnormal rate <15%, and concentration ≥2.5 × 10^9 spermatozoa/mL. The present study consisted of 6 trials and 2 stages because of the large number of groups. In the first stage, the first 8 groups were tested.

The semen from 4 rams were pooled and used for each process. The pooled ejaculate was divided into 100- to 120-µL/mL aliquots [for 8 groups and 5 times (0, 24, 48, 72, and 96 h), 8 × 5 = 40 samples prepared] in a 1.5-mL centrifuge tube and diluted with 1 mL of the extenders (final concentration of approximately 400 × 10^6 spermatozoa) at 37 °C. After the dilution, the samples were placed in a refrigerator at 4 °C. For sperm analysis, 300 µL of 1-mL samples were placed in 1.5-mL tubes, which were incubated in a water bath at 37 °C. The remaining 700 µL of samples were directly stored at −20 °C until oxidative stress analysis. The samples were analyzed at 0, 24, 48, 72, and 96 h for motility, membrane integrity, acrosome integrity, mitochondrial membrane potential, and oxidative stress parameters.

Extender groups

1. TRIS-1: TRIS + 20% EY (egg yolk) - Control
2. TRIS-2: TRIS + 20% CEY (centrifuged egg yolk)
3. TRIS-3: TRIS + 20% CEY + 0.75% EP (Equex paste)
4. TRIS-4: TRIS + 20% CEY + 0.75% EP + EDTA + BSA
5. TL-HEPES-1: TL-HEPES + 20% CEY
6. TL-HEPES-2: TL-HEPES + 20% CEY + 0.75% EP
7. TL-HEPES-3: TL-HEPES + 20% CEY + 0.75% EP + glucose
8. TL-HEPES-4: TL-HEPES + 20% CEY + 0.75% EP + glucose + EDTA + BSA
9. TEST-1: TEST + 20% CEY
10. TEST-2: TEST + 20% CEY + 0.75% EP
11. TEST-3: TEST + 20% CEY + 0.75% EP + glucose
12. TEST-4: TEST + 20% CEY + 0.75% EP + glucose + EDTA + BSA
13. PBS-1: PBS + 20% CEY
14. PBS-2: PBS + 20% CEY + 0.75% EP
15. PBS-3: PBS + 20% CEY + 0.75% EP + glucose
16. PBS-4: PBS + 20% CEY + 0.75% EP + glucose + EDTA + BSA

2.2. Preparation of extenders

Four extenders—TL-HEPES (36), Tris-citrate (1), TES-Tris (25), and PBS (Invitrogen)—were used. The osmolalities of the extenders were determined using a vapor pressure osmometer (Vapro 5600, Wescor, Logan, UT, USA).

The pH and osmolality of each extender were adjusted to approximately 6.8 to 7.4 and 330 to 380 mOsm, respectively. Centrifuged egg yolk (20%; v/v) was added to the extenders and mixed by placing the tube in an orbital shaker for 10 min and centrifuging at 15,000 × *g* for 60 min, and the supernatant was filtered through a 0.45-mm membrane filter (VWR, Lutterworth, UK). Supplements were added to the diluent at a ratio of glucose 27 mM, EDTA 2 g/L, BSA Fraction-V 3 mg/mL, and 0.75% (v/v) EP (Minitüb, Tiefenbach, Germany).

2.3. Evaluation of sperm motility

Motility was evaluated using a phase-contrast microscope (Olympus BX51, Tokyo, Japan) with a warm stage (37 °C). Five-microliter drops of semen from 100-µL samples were placed on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields (20).

2.4. Fluorescent staining

Three hundred-microliter samples were divided in two parts: 200 µL used for fluorescein staining and 100 µL used for motility examination.

2.4.1. Evaluation of sperm plasma membrane and acrosomal integrity

SYBR-14/Propidium iodide (Live/Dead Sperm Viability Kit, catalog no: L-7011, Molecular Probes, Eugene, OR, USA) and Alexa Fluor-488-PNA (catalog no: L-21,409, Molecular Probes) conjugate were used to determine plasma membrane and acrosome integrity, respectively. For plasma membrane integrity, 200-µL sperm samples were centrifuged at 200 × *g* for 3 min. Then the supernatant was removed and 1000 µL of PBS was added to the tube containing the sperm pellet to resuspend the sperm pellet by rotation of the tube. One hundred microliters of resuspended sperm (approximately 80 × 10^6 spermatozoa/mL) was incubated with 10 µL of SYBR- 14 (1 µM final concentration) at 37 °C for 10 min and 5 µL of PI (1 µM final concentration). After staining, 3 µL of Hancock solution

was added to specimens to stop the sperm movement, and 5-µL sperm samples were observed under the fluorescence microscope (Olympus BX51, using a dual fluorescence filter: Olympus U-M51009). The categorization of spermatozoa has been described previously (25).

For acrosomal integrity, 10-µL sperm samples (prepared before fluorescence staining) were smeared onto microscope slide and air dried. The specimens were fixed with 99% methanol and kept at −20 °C until fluorescence staining (fixation of sperm cannot prevent acrosome damage; for this reason, keeping specimens at −20 °C is important). Staining and description of spermatozoa have been described previously (25).

2.4.2. Evaluation of sperm mitochondrial membrane potential (MMP)

The sperm MMP was evaluated using JC-1 fluorescent dye (M34152, Molecular Probes). This dye was used to distinguish spermatozoa with poorly and highly functional mitochondria. In poorly functional mitochondria, JC-1 fluoresces green. However, in highly functional mitochondria, JC-1 forms aggregate that fluoresces orange. For evaluation of MMP in spermatozoa, 100 µL of the washed (prepared before membrane integrity analysis) sperm suspensions (80×10^6 spermatozoa/mL) were mixed with 10 µL of JC-1 (10-µg final concentration). The mixture was incubated at 37 °C for 30 min, and then sperm movement was stopped with 3 µL of Hancock solution. One hundred sperm per sample were analyzed under a fluorescent microscope (25).

2.5. Analyses of oxidant and antioxidant parameters

The 700-µL specimens were stored at −20 °C in a freezer and transferred to the laboratory in cold chain for analysis.

Eight parameters, i.e. lipid hydroperoxide, total oxidant status, oxidative stress index, total antioxidant status, sulfhydryl groups, ceruloplasmin, paraoxonase, and arylesterase, were analyzed in the semen samples. Oxidative and antioxidative parameters were measured in the tissue samples using an Aeroset automated analyzer (Abbott, IL, USA) and a spectrophotometer (Cecil 3000, Cambridge, UK). The lipid hydroperoxide level (LOOH) was evaluated by fluorometric method based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (39). All analyses, described previously (39), were performed at the Biochemistry Laboratory of the Faculty of Medicine at Harran University.

2.6. Statistical analysis

Statistical analyses were performed using SPSS (version 23 for Mac; SPSS, Inc., Chicago, IL, USA). The data were analyzed to determine the effects of extenders and time on motility, membrane and acrosome plasma integrity, MMP, and oxidative stress parameters. Parametric data were analyzed by analysis of variance (two-way ANOVA), and if there were significant differences, Duncan's test for multiple comparisons was used for post hoc analysis. The nonparametric data were analyzed by the Kruskal–Wallis test, and if there were significant differences between groups, the Mann–Whitney test was used to determine the differences in groups. Statistical significance was set at $P < 0.05$. Values were presented as the mean ± standard error of the mean (SEM).

3. Results

In the present study, mean motility, amount, density, and acrosome integrity of the native sperm were 79.9%, 1.3 mL, 3.7×10^9, and 97%, respectively.

At 0 h postextension, no differences in motility were detected between the extender groups ($P > 0.05$). The significantly highest motility at 24, 48, 72, and 96 h was measured in PBS-1, TRIS-3, TEST-4, and TEST-3, respectively. In terms of motility, at 96 h, it was found that EDTA and BSA added to TL-HEPES and PBS caused a decrease in motility; however, this decrease was not observed in TEST or TRIS. Centrifuged egg yolk and EP, although not statistically significant, had positive effects on the effectiveness of TRIS and TEST. Between 0 and 96 h, no statistically significant decrease in motility was detected in TEST-3 solution.

At 0 h postextension, no difference in membrane integrity was detected between the extender groups ($P > 0.05$). No statistically significant loss of membrane integrity was detected between 0 and 96 h in TL-HEPES-3, TEST-2, or TEST-3. Membrane integrity was between 49% and 58% in TRIS-based solutions and between 50% and 58% in TES/TRIS-based solutions at 96 h. Membrane integrity greater than 50% could not be achieved in PBS-based extenders. Glucose added to TL-HEPES was effective in protecting sperm motility and membrane integrity up to 96 h (Tables 1 and 2), but BSA and EDTA had negative effects on motility and membrane integrity in TL-HEPES and PBS extenders.

The results obtained from the mitochondria potential assay were close to those obtained from the motility and membrane integrity assays; however, significant decreases were detected up to 96 h in all solutions ($P < 0.05$). In the TL-HEPES and PBS groups that had low motility and membrane integrity, MMP levels were also low, as expected. However, unlike the results of motility and membrane integrity, the highest MMP level was obtained with TRIS-1 at 96 h (Table 3).

Based on the fluorescent assessment, acrosome damage in the native sperm was 3%. With the addition of extenders, this can reach up to 5%. It was observed that after 96 h of incubation, damage did not exceed 10% in any solution. The data obtained in the present study showed that in short-term storage significant acrosome damage did not occur ($P > 0.05$; Table 4).

Table 1. Motility (%) of ram sperm liquid storage at 4 °C up to 96 h in different extenders (n = 6).

Groups	0 h	24 h	48 h	72 h	96 h	Significance
TRIS-1	80.0 ± 0.0	63.3 ± 4.2^{abcde}	56.7 ± 4.2^{bcde}	56.7 ± 2.1^{cd}	50.0 ± 0.0^{bc}	*
TRIS-2	80.0 ± 0.0	65.0 ± 3.4^{abcde}	58.3 ± 3.1^{bcde}	55.0 ± 3.4^{cd}	55.0 ± 3.2^{bc}	*
TRIS-3	80.0 ± 0.0	73.3 ± 3.3^{de}	71.7 ± 1.7^{e}	63.3 ± 3.3^{d}	60.0 ± 4.5^{bc}	*
TRIS-4	80.0 ± 0.0	56.7 ± 6.1^{abcd}	58.3 ± 5.4^{cde}	53.3 ± 8.4^{bcd}	49.2 ± 2.0^{b}	*
TL-HEPES-1	76.7 ± 3.3	51.7 ± 5.4^{ab}	43.3 ± 4.2^{abc}	44.2 ± 3.7^{bcd}	35.0 ± 6.6^{a}	*
TL-HEPES-2	73.3 ± 3.3	43.3 ± 8.8^{ab}	23.3 ± 4.9^{a}	33.3 ± 6.7^{b}	17.5 ± 4.4^{a}	*
TL-HEPES-3	75.0 ± 5.0	58.3 ± 6.5^{abcd}	56.7 ± 6.1^{bcde}	61.7 ± 4.0^{cd}	53.3 ± 6.1^{bc}	*
TL-HEPES-4	76.7 ± 2.1	50.0 ± 9.3^{abc}	37.5 ± 10.9^{abcd}	24.2 ± 8.4^{ab}	22.2 ± 7.7^{a}	*
TEST-1	73.3 ± 2.1	60.0 ± 2.6^{abc}	60.0 ± 5.2^{cde}	53.3 ± 4.9^{bcd}	53.3 ± 3.1^{bc}	*
TEST-2	75.0 ± 2.2	70.0 ± 2.6^{bcde}	65.8 ± 2.0^{de}	59.2 ± 6.4^{cd}	61.7 ± 3.3^{bc}	*
TEST-3	75.0 ± 3.4	66.7 ± 2.1^{abcde}	60.8 ± 3.3^{cde}	59.2 ± 10.4^{cd}	64.2 ± 3.7^{c}	-
TEST-4	78.3 ± 1.7	65.0 ± 3.4^{abcde}	64.2 ± 2.7^{cde}	65.0 ± 4.3^{d}	61.7 ± 3.1^{bc}	*
PBS-1	78.3 ± 1.7	73.3 ± 2.1^{e}	45.8 ± 7.1^{abcd}	38.3 ± 3.1^{b}	30.8 ± 3.3^{a}	*
PBS-2	76.7 ± 3.3	73.3 ± 2.1^{cde}	50.0 ± 4.8^{abcde}	38.3 ± 4.0^{bc}	22.8 ± 5.2^{a}	*
PBS-3	75.0 ± 2.2	73.3 ± 3.3^{de}	43.3 ± 6.1^{abcd}	29.2 ± 6.1^{ab}	23.5 ± 8.0^{a}	*
PBS-4	73.3 ± 2.1	41.7 ± 7.4^{a}	23.3 ± 8.3^{ab}	9.5 ± 3.4^{a}	6.8 ± 2.0^{a}	*

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences (P < 0.05)
*Differences within the rows were significant
-Differences within the rows were not significant

Table 2. Membrane integrity (%) of ram sperm liquid storage at 4 °C up to 96 h in different extenders (n = 6).

Groups	0 h	24 h	48 h	72 h	96 h	Significance
TRIS-1	72.5 ± 1.5	65.2 ± 3.4^{ab}	66.0 ± 3.9^{ab}	57.7 ± 4.2^{bcd}	53.8 ± 5.3^{bcd}	*
TRIS-2	74.8 ± 2.3	67.2 ± 3.8^{ab}	59.2 ± 3.7^{ab}	62.7 ± 3.4^{cd}	58.3 ± 2.6^{d}	*
TRIS-3	70.8 ± 3.8	64.7 ± 4.7^{ab}	65.8 ± 3.0^{b}	57.3 ± 6.5^{bcd}	49.2 ± 5.2^{bcd}	*
TRIS-4	75.3 ± 1.1	66.7 ± 5.6^{ab}	63.5 ± 5.4^{ab}	54.8 ± 2.8^{bcd}	51.0 ± 2.8^{bcd}	*
TL-HEPES-1	65.7 ± 3.6	49.3 ± 5.2^{ab}	45.8 ± 5.0^{ab}	45.8 ± 4.1^{bc}	42.8 ± 5.9^{bcd}	*
TL-HEPES-2	62.7 ± 4.7	63.3 ± 3.4^{ab}	61.3 ± 3.7^{ab}	53.8 ± 4.3^{bcd}	40.5 ± 6.7^{bcd}	*
TL-HEPES-3	64.2 ± 3.9	53.2 ± 4.0^{ab}	52.3 ± 4.1^{ab}	52.0 ± 6.2^{bcd}	53.0 ± 1.4^{cd}	-
TL-HEPES-4	67.0 ± 3.1	52.7 ± 6.7^{ab}	56.5 ± 6.3^{ab}	42.7 ± 7.5^{bcd}	35.3 ± 7.5^{bcd}	*
TEST-1	74.5 ± 2.3	58.5 ± 2.7^{ab}	62.5 ± 4.6^{ab}	55.2 ± 3.8^{bcd}	52.5 ± 4.2^{bcd}	*
TEST-2	69.0 ± 2.7	66.2 ± 2.8^{ab}	58.5 ± 3.2^{ab}	58.0 ± 3.5^{bcd}	58.2 ± 4.0^{cd}	-
TEST-3	69.0 ± 2.8	65.0 ± 3.2^{ab}	67.2 ± 2.9^{ab}	67.8 ± 3.2^{d}	58.0 ± 8.0^{cd}	-
TEST-4	75.2 ± 2.2	68.2 ± 3.8^{ab}	63.5 ± 2.5^{ab}	57.8 ± 3.9^{bcd}	50.7 ± 3.8^{bcd}	*
PBS-1	70.8 ± 3.8	66.8 ± 3.2^{b}	61.0 ± 2.2^{ab}	62.0 ± 3.2^{cd}	50.7 ± 3.7^{bcd}	*
PBS-2	70.5 ± 2.9	65.5 ± 2.2^{ab}	60.3 ± 1.9^{ab}	46.3 ± 6.2^{bcd}	34.5 ± 2.7^{b}	*
PBS-3	66.8 ± 3.6	61.8 ± 3.9^{ab}	53.5 ± 4.7^{ab}	32.5 ± 5.8^{b}	28.3 ± 7.8^{bc}	*
PBS-4	70.8 ± 3.9	32.7 ± 11.9^{a}	27.0 ± 11.5^{a}	8.0 ± 1.1^{a}	8.0 ± 1.9^{a}	*

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences (P < 0.05)
*Differences within the rows were significant
-Differences within the rows were not significant

Table 3. Mitochondrial membrane integrity (%) of ram sperm liquid storage at 4 °C up to 96 h in different extenders (n = 6).

Groups	0 h	24 h	48 h	72 h	96 h	Significance
TRIS-1	90.2 ± 2.7	75.2 ± 6.1^{ab}	76.5 ± 3.9^{cde}	68.2 ± 5.1^{cd}	66.2 ± 7.0^{e}	*
TRIS-2	85.5 ± 2.6	79.2 ± 6.9^{ab}	73.0 ± 7.1^{abcde}	61.3 ± 8.2^{abcd}	61.5 ± 9.5^{bcde}	*
TRIS-3	84.0 ± 2.6	74.8 ± 4.6^{ab}	73.7 ± 5.8^{abcde}	63.5 ± 4.5^{bcd}	55.7 ± 5.0^{bcde}	*
TRIS-4	79.5 ± 2.9	67.8 ± 7.6^{ab}	55.3 ± 9.6^{abcde}	42.5 ± 11.9^{abcd}	38.0 ± 11.1^{bcde}	*
TL-HEPES-1	76.2 ± 6.2	50.8 ± 5.4^{a}	46.7 ± 5.9^{a}	44.2 ± 3.8^{ab}	45.2 ± 3.0^{bcde}	*
TL-HEPES-2	77.8 ± 4.0	69.7 ± 5.8^{ab}	58.7 ± 6.6^{abcde}	48.3 ± 4.2^{abcd}	43.0 ± 6.7^{bcde}	*
TL-HEPES-3	81.7 ± 3.8	68.2 ± 6.3^{ab}	57.5 ± 7.2^{abcd}	51.0 ± 6.4^{abcd}	44.2 ± 6.4^{bcde}	*
TL-HEPES-4	75.7 ± 5.4	51.0 ± 10.1^{ab}	43.3 ± 9.5^{ab}	36.8 ± 8.4^{ab}	33.5 ± 9.3^{bcd}	*
TEST-1	83.7 ± 1.5	74.7 ± 3.1^{ab}	74.2 ± 5.0^{abcde}	67.2 ± 3.1^{bcd}	62.7 ± 5.6^{cde}	*
TEST-2	79.0 ± 1.9	76.0 ± 1.8^{ab}	78.5 ± 2.7^{e}	71.8 ± 3.2^{d}	63.3 ± 5.0^{de}	*
TEST-3	84.8 ± 2.9	82.3 ± 3.2^{b}	77.2 ± 3.1^{de}	70.8 ± 4.2^{cd}	63.5 ± 6.3^{cde}	*
TEST-4	80.8 ± 3.0	71.0 ± 5.2^{ab}	60.2 ± 5.1^{abcde}	61.8 ± 4.8^{abcd}	52.3 ± 1.0^{bcde}	*
PBS-1	86.7 ± 3.4	80.4 ± 2.6^{ab}	74.9 ± 6.0^{bcde}	59.7 ± 6.0^{abcd}	47.6 ± 6.4^{bcde}	*
PBS-2	79.4 ± 2.8	71.4 ± 3.5^{ab}	52.4 ± 11.3^{abcd}	51.2 ± 8.7^{abcd}	39.0 ± 4.2^{bc}	*
PBS-3	81.2 ± 3.5	72.2 ± 4.8^{ab}	57.0 ± 4.5^{abc}	42.3 ± 8.4^{abc}	24.2 ± 8.5^{ab}	*
PBS-4	83.2 ± 3.7	34.3 ± 14.7^{ab}	31.5 ± 14.1^{a}	19.5 ± 11.2^{a}	9.0 ± 1.9^{a}	*

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences (P < 0.05)
*Differences within the rows were significant
-Differences within the rows were not significant

Table 4. Acrosome integrity (%) of ram sperm liquid storage at 4 °C up to 96 h in different extenders (n = 6).

Groups	0 h	24 h	48 h	72 h	96 h	Significance
TRIS-1	95.5 ± 0.6	93.3 ± 1.8	95.7 ± 0.8	93.8 ± 0.9	93.5 ± 1.0	-
TRIS-2	95.0 ± 0.3	96.2 ± 0.6	95.5 ± 0.8	93.8 ± 0.7	93.0 ± 0.6	-
TRIS-3	95.8 ± 0.7	94.0 ± 0.7	93.7 ± 0.8	93.3 ± 0.8	92.5 ± 0.4	-
TRIS-4	95.5 ± 0.3	94.7 ± 1.2	94.8 ± 0.8	95.5 ± 1.0	92.0 ± 0.9	-
TL-HEPES-1	95.7 ± 0.9	94.3 ± 0.8	94.2 ± 1.1	94.2 ± 0.9	92.2 ± 0.8	-
TL-HEPES-2	94.8 ± 0.9	94.3 ± 1.1	91.8 ± 1.7	93.3 ± 0.3	93.0 ± 0.8	-
TL-HEPES-3	96.0 ± 0.8	93.5 ± 0.6	95.3 ± 0.9	94.7 ± 0.5	93.7 ± 1.0	-
TL-HEPES-4	95.3 ± 0.6	95.3 ± 0.6	92.8 ± 0.7	94.3 ± 1.0	93.7 ± 1.2	-
TEST-1	96.0 ± 0.4	95.3 ± 0.5	94.8 ± 0.3	95.7 ± 0.4	93.2 ± 0.7	-
TEST-2	94.5 ± 1.1	94.2 ± 1.1	95.3 ± 0.7	95.0 ± 1.0	95.0 ± 0.9	-
TEST-3	96.3 ± 0.6	95.2 ± 1.2	96.0 ± 0.6	94.2 ± 1.1	95.2 ± 0.9	-
TEST-4	96.5 ± 0.5	95.0 ± 0.4	95.0 ± 0.5	94.3 ± 0.8	95.0 ± 1.0	-
PBS-1	97.0 ± 0.5	95.7 ± 0.6	94.6 ± 1.1	94.1 ± 0.3	93.9 ± 1.1	-
PBS-2	95.2 ± 0.7	95.0 ± 0.9	94.0 ± 0.6	95.2 ± 0.7	93.6 ± 0.6	-
PBS-3	96.5 ± 0.5	95.5 ± 0.8	94.2 ± 0.9	94.8 ± 0.9	94.2 ± 0.9	-
PBS-4	96.7 ± 0.6	97.0 ± 0.5	92.5 ± 1.1	94.2 ± 1.0	93.0 ± 1.1	-

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences (P < 0.05)
-Differences within the rows were not significant

Oxidative stress parameters after postextending the sperm samples using the solutions are given in Table 5. While there were statistically significant differences between the groups in terms of SH, TAS, LOOH, OSI, and TOS ($P < 0.05$), no difference was found in terms of TAS or OSI ($P > 0.05$). The ceruloplasmin (CP) level was significantly high in TL-HEPES-4, in which BSA and EDTA were used.

Oxidative stress parameters of sperm samples incubated at 4 °C for 24 h after being extended with various solutions are shown in Table 6. While significant differences were found between the groups in terms of SH, TAS, LOOH, TOS, and OSI ($P < 0.05$), no difference was detected in terms of CP ($P > 0.05$). The observed difference in CP levels after extending (hour 0) in groups was not observed after 24 h of storage despite the extreme difference between the data. While no significant change was detected in TAS value after dilution and 24 h of incubation, the increases in LOOH and TOS values in the same extenders are worth noting. The LOOH levels were lowest both after extending and after 24 h of incubation in TEST-4 extender (Table 6).

No paraoxonase or arylesterase activity was detected in sperm samples from 0 to 96 h.

Oxidative stress parameters of the sperm samples incubated at 4 °C for 48 h are shown in Table 7. While a significant difference was detected between the groups in terms of SH and OSI ($P < 0.05$), no difference was detected in terms of CP, TAS, LOOH, or TOS ($P > 0.05$). LOOH and TOS levels in the TL-HEPES-4, TEST-4, and PBS-4 groups after 48 h of incubation were lower than those of the other groups ($P > 0.05$). These low values were significant at 24 h, whereas they were not significant at 48 h, but were still low. The increase in TAS after 24 h of incubation was not observed in incubation times between 24 and 48 h (Table 7).

Oxidative stress parameters of sperm samples incubated at 4 °C for 72 and 96 h are shown in Tables 8 and 9. While a significant difference was found between the groups in terms of CP, SH, LOOH, TOS, and OSI ($P < 0.05$) at 72 and 96 h, no difference was detected in terms of TAS ($P > 0.05$). Although the total antioxidant values were low, the sperm retained their properties during 96 h of incubation. The low levels of LOOH and TOS observed in the TL-HEPES-4, TEST-4, and PBS-4 groups after extension continued until the end of incubation (Tables 8 and 9). Although the total oxidant levels were low when the 4 extenders were used (except TEST-4), they did not have any beneficial effects on the motility, membrane, or MMP values (Tables 8 and 9). High levels of total oxidants are known to damage sperm and, therefore, low TOL is desired. After 96 h of incubation, the lowest TOS levels were detected in PBS, TEST-4, and TL-HEPES-4, whereas the best motility was found in TEST-3, the best membrane integrity in TRIS-2, and the best MMP in TRIS-1.

4. Discussion

Liquid storage of sperm up to 2–4 days is the main goal of artificial insemination in sheep breeding programs (38). However, decrease in sperm fertility in durations longer than 24 h of liquid storage (1) is the most important problem. For this purpose, improvements in both the artificial insemination techniques and the storage techniques are required. Extenders play an important role in liquid storage (1,40), and the aim of the present study was to achieve better sperm storage conditions by using different extenders and additives.

Mitochondria of spermatozoa are different from those of the somatic cells in terms of morphology and biochemistry. Mitochondrial energy metabolism plays a vital role in the continuation of sperm functions. In liquid storage, spermatozoa need to be able to maintain their energy reserves and their mitochondria function fully to survive for an extended period of time without losing their motility. In the present study, some of the most remarkable data have been the MMP values. While no differences were found in the motility and membrane integrity of the sperm stored in TEST-3 solution up to 96 h ($P > 0.05$), decreases in MMP levels suggest that mitochondria can play a role in sperm aging or loss of fertility. While no decrease between 0 and 96 h of storage was detected in motility in TEST-3, membrane integrity in TL-HEPES, TEST-2, and TEST-3 ($P > 0.05$), and decreases in MMP in all extenders were notable ($P < 0.05$).

In a study conducted by Maxwell and Salamon (41), it was reported that more than 24 h of storage rapidly decreased fertility. The rate of decline in fertility was between 10% and 15% per day. However, decline in fertility in long-term storage was not detected in boar sperm (42); Martinez et al. (43) reported that they observed 83.8% fertility on day 5, and Ratto and Joking (44) observed 80.4% fertility. These results suggest that ram sperm has its own problems that reduce the fertility rate after 24 h of incubation. In our study, we found that motility data alone are not sufficient to decide on sperm quality. In particular, although there was no significant decline in motilities in TEST extenders up to 96 h, the decrease in MMP levels and the increase in TOS and lipid peroxidation were remarkable.

In the present study, based on the parameters of semen analyses, the most successful extenders were TRIS, TES, and TL-HEPES. Similar to the present study, Paulenz et al. (45) confirmed that Tris-based diluents are effective. In another study, it was reported that BioXcell was better than AndroMed in liquid storage up to 36 h, and a 75% fertility rate could be achieved (40). In fertility comparisons, it was reported that milk-based extenders provided 10% more fertility than Tris-based extenders (46). Mata-Campuzano et al. (47) used Tris-citrate-fructose (TCF), TES-Tris-

Table 5. Oxidative status of sperm specimens after dilution with extenders.

Groups	CP (U/g protein)	SH (mmol g^{-1} protein)	TAS (mmol trolox eq. g^{-1} protein)	LOOH (µmol g^{-1} protein)	TOS (µmol H_2O_2 eq. g^{-1} protein)	OSI (arbitrary units)
TRIS-1	13.8 ± 1.4^{abc}	0.01 ± 0.0^{a}	0.05 ± 0.4	3.8 ± 0.8^{b}	20.8 ± 4.7^{b}	31.3 ± 8.2
TRIS-2	11.6 ± 4.1^{abc}	0.02 ± 0.0^{a}	0.05 ± 0.1	3.0 ± 0.4^{b}	5.8 ± 0.8^{ab}	39.2 ± 10.8
TRIS-3	26.7 ± 9.5^{bcd}	0.02 ± 0.0^{a}	0.05 ± 0.0	2.4 ± 0.3^{b}	18.1 ± 4.6^{b}	35.0 ± 5.7
TRIS-4	20.9 ± 9.8^{abcd}	0.02 ± 0.0^{a}	0.03 ± 0.0	4.2 ± 0.8^{b}	19.7 ± 7.8^{b}	66.1 ± 15.0
TL-HEPES-1	8.2 ± 4.6^{ab}	0.03 ± 0.0^{a}	0.4 ± 0.0	2.0 ± 0.1^{ab}	15.2 ± 5.2^{b}	46.2 ± 18.5
TL-HEPES-2	10.5 ± 6.7^{abc}	0.02 ± 0.0^{a}	0.04 ± 0.0	3.5 ± 1.3^{b}	16.8 ± 4.6^{b}	44.5 ± 12.9
TL-HEPES-3	4.9 ± 4.0^{a}	0.02 ± 0.0^{a}	0.05 ± 0.0	3.7 ± 1.6^{b}	13.3 ± 4.3^{b}	33.3 ± 14.0
TL-HEPES-4	39.2 ± 5.4^{d}	0.02 ± 0.0^{a}	0.04 ± 0.0	0.9 ± 0.6^{ab}	4.7 ± 0.5^{a}	12.6 ± 2.1
TEST-1	14.6 ± 3.6^{abc}	0.02 ± 0.0^{a}	0.4 ± 0.0	4.4 ± 1.1^{b}	29.7 ± 8.4^{b}	35.2 ± 8.5
TEST-2	10.3 ± 3.0^{abc}	0.02 ± 0.0^{a}	0.03 ± 0.0	4.2 ± 1.5^{b}	20.7 ± 5.4^{b}	26.1 ± 6.9
TEST-3	8.7 ± 5.1^{ab}	0.02 ± 0.0^{a}	0.05 ± 0.0	3.5 ± 1.2^{b}	21.7 ± 7.6^{b}	43.1 ± 11.8
TEST-4	29.8 ± 7.8^{bcd}	0.02 ± 0.0^{a}	0.06 ± 0.0	0.7 ± 0.3^{a}	13.3 ± 4.1^{b}	23.8 ± 8.1
PBS-1	11.8 ± 6.1^{abc}	0.02 ± 0.0^{a}	0.02 ± 0.0	5.1 ± 1.3^{b}	20.7 ± 6.9^{b}	40.1 ± 16.7
PBS-2	4.5 ± 2.2^{a}	0.03 ± 0.0^{a}	0.04 ± 0.0	2.5 ± 0.4^{b}	20.8 ± 7.1^{b}	49.1 ± 14.2
PBS-3	11.3 ± 3.2^{abc}	0.03 ± 0.0^{a}	0.04 ± 0.0	3.5 ± 1.2^{b}	20.7 ± 6.8^{b}	27.8 ± 7.0
PBS-4	36.6 ± 8.6^{d}	0.05 ± 0.1^{b}	0.08 ± 0.0	1.6 ± 0.6^{ab}	12.5 ± 6.7^{ab}	26.4 ± 17.7

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences ($P < 0.05$)

Table 6. Oxidative status of sperm specimens diluted with extenders and stored at 4 °C for 24 h.

Groups	CP (U/g protein)	SH (mmol g^{-1} protein)	TAS (mmol trolox eq. g^{-1} protein)	LOOH (µmol g^{-1} protein)	TOS (µmol H_2O_2 eq. g^{-1} protein)	OSI (arbitrary units)
TRIS-1	15.0 ± 2.7	0.02 ± 0.0^{ab}	0.03 ± 0.0^{abc}	14.6 ± 5.1^{bc}	24.7 ± 6.6^{ab}	38.4 ± 8.4^{abc}
TRIS-2	13.2 ± 3.1	0.02 ± 0.0^{ab}	0.02 ± 0.0^{abc}	14.1 ± 6.7^{bc}	20.0 ± 5.5^{ab}	80.0 ± 5.2^{c}
TRIS-3	18.3 ± 2.3	0.03 ± 0.0^{ab}	0.02 ± 0.0^{ab}	14.0 ± 5.8^{bc}	21.7 ± 6.8^{ab}	48.1 ± 7.4^{bc}
TRIS-4	14.0 ± 1.3	0.04 ± 0.0^{b}	0.02 ± 0.0^{a}	13.8 ± 7.3^{bc}	22.0 ± 7.4^{ab}	55.5 ± 5.8^{bc}
TL-HEPES-1	15.5 ± 3.3	0.03 ± 0.0^{ab}	0.05 ± 0.0^{abc}	14.2 ± 3.8^{bc}	22.9 ± 5.2^{ab}	32.2 ± 4.6^{abc}
TL-HEPES-2	10.5 ± 4.0	0.02 ± 0.0^{ab}	0.05 ± 0.0^{bc}	13.6 ± 6.7^{abc}	18.0 ± 5.9^{ab}	47.2 ± 16.0^{abc}
TL-HEPES-3	15.6 ± 4.9	0.02 ± 0.0^{ab}	0.04 ± 0.0^{abc}	9.3 ± 2.9^{abc}	17.1 ± 3.4^{ab}	28.5 ± 7.4^{abc}
TL-HEPES-4	31.3 ± 6.6	0.02 ± 0.0^{ab}	0.04 ± 0.0^{abc}	3.1 ± 2.2^{ab}	8.8 ± 4.2^{ab}	15.4 ± 4.2^{ab}
TEST-1	15.6 ± 1.6	0.01 ± 0.0^{ab}	0.02 ± 0.0^{abc}	23.9 ± 5.8^{bc}	38.5 ± 9.4^{b}	30.1 ± 8.6^{abc}
TEST-2	35.4 ± 10.6	0.01 ± 0.0^{a}	0.04 ± 0.0^{abc}	28.4 ± 5.7^{c}	38.9 ± 7.2^{b}	40.4 ± 12.4^{abc}
TEST-3	35.0 ± 14.0	0.03 ± 0.0^{ab}	0.05 ± 0.0^{abc}	21.3 ± 7.2^{bc}	30.9 ± 10.7^{ab}	48.2 ± 20.1^{abc}
TEST-4	47.1 ± 13.6	0.02 ± 0.0^{ab}	0.05 ± 0.0^{abc}	0.9 ± 0.1^{a}	6.9 ± 1.1^{a}	15.7 ± 3.3^{abc}
PBS-1	17.6 ± 3.3	0.01 ± 0.0^{ab}	0.06 ± 0.0^{abc}	15.2 ± 4.9^{bc}	25.1 ± 5.6^{ab}	46.1 ± 13.7^{abc}
PBS-2	24.9 ± 11.8	0.02 ± 0.0^{ab}	0.04 ± 0.0^{abc}	20.8 ± 7.4^{bc}	31.4 ± 8.8^{ab}	22.6 ± 6.5^{abc}
PBS-3	17.7 ± 3.0	0.02 ± 0.0^{ab}	0.05 ± 0.0^{abc}	14.6 ± 8.4^{abc}	21.8 ± 8.3^{ab}	45.3 ± 14.4^{abc}
PBS-4	34.4 ± 4.5	0.03 ± 0.0^{ab}	0.07 ± 0.0^{c}	5.3 ± 4.5^{abc}	12.6 ± 6.8^{ab}	12.6 ± 4.1^{a}

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences ($P < 0.05$)

Table 7. Oxidative status of sperm specimens diluted with extenders and stored at 4 °C for 48 h.

Groups	CP (U/g protein)	SH (mmol g^{-1} protein)	TAS (mmol trolox eq. g^{-1} protein)	LOOH (µmol g^{-1} protein)	TOS (µmol H_2O_2 eq. g^{-1} protein)	OSI (arbitrary units)
TRIS-1	27.6 ± 11.8	0.02 ± 0.0^{abcd}	0.03 ± 0.0	9.9 ± 3.6	18.5 ± 4.5	64.8 ± 9.8^{b}
TRIS-2	13.6 ± 3.1	0.02 ± 0.0^{abcd}	0.02 ± 0.0	12.1 ± 3.5	21.9 ± 3.8	44.6 ± 16.2^{ab}
TRIS-3	23.7 ± 5.0	0.03 ± 0.0^{abcd}	0.05 ± 0.1	13.1 ± 6.1	22.4 ± 7.9	32.2 ± 12.9^{ab}
TRIS-4	12.8 ± 0.2	0.03 ± 0.0^{d}	0.06 ± 0.02	17.8 ± 10.0	18.6 ± 5.8	35.2 ± 11.7^{ab}
TL-HEPES-1	31.4 ± 12.5	0.02 ± 0.0^{bcd}	0.03 ± 0.01	13.9 ± 4.0	19.8 ± 4.9	32.2 ± 8.5^{ab}
TL-HEPES-2	24.3 ± 12.0	0.02 ± 0.0^{abcd}	0.04 ± 0.0	10.6 ± 2.1	23.8 ± 5.1	39.8 ± 8.1^{ab}
TL-HEPES-3	13.9 ± 4.1	0.02 ± 0.0^{abcd}	0.05 ± 0.0	12.9 ± 3.1	21.6 ± 3.8	38.9 ± 6.5^{ab}
TL-HEPES-4	31.0 ± 7.2	0.03 ± 0.0^{bcd}	0.04 ± 0.0	1.8 ± 0.9	9.5 ± 4.5	16.9 ± 8.1^{a}
TEST-1	24.8 ± 8.0	0.01 ± 0.0^{ab}	0.05 ± 0.0	21.1 ± 6.6	32.1 ± 8.2	39.7 ± 13.7^{ab}
TEST-2	28.9 ± 7.6	0.01 ± 0.0^{a}	0.03 ± 0.0	24.4 ± 5.6	36.6 ± 7.5	49.0 ± 10.4^{ab}
TEST-3	36.0 ± 11.8	0.01 ± 0.0^{abc}	0.05 ± 0.0	23.8 ± 5.9	36.2 ± 5.4	80.2 ± 15.4^{ab}
TEST-4	28.0 ± 8.9	0.02 ± 0.0^{abcd}	0.06 ± 0.01	9.1 ± 8.2	17.9 ± 10.8	46.2 ± 32.9^{ab}
PBS-1	19.8 ± 6.5	0.03 ± 0.0^{d}	0.06 ± 0.02	14.0 ± 6.0	22.6 ± 5.8	60.8 ± 32.2^{ab}
PBS-2	19.8 ± 8.6	0.02 ± 0.0^{abcd}	0.03 ± 0.0	20.1 ± 6.9	32.7 ± 8.9	161.0 ± 60.6^{b}
PBS-3	15.3 ± 6.8	0.02 ± 0.0^{abcd}	0.05 ± 0.01	20.2 ± 7.3	26.6 ± 7.1	90.8 ± 45.7^{ab}
PBS-4	40.6 ± 11.9	0.04 ± 0.0^{cd}	0.09 ± 0.02	5.4 ± 3.5	9.6 ± 4.2	15.5 ± 6.9^{ab}

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences ($P < 0.05$)

Table 8. Oxidative status of sperm specimens diluted with extenders and stored at 4 °C for 72 h.

Groups	CP (U/g protein)	SH (mmol g^{-1} protein)	TAS (mmol trolox eq. g^{-1} protein)	LOOH (µmol g^{-1} protein)	TOS (µmol H_2O_2 eq. g^{-1} protein)	OSI (arbitrary units)
TRIS-1	33.5 ± 13.1^{ab}	0.01 ± 0.0^{ab}	0.05 ± 0.0	11.02 ± 3.4^{b}	13.4 ± 3.2^{bc}	33.3 ± 7.4^{b}
TRIS-2	12.2 ± 2.6^{ab}	0.01 ± 0.0^{ab}	0.02 ± 0.0	12.2 ± 2.4^{b}	17.2 ± 2.4^{bc}	42.9 ± 14.0^{ab}
TRIS-3	25.5 ± 6.2^{ab}	0.03 ± 0.0^{de}	0.03 ± 0.0	14.8 ± 5.2^{b}	20.3 ± 5.7^{bc}	21.1 ± 6.8^{ab}
TRIS-4	43.7 ± 26.7^{ab}	0.02 ± 0.0^{bcde}	0.03 ± 0.0	17.1 ± 9.0^{b}	21.8 ± 8.9^{bc}	36.2 ± 15.3^{ab}
TL-HEPES-1	36.6 ± 13.9^{ab}	0.03 ± 0.0^{cde}	0.04 ± 0.0	15.4 ± 3.1^{b}	22.3 ± 2.9^{bc}	47.1 ± 12.8^{b}
TL-HEPES-2	25.4 ± 12.6^{ab}	0.02 ± 0.0^{abc}	0.04 ± 0.0	13.0 ± 2.8^{b}	24.7 ± 3.4^{bc}	39.9 ± 12.1^{b}
TL-HEPES-3	15.2 ± 3.7^{ab}	0.02 ± 0.0^{abcd}	0.05 ± 0.0	12.2 ± 3.6^{b}	24.8 ± 4.8^{bc}	25.8 ± 4.5^{ab}
TL-HEPES-4	47.7 ± 10.9^{b}	0.02 ± 0.0^{bcde}	0.04 ± 0.0	0.93 ± 0.5^{a}	4.8 ± 0.4^{a}	13.9 ± 3.2^{ab}
TEST-1	12.5 ± 2.3^{ab}	0.01 ± 0.0^{ab}	0.03 ± 0.0	22.7 ± 6.0^{b}	30.9 ± 7.6^{bc}	36.3 ± 17.4^{ab}
TEST-2	32.6 ± 10.4^{ab}	0.01 ± 0.0^{a}	0.04 ± 0.0	22.5 ± 6.8^{b}	29.6 ± 7.2^{bc}	19.0 ± 5.0^{ab}
TEST-3	25.8 ± 10.4^{ab}	0.01 ± 0.0^{ab}	0.03 ± 0.0	20.9 ± 6.9^{b}	30.9 ± 8.6^{bc}	27.7 ± 6.6^{ab}
TEST-4	44.7 ± 12.3^{ab}	0.01 ± 0.0^{ab}	0.07 ± 0.0	4.2 ± 3.4^{ab}	10.2 ± 4.0^{abc}	21.9 ± 11.4^{ab}
PBS-1	9.5 ± 6.8^{a}	0.02 ± 0.0^{abcd}	0.06 ± 0.0	18.7 ± 5.3^{b}	30.9 ± 7.1^{bc}	49.4 ± 9.7^{ab}
PBS-2	11.5 ± 3.3^{ab}	0.02 ± 0.0^{ab}	0.03 ± 0.0	23.5 ± 7.7^{b}	33.7 ± 9.2^{bc}	42.9 ± 12.1^{b}
PBS-3	16.6 ± 6.0^{ab}	0.02 ± 0.0^{abc}	0.06 ± 0.0	26.8 ± 8.1^{b}	34.6 ± 7.8^{b}	42.3 ± 9.5^{b}
PBS-4	38.9 ± 7.5^{ab}	0.07 ± 0.0^{e}	0.10 ± 0.0	4.3 ± 3.2^{ab}	9.0 ± 4.5^{ab}	11.1 ± 5.4^{a}

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences ($P < 0.05$)

Table 9. Oxidative status of sperm specimens diluted with extenders and stored at 4 °C for 96 h.

Groups	CP (U/g protein)	SH (mmol g^{-1} protein)	TAS (mmol trolox eq. g^{-1} protein)	LOOH (μmol g^{-1} protein)	TOS (μmol H_2O_2 eq. g^{-1} protein)	OSI (arbitrary units)
TRIS-1	12.8 ± 1.0[b]	0.01 ± 0.0[abc]	0.08 ± 0.0	15.6 ± 2.9[b]	22.0 ± 2.6[b]	49.4 ± 10.5[abc]
TRIS-2	11.4 ± 2.1[a]	0.02 ± 0.0[abc]	0.03 ± 0.0	15.0 ± 2.0[b]	24.4 ± 1.9[b]	32.4 ± 10.8[abc]
TRIS-3	52.2 ± 36.4[ab]	0.04 ± 0.0[bc]	0.04 ± 0.0	16.1 ± 5.8[b]	25.1 ± 7.1[b]	25.5 ± 5.1[abc]
TRIS-4	16.2 ± 0.3[ab]	0.04 ± 0.0[c]	0.06 ± 0.0	24.5 ± 16.3[b]	28.3 ± 14.4[b]	28.0 ± 14.0[abc]
TL-HEPES-1	20.1 ± 3.2[ab]	0.02 ± 0.0[abc]	0.04 ± 0.0	22.8 ± 3.8[b]	29.9 ± 4.2[b]	39.8 ± 10.9[abc]
TL-HEPES-2	16.8 ± 2.2[ab]	0.02 ± 0.0[abc]	0.05 ± 0.0	17.5 ± 5.4[b]	27.3 ± 7.4[b]	43.3 ± 14.2[abc]
TL-HEPES-3	27.4 ± 7.4[ab]	0.02 ± 0.0[abc]	0.05 ± 0.0	24.5 ± 4.6[b]	32.4 ± 5.4[b]	51.7 ± 11.2[bc]
TL-HEPES-4	29.2 ± 9.2[ab]	0.02 ± 0.0[abc]	0.05 ± 0.0	1.6 ± 0.7[a]	4.7 ± 0.3[a]	11.1 ± 2.2[ab]
TEST-1	22.4 ± 3.7[ab]	0.02 ± 0.0[abc]	0.03 ± 0.0	23.0 ± 4.3[b]	34.2 ± 5.8[b]	56.1 ± 10.6[bc]
TEST-2	38.6 ± 14.0[ab]	0.01 ± 0.0[ab]	0.06 ± 0.0	22.9 ± 7.8[b]	32.4 ± 7.1[b]	36.3 ± 10.9[abc]
TEST-3	28.2 ± 7.4[ab]	0.01 ± 0.0[a]	0.03 ± 0.0	22.0 ± 7.6[b]	29.0 ± 9.1[b]	28.9 ± 10.1[abc]
TEST-4	38.7 ± 12.2[ab]	0.01 ± 0.0[a]	0.04 ± 0.0	0.6 ± 0.4[a]	6.6 ± 0.8[a]	20.3 ± 4.8[abc]
PBS-1	25.0 ± 9.2[ab]	0.02 ± 0.0[abc]	0.06 ± 0.0	5.1 ± 2.3[ab]	9.2 ± 3.1[ab]	21.4 ± 8.0[abc]
PBS-2	15.6 ± 4.0[ab]	0.01 ± 0.0[a]	0.04 ± 0.0	26.6 ± 5.7[b]	36.7 ± 7.5[b]	41.8 ± 10.1[abc]
PBS-3	13.0 ± 3.0[ab]	0.01 ± 0.0[a]	0.04 ± 0.0	25.0 ± 6.3[b]	33.0 ± 6.6[b]	129.8 ± 49.6[c]
PBS-4	34.1 ± 3.6[b]	0.02 ± 0.0[abc]	0.08 ± 0.0	0.7 ± 0.3[a]	5.4 ± 1.0[a]	9.2 ± 2.8[a]

Values are mean percentages ± SEM (n = 6)
Different superscripts within the same column denote significant differences ($P < 0.05$)

fructose with 10% egg yolk (TTF-EY), and INRA 96 and reported that they obtained 60% to 65% motility and 15% to 25% progressive motility at 96 h. Similar to the present study, there were no significant differences between TES-Tris and Tris-based diluents. Mata-Campuzano et al. (47) reported that MITO levels were very low compared to those in other diluents when 3.5% soybean lecithin was added to TTF-EY diluent at 96 h. Although TRIS- and TES-based extenders are sufficient for acceptable motility in liquid storage, their effectiveness in maintaining the fertility rate remains unclear.

In the present study, glucose (27 mM) as an energy source, and EDTA (2 mg/mL) and BSA (3 mg/mL) as adjuvants were added to the final version of TRIS, TEST, TL-HEPES, and PBS extenders, and the activity of the extenders was evaluated. The highest motility and membrane integrity up to 24 h were observed in PBS-1. However, after 24 h, the values declined rapidly. In a study on rat sperm, a similar decline in semen parameters was observed. However, in the mentioned study, PBS was successful at room temperature (20). In our study, although PBS was strengthened with glucose, EDTA, BSA, and egg yolk, it did not have any protective effect in incubations longer than 24 h. Very successful results were obtained with TL-HEPES compared to those with PBS. In particular, with the formulation supported by egg yolk, glucose, and EP, at the end of 96 h, motility was higher than 50% (Table 1). Lower semen parameters were obtained with other formulations. While 17.5% motility was obtained at 96 h with the TL-HEPES + 0.75% EP formulation, 53.3% motility was obtained with the TL-HEPES + 0.75% EP + glucose formulation, and it was found to be as effective as TEST and TRIS.

In general, similar spermatological values were obtained with TEST 1-4, TRIS 1-4, and TL-HEPES-3 extenders. However, although the highest values are not statistically significant, they have small variations depending on spermatological parameters. While the highest motility at 96 h was achieved with TEST-3, the highest MB was achieved with TRIS-2, and the highest MMP level was achieved with TRIS-1. These 3 parameters ranged between 50% and 60% in TRIS and TEST solutions. As mentioned earlier, motility alone is not enough to predict the fertility of the sperm, and it is unable to explain the fertility decline over a period of more than 24 h in liquid storage (1). When we took the average of these 3 parameters of motility, membrane integrity, and MMP to determine the best extender, the solutions with the highest

average were TEST-3 with 61.9% and TEST-2 with 61.1%. When we consider the oxidative stress parameters, these results suggest different conclusions. It is noteworthy that lipid peroxidation and OS values of PBS-4, TL-HEPES-4, and TEST-4, which are EDTA- and BSA-added groups, were significantly lower than those of the others (Table 9). Considering that BSA delays time-dependent sperm aging (1,4), and that the number of oxidants exceeding the capacity of the antioxidant defense system causes infertility defects (48), sperm aging observed in periods longer than 24 h (1,4) can be due to LOOH and TOS, and EDTA and BSA may reduce this effect.

Egg yolk, which is widely used for long-term and liquid sperm storage, protects the spermatozoa membrane against cold shock and prevents the loss of or restores the membrane phospholipids. Egg yolk has a protective effect on the membrane (49) because it contains proteins, vitamins, phospholipids, glucose, and antioxidants. It has been reported that detergents such as STM paste, which are added to the egg yolk, increase the efficiency of egg yolk (50). In the present study, the protective effects of egg yolk and EP against cold shock were demonstrated. When the egg yolk is added directly to the extender, due to the macromolecules it contains, it restricts the field of view of the microscope and particularly prevents the use of computer-assisted semen analysis (CASA). There is also the risk of contaminating the extender due to the animal origin of egg yolk. However, centrifuged egg yolk (CEY) becomes a clear liquid and, therefore, does not prevent sperm analysis with CASA and the risk of contamination is reduced as it can pass through the microfilters. In our study, the positive and negative effects of both forms of egg yolk on ram sperm were observed. While standard egg yolk was used in TRIS-1, centrifuged egg yolk was used in TRIS-2, and no difference was found in terms of spermatological parameters. The addition of 0.75% EP to the solutions did not have a statistically significant effect, although it is useful in obtaining a clearer solution. However, in preliminary trials, addition of 1.5% to 3% EP caused a sharp decline in spermatological parameters, and thus it was not included in the present study. Different results were obtained in studies investigating the effect of EP. It was reported to protect acrosome integrity in the freezing process of cat sperm (29), whereas it was reported to shorten the life span of the sperm at 5 °C by initiating sperm activity in dogs (51). In Awassi sheep, it was found to significantly increase the life span of sperm during 6 h of incubation at 37 °C after freezing. In parallel with our study, it is seen as a chemical that contributes to the freezing process by extending the life span of sheep sperm both during and after the freezing process (32).

The data obtained in the present study suggest that ram sperm can be successfully stored for up to 96 h using TEST, TRIS, and TL-HEPES-3. It was observed that in some solutions adding centrifuged egg yolk and EP to the extender positively contributes to sperm parameters. It was also determined that high oxidative stress parameters were not directly related to motility, MB integrity, or MMP, and BSA and EDTA had a reductive effect on oxidative stress parameters.

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