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Research Article

Effect of Equex on Ram Semen in Different Freezing Extenders [1]

Ebru ŞENGÜL [1 (*)] Cemal DAYANIKLI [1] Bilge ALPCAN KÜNYELİ [1] İsmail ÇOBAN [1] Bülent BÜLBÜL [2] Burcu ÜSTÜNER [3] Zekariya NUR [3]

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[1] Department of Breeding Techniques, Sheep Breeding Research Institute, TR-10200 Bandırma, Balıkesir - TÜRKİYE
[2] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Dokuz Eylül University, TR-35890 Kiraz, İzmir - TÜRKİYE
[3] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Bursa Uludağ University, TR-16059 Görükle, Bursa - TÜRKİYE

[(*)] **Corresponding author:** Ebru ŞENGÜL

Tel: +90 266 738 0080
Cellular phone: +90 505 370 4094
Fax: +90 266 738 0083

E-mail: ebru.sengul@tarimorman.gov.tr

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Abstract

This study was carried out to determine the effects of Equex on ram semen freezability in three different extenders. Semen samples were collected from five rams and divided into six equal aliquots. At first, three non-commercial base extenders **(E1:** Tris-based extender, E2: Tris based extender+ Cystein and E3: Skim Milk Powder (SMP)) were prepared. Each base extender was divided into two parts; a base extender (E1, E2 and E3) or fortified with Equex (E1E, E2E and E3E). The pooled semen was diluted according to the two-step dilution method, and cryopreserved using a programmable freezing machine. Sperm motility parameters were examined by CASA. The morphological properties were evaluated by flow cytometer. It was observed that the addition of Equex had a positive effect on post-thaw motility and live spermatozoa ($P<0.001$), but had no effect on the LIN and Polarized mitochondria. While the Equex addition decreased post-thaw VCL, VSL, VAP and WOB values, it didn't have effect on the post-thaw intact acrosome rates ($P<0.05$). Furthermore, it was observed that Equex had a cryoprotective effect on post-thaw progressive motility in E1E and E3E groups ($P<0.05$), but was not observed in E2E group. In conclusion there was an interaction between Equex and extender ingredient. The cryoprotective effect of Equex was more evident in skim milk-based extender than that in tris-based extender.

Keywords: Cryopreservation, Equex, Kinematic parameters, Ram remen

Introduction

In livestock, artificial insemination (AI) with cryopreserved semen is one of the most important tools to improve the reproductive efficiency. Freeze-thaw process results in biological and functional changes that adversely impairs fertilizing ability by altering sperm mitochondria, acrosome and plasma membrane functional integrity [1]. The composition of semen freezing extenders helps to stabilize the cells during the cooling and storage process. Ram semen has been diluted with various extender ingredients, both defined and undefined, to maintain sperm fertilizing ability during processing and storage [2-6].

Semen collection procedures, extender composition, dilution, cooling, and freezing rates [2,7,8] as well as the type and concentration of cryoprotective agents used [3,5,6], temperature at which cryoprotectant is added to the semen [9], equilibration period [7], and thawing rate [10] can all result in the loss of post-thaw sperm fertilizing ability. Some researchers have attributed this decline in fertility at post-thaw stage to reduced transport ability and survival of ram spermatozoa in the female reproductive tract, along with diminished viability, morphology, and DNA integrity during freezing and thawing procedures [3,6,11]. Another reason for reduced fertility in sheep intra-cervical inseminated with frozen-thawed semen is the anatomical structure of the ewe's cervix and the passage of viable spermatozoa through the cervix. Therefore, there is still a need to optimize cryopreservation and breeding protocols for ram semen [12-14].

Semen extenders have been designed to protect and maintain spermatozoa during the processing and storage

of semen [15]. Various homemade or commercially available extenders have been developed and they are supplemented with different chemicals that reduce cryodamage or oxidative stress with varying levels of success. Skim milk or Tris-based extender are widely used components of semen extenders in most species, showing successful results both *in vitro* and *in vivo* [16,17]. The protective effect of skim milk is provided by the different components that present in the milk [18].

The semen freezing extenders and additives protect spermatozoa metabolism in many ways, including stabilizing the plasma membrane and maintaining intracellular and intramembrane ionic concentrations, thus reducing cold shock damage and osmotic shock. Tris-based extenders with some additives (citrate, glucose, and trehalose, etc) are widely used for liquid/frozen mammalian semen storage [5,6,15,19-24]. The success of Tris-based extender has been attributed to its buffering capacity against changes in pH and tonicity [25]. The other extender components are fructose, citric acid and egg- yolk which are energy sources and protect the cell membrane during cooling process [25,26]. Aisen et al.[20] enriched Salamon and Ritar's Tris based ram semen extender by adding trehalose, and improved the cryo-protective properties of this extender by cystein additives [4].

Equex is a non-permeating cryoprotective agent and a surfactant stabilizes the acrosomal membranes and protects spermatozoa against freeze-thaw toxic effects [27-29]. How Equex stabilizes sperm cell membranes during freeze-thaw process is still being investigated [28]. Equex is a well-known supplement for semen freezing and it has been extensively studied in bull [30], dog [31], stallion [32], boar [27], and gazelle [33]. However, there is a lack of research on the effect of Equex on post-thaw quality of ram semen diluted with various extenders. Also, such a wide range of spermatological features have not been evaluated together in previously published studies [28,34]. Furthermore, in the literature review conducted, no study was found on the effect of Equex in a skim milk-based extender.

The aim of this study, therefore, was to evaluate the effects of Equex on post-thaw motility and motion parameters, plasma membrane and acrosome integrity, and mitochondrial membrane potential (MMP) of ram spermatozoa in extender formulas, that were documented as successful for ram semen freezing.

Material and Methods

Ethical Statement

All procedures were approved by the local ethics committee of Sheep Breeding Research Institute, Ethics Committee on Animal Research (25/05/2018,78255852-050.01.04/0149/1331374).

Animal Management and Semen Collection

The study was carried out during breeding season (September and October) in the Laboratory of Sheep Breeding Research Institute, Bandırma, Türkiye. All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA). Five adult Karacabey Merino rams from Sheep Breeding Research Institute (40.32 latitude, 27.91 longitude), fertility proven aged 2-3 years were used for semen collection. All rams were hosted under uniform management conditions.

Semen Collection and Freezing

Semen samples were collected from five rams by artificial vagina every other day in five replicates. Obtained ejaculates were primarily evaluated for colour, volume, wave motion and initial motility [6]. Normospermic semen (volume ≥0.9 mL, concentrations ≥1800 x 10^6 spz/mL (Photometre Ovine Acucell®- IVM Technologies, France), wave motion ≥4 (1-5), progressive motility ≥70%) were pooled to avoid individual variations.

All the diluent formulas used in the study were prepared according to the formulas stated in the previous studies of Aisen et al.[20], Alçay et al.[4] and Kulaksız et al.[17]. Pooled semen was divided into six equal aliquots. Each aliquot was diluted with one of the extenders below at 400×10^6 spz/mL according to two-step dilution technique [6]. Three base extenders; E1: Tris based extender (Sigma® T6791 Tris 2.71 g, Sigma® F3510 D-Fructose 1.4 g, Sigma® C2404 Citric acid 1.0 g, %20 eggyolk) + Trehalose 50 mM Sigma® T0167) [20], E2: Tris based extender (Sigma® T6791 Tris 2.71 g, Sigma® F3510 D-Fructose 1.4 g, Sigma® C2404 Citric acid 1.0 g, 20% eggyolk, Sigma® T0167 Trehalose 50 mM) +1 mM Cystein (Sigma® C7352) [4] and E3: Skim Milk Powder (SMP) (10 g SMP (Sigma® 70166), 1 g of glucose (Sigma® G7021), 5% egg yolk, 5% glycerol) [17] were used. Each extender was divided into two parts; a base extender (E1, E2 and E3) or fortified with equex (Equex-Paste, Minitube, Tieffenbach Germany, REF.13560/0030) (E1E, E2E and E3E) at a ratio of 0.75% v/v. Diluted semen was cooled to +4°C at a rate of -0.3°C/min in 2 h. Second step dilution was at +4°C at 5 times with 10 min intervals [6].

Then, the samples were allowed to equilibrate for 2 h. Equilibrated semen was filled in 0.25 mL straws (MRS-1 Dual® IMV, France) and frozen (Mini-Digitcool® IMV, France) using IMV advised freezing protocol (4°C to -10°C 5°C/min, -10°C to -55°C 80°C/min, -55°C to -100°C 40°C/min occurred at a rate of 20°C/min from -100°C to -140°C). Frozen straws were stored in liquid nitrogen (-196°C) until thawing for further evaluation. Minimum one week after freezing, at least 3 straws from each group of the independent replicates were thawed at 37°C for 30 sec. for post-thaw semen evaluation.

Post-thaw Semen Evaluation

Sperm Motility: Sperm motility was examined by computer-assisted sperm analyser (CASA) (SCA®, Version 6.3; Microptic, Spain). Tris-based egg yolk free extender at the ratio of $16x10^6$ spz/mL was used to re-dilute semen samples. Progressive motility and total motility values were determined by CASA after 3 µL of re-diluted sample was placed on the slide recommended by the manufacturer (Leja®, Ref. 025107, IMV Technologies, France). While evaluating spermatozoon movement characteristics, field setting was as; min-max 15-70 μm^2, speed settings; set to static <10 µm/s, slow to medium >45 µm/s, fast >75 µm/s, progressive (STR>80). The examined parameters were; total, progressive, rapid and slow motility and the kinematic parameters (linearity (LIN), straightness (STR), curvilinear velocity (VCL), rectilinear velocity (VSL), average path velocity (VAP), wobble (WOB) and amplitude of lateral head displacement (ALH)).

Flow Cytometry Analysis: Plasma and Acrosome Membrane Integrity and Mitochondrial Membrane Potential were analysed by Guava EasyCyte flow cytometry (Guava Technologies Inc., Hayward, CA, USA; distributed by IMV Technologies). At least 5000 sperm cells were counted for each sample. The analyser was routinely checked by the Guava Check kit daily in terms of accuracy (Guava Technologies, Inc., Millipore, Billerica, MA, USA).

Viability and Acrosome Membrane Integrity: The sperm viability and acrosome membrane integrity were evaluated by Easykit 5 (ref. 025293; IMV Technologies) according to the manufacturer recommendations. Briefly, approximately 40000 spermatozoa were added in each well and incubated with 200 µL of EasyBuffer B (Ref. 023862; IMV Technologies) at 37°C for 45 min in the dark. The proportion of intact or damaged sperm membranes was calculated by the software program (EasySoft, ref. 024842; IMV Technologies).

Mitochondrial Membrane Potential: EasyKit 2 (ref. 024864; IMV Technologies) was used to analyse sperm mitochondrial potential according to the manufacturer recommendations. In summary, at the beginning, 10 µL of ethanol was added to the 96 ready-to-use wells to dissolve the fluorochrome. Next, 190 µL of Easybuffer B (Ref. 023862; IMV Technologies) and semen were added, for a total of 50000 sperm. After incubation of the sample in the dark for 30 min at 37°C, analyses were performed until 5000 spermatozoa were counted.

Statistical Analysis

All sperm parameters were considered as Mean ± Standard Error. The data obtained after thawing were analysed by one-way analysis of variance (ANOVA). Tukey comparison method was used to compare the differences between the groups and it was considered significant at the $P<0.05$ level. All statistical analyses were analysed in the SPSS package program.

Results

The effects of Equex in three different freezing extenders were evaluated in five replicates. Pooled semen mean volume, TM, PM and sperm concentration were 4.7 mL, 89.6%, 75.4% and 4.3×10^9 spermatozoa/mL, respectively. There was no statistical difference observed between the mean TM, PM and live sperm with intact acrosome parameters in different extenders E1, E1E, E2, E2E, E3, E3E after dilution, cooling and equilibration.

The effects of different extenders on post-thaw sperm motility-related parameters (TM, PM, RPM, MPM and SPM) are shown in *Table 1*. Freeze-thaw process resulted in reduced TM ($P<0.01$), PM ($P<0.01$) and live sperm with intact acrosome rate ($P<0.001$). The post-thaw TM was not significantly, but numerically higher in the E1 and E2 groups compared to the E3 group. For the post-thaw progressivity (PM, Rapid, Medium and Slow), there was no difference between base extender groups except medium progressivity ($P<0.05$).

Table 1. *The effect of extender and Equex on the post-thaw sperm motility related parameters (Mean±SE)*

Item	n	Total Motil (%)	Progressive Motil (%)	Rapid Motil (%)	Medium Motil (%)	Slow Motil (%)
E1	15	58.0±2.1^{bc}	44.5±1.9^{b}	36.6±1.7^{b}	15.4±1.0^{a}	6.0±0.4
E1E	15	68.2±3.0^{ab}	48.3±2.8^{ab}	40.2±2.3^{b}	19.4±1.6^{a}	8.7±0.9
E2	15	64.3±2.6^{abc}	51.5±2.3^{ab}	42.3±2.1^{b}	14.8±1.3^{a}	7.2±0.8
E2E	15	69.4±2.4^{a}	49.9±2.2^{ab}	44.2±1.9^{ab}	16.7±1.4^{a}	8.5±0.8
E3	15	54.8±2.8^{c}	45.3±2.4^{ab}	42.2±2.2^{b}	5.9±0.7^{b}	6.7±0.8
E3E	15	65.5±2.4^{ab}	54.2±2.0^{a}	50.9±2.0^{a}	7.8±0.9^{b}	6.7±0.5
Total	90	63.4±1.2	49.0±1.0	42.7±0.9	13.3±0.7	7.3±0.3

^{a,b,c} *Different letters in the same column at post-thaw stage indicates significant difference between groups (P<0.05);* ***E1:*** *Tris based extender (Tris 2.71 g, D-Fructose 1.4 g, Citric acid 1.0 g, 20% eggyolk, Trehalose 50 mM);* ***E1E:*** *E1 + Equex at a ratio of 0.75% v/v;* ***E2:*** *Tris based extender + 1 mM Cystein;* ***E2E:*** *E2+ Equex at a ratio of 0.75% v/v;* ***E3:*** *Skim Milk Powder (SMP);* ***E3E:*** *E3 + Equex at a ratio of 0.75% v/v*

***Table 2**. The effect of Equex on the post-thaw kinematics in different extenders (Mean±SE)*

Item	n	LIN	STR	VCL	VSL	VAP	WOB	ALH
E1	15	58.7±1.6^{ab}	67.0±1.8^{a}	98.6±1.5^{b}	55.4±1.8^{ab}	85.7±1.5^{bc}	86.4±0.6^{a}	2.5±0.1^{c}
E1E	15	55.1±1.8^{bc}	63.8±1.8^{ab}	93.2±1.4^{b}	49.4±2.3^{bc}	80.5±1.8^{c}	85.5±0.8^{a}	2.4±0.0^{c}
E2	15	61.6±1.7^{a}	70.6±1.8^{a}	97.0±1.6^{b}	58.8±2.1^{a}	84.5±2.0^{bc}	85.9±0.9^{a}	2.4±0.1^{c}
E2E	15	49.4±1.8^{cd}	58.2±1.9^{b}	94.5±1.5^{b}	44.5±1.8^{c}	80.5±2.0^{c}	84.4±1.0^{a}	2.6±0.1^{c}
E3	15	44.4±1.2^{de}	58.1±1.2^{b}	134.0±2.6^{a}	55.6±1.8^{ab}	101.3±2.2^{a}	74.5±0.6^{b}	4.2±0.1^{b}
E3E	15	41.6±1.2^{e}	59.9±1.9^{b}	132.4±1.8^{a}	51.2±1.9^{abc}	91.1±2.7^{b}	68.8±1.3^{c}	4.6±0.1^{a}
Total	90	51.8±1.0	62.9±0.9	108.3±2.0	52.5±0.9	87.3±1.1	80.9±0.8	3.1±0.1

^{a,b,c,d,e} *Different letters in the same column at post-thaw stage indicates significant difference between groups (P<0.05);* ***E1:*** *Tris based extender (Tris 2.71 g, D-Fructose 1.4 g, Citric acid 1.0 g, 20% eggyolk, Trehalose 50 mM);* ***E1E:*** *E1 + Equex at a ratio of 0.75% v/v;* ***E2:*** *Tris based extender +1 mM Cystein;* ***E2E:*** *E2 + Equex at a ratio of 0.75% v/v;* ***E3:*** *Skim Milk Powder (SMP);* ***E3E:*** *E3 + Equex- at a ratio of 0.75% v/v*

The effects of Equex on the post-thaw sperm motility-related parameters in different extenders are presented in *Table 2*. In general, there was no statistical difference between TM, PM, and slow progressive motility of Equex supplemented groups except for medium and rapid progressive motility (P<0.05). The medium progressive motility of the E3E group was lower and rapid progressive motility was higher than the E1E and E2E groups (P<0.05).

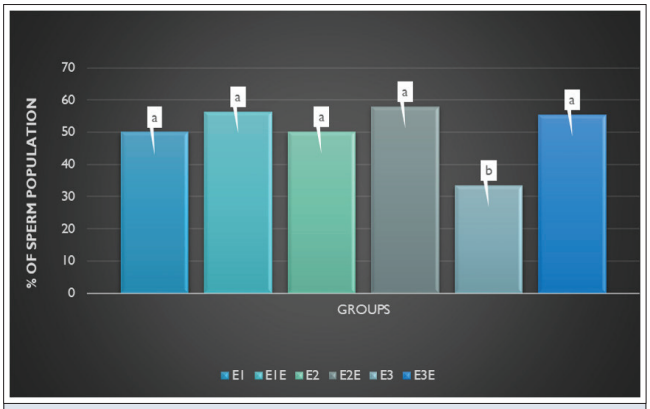

Fig 1. The percentage (%) of sperm with live and intact acrosome parameters at post-thaw stage. ^{a,b}: The difference between groups at post-thaw stage is significant (P<0.05); **E1:** Tris based extender (Tris 2.71 g, D-Fructose 1.4 g, Citric acid 1.0 g, 20% eggyolk, Trehalose 50 mM); **E1E:** E1+ Equex at a ratio of 0.75% v/v; **E2:** Tris based extender + 1 mM Cystein; **E2E:** E2+ Equex at a ratio of 0.75% v/v; **E3:** Skim Milk Powder (SMP); **E3E:** E3 + Equex at a ratio of 0.75% v/v

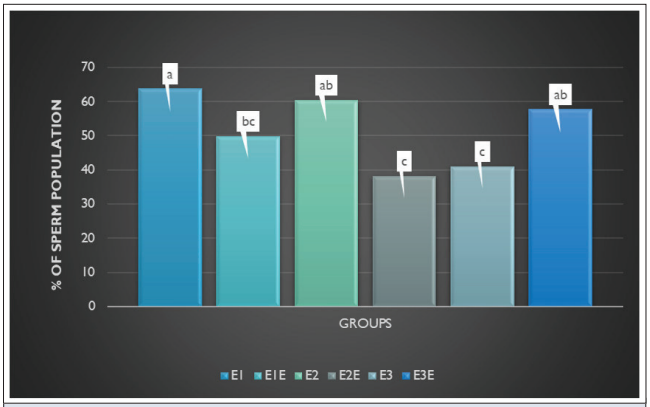

Fig 2. The percentage (%) of sperm with polarized mitochondria at post-thaw stage. Polarized mitochondria is a sperm with high fluoroscense (with Δψm high). ^{a,b,c}: The difference between groups at post-thaw stage is significant (P<0.05); **E1:** Tris based extender (Tris 2.71 g, D-Fructose 1.4 g, Citric acid 1.0 g, 20% eggyolk, Trehalose 50 mM); **E1E:** E1+ Equex at a ratio of 0.75% v/v; **E2:** Tris based extender + 1 mM Cystein; **E2E:** E2+ Equex at a ratio of 0.75% v/v; **E3:** Skim Milk Powder (SMP); **E3E:** E3 + Equex at a ratio of 0.75% v/v

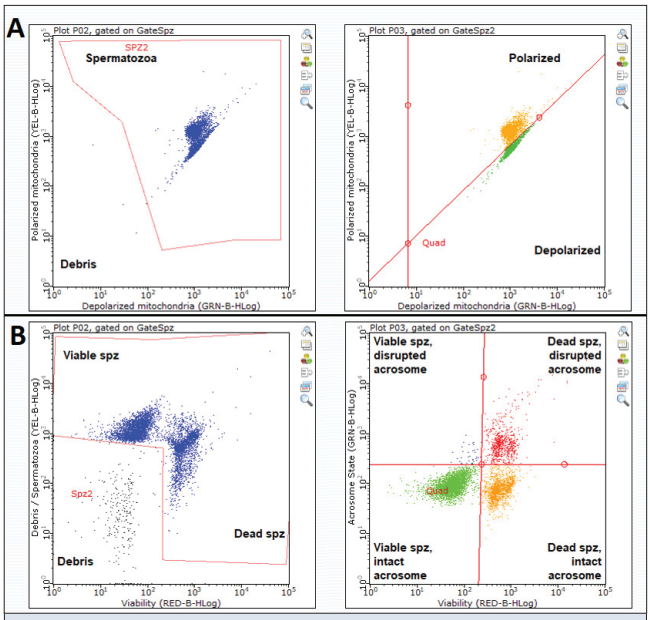

Fig 3. An example for the dotplots of flow cytometer analyses**.** A- Flow cytometry analyses of one experiment showing cells sorted dotplots using Easy kit-2 (mitochondrial activity). Polarized mitochondria is a sperm with high fluoroscense (with Δψm high). At the right dotplot; Orange population; Polarized mitochondria/High Mitochondrial Potential, Green population; Depolarized mitochondria/Low Mitochondrial Potential; B-Flow cytometry analyses of one experiment showing cells sorted dotplots using Easy kit-5 (viability & acrosome integrity). At the right dotplot; Green population: Viable and intact acrosome, Orange population: Dead and intact acrosome, Red population: Dead and damaged acrosome, Blue points: Viable but damage acrosome

The post-thaw mitochondrial activity and live sperm with intact acrosome in function of extender and Equex are represented in *Fig.1*, *Fig. 2*. The extenders ingredients affected post-thaw percentage of sperm with polarized mitochondria and live sperm with intact acrosome. The post-thaw percentage of sperm with polarized

mitochondria and live sperm with intact acrosome of Tris based extender groups (E1 and E2) was higher than that of skim milk based extender group (E3) (P<0.05). It was observed that Equex addition resulted in higher post-thaw live sperm with intact acrosome rates in E1E (proportional), E2E (proportional) and E3E (significant, P<0.05) groups. While the addition of Equex decreased the post-thaw polarized mitochondria rates in the tris based diluent groups (E1E and E2E), it increased in the milk-based extender group (E3E) (P<0.05).

There was an interaction between extenders ingredients and post-thaw sperm LIN and STR *(Table 2)*. The LIN and STR of the E1 and E2 groups were better than that of the E3 group (P<0.05). The Equex supplementation of freezing extender decreased sperm LIN and STR all groups. There was a significant decrease in LIN and STR of the E2E group compared to the E2 group (P<0.05). The post-thaw VCL, WAP and ALH of skim milk based extender group (E3) were higher than that of tris based extender groups (E1 and E2) (P<0.05). The Equex addition was decreased post-thaw VCL, VSL, VAP and WOB values. The ALH values of skim milk based extender groups (E3 and E3E) were higher than that of all the tris based extender groups (P<0.05).

Discussion

In the present study, we investigated the effect of Equex in different laboratory prepared extenders to preserve sperm quality during freeze-thawing. We evaluated sperm motility and motion parameters, live sperm with intact acrosome integrity, and mitochondrial activity. The mean volume of the pooled semen (4.7 mL), the percentage of motile sperm (89.6%), the percentage of progressively motile sperm (75.4%) and the sperm concentration (4.3 × 10^9 spermatozoa/mL) were similar to the results of previous studies [5,19,35,36].

The ingredients of extenders interact with different parts of spermatozoa leading to osmotic and toxic stresses and inducing biochemical changes in cell metabolism immediately after dilution [9,37,38]. Semen extenders provide energy for maintaining sperm motility [39]. As expected, the diluted sperm had slightly higher TM and PM than the pooled semen. There were no differences between the mean TM, PM, and live sperm with intact acrosome parameters in the different extenders (E1, E1E, E2, E2E, E3, E3E) after dilution, cooling, and equilibration.

The freeze-thaw process can damage spermatozoa, causing unfavorable changes in their membrane lipid composition, acrosome status, motility, motion parameters, morphology, and viability [39,40]. In our study, freeze-thaw process resulted in reduced TM (P<0.01), PM (P<0.01) and live sperm with intact acrosome rate (P<0.001) in all groups. So many studies have been conducted using different semen extenders and additives for protecting ram sperm fertilizing ability during freeze-thawing process [5,6]. Tris and Test buffers have been commonly incorporated into extender and help in sustaining sperm motility and fertilizing ability [21-24]. In addition, skim milk and egg yolk based extenders are widely used for preserving ram semen [17,36]. In liquid storage of ram semen, usage of the skim milk-based extender is superior to Tris-based extender [41]. The Tris based extender groups (E1 and E2) resulted in higher post-thaw TM (P>0.05), and live sperm with intact acrosome compared to the skim milk based extender (E3) group (P<0.05). For the post-thaw progressivity related parameters (PM, Rapid, Medium and Slow), there was no difference between groups except E3 medium progressivity (P<0.05).

Equex addition to the extender (0.7 percent Equex in Fiser's extender) improves post-thaw ram sperm motility [34]. It acts as an emulsifier to the egg yolk particles of the extender, and promotes the cryoprotective effect by facilitating the interaction of egg yolk proteins with the sperm plasma membrane [11,42]. Spermatozoa change their behaviour in response to environmental changes and storage. Therefore, it is likely that obtained changes in the sperm motions pattern, reflect the distinctions of the process [33]. The addition of Equex in semen freezing extenders increased post-thaw TM, rapid medium and slow progressive motility numerical in E1E and E2E groups, and significantly in E3E (P<0.05) group.

Sperm motility and membrane integrity are positively correlated with sperm fertilization ability [43]. The extender ingredients and freeze-thaw process modify the structure of the sperm cell membrane that results in mitochondrial damage, and alters the metabolic functions of spermatozoa by reducing the ATP-producing capacity of mitochondria [44]. In our study, post-thaw sperm polarized mitochondria was higher in Tris based extender (E1 and E2) than skim milk based extender (E3) (P<0.05). Equex acts as a non-permeating cryoprotectant agent [11,42]. For the Equex supplemented groups, post-thaw polarized mitochondria rates in the milk-based extender group (E3E) was higher than Tris based extender groups (E2E) (P<0.05). This could be explained by the fact that Equex facilitates the interaction of milk powder proteins such as egg yolk proteins with the sperm plasma membrane [11,42].

The sperm plasma and acrosome membrane integrity is necessary to maintain sperm functionality during storage and also in the female reproductive tract [13]. It was observed that live sperm with intact acrosome in Tris-based extender groups (E1 and E2) was higher than in the skim milk based extender group (E3) (P<0.05). The mechanism whereby Equex exerts its protective effect on the sperm remains unclear [45]. The active ingredient of Equex is sodium dodecyl sulfate (SDS), which has a

detergent property. SDS is known to have a toxic effect on sperm membranes and viability in bull [29]. Extender supplementation with Equex has been reported to promote the post-thaw recovery of motile sperm and intact acrosome in ram [34] and cat epidymal spermatozoa [45]. Equex addition resulted in higher post-thaw live sperm with intact acrosome rates in E1E (proportional), E2E (proportional) and in E3E (significant, P<0.05) groups.

When assessing the potential fertilizing capacity of sperm, it is imperative to keep in mind that each spermatozoon is a multi-compartmental cell required to have various features in order to fertilize an oocyte [46]. For many years, the CASA analysis has been one standard in the laboratory for motility and kinematic parameter assessment [47]. There was an interaction between extender ingredients and post-thaw sperm LIN and STR parameters in our study. The LIN and STR of the tris based extenders groups (E1 and E2) were higher than the skim milk based extender group (E3) (P<0.05). The Equex supplementation of the freezing extender decreased sperm LIN and STR in all groups. In addition, Equex decreased post-thaw VCL, VSL, VAP and WOB values. These results might be due to the increased osmotic environment and the altered sperm membrane functional integrity by the Equex.

Sperm kinematic parameters such as VCL, VSL, ALH, VAP, LIN, STR and WOB are associated with fertility [48,49]. The post-thaw VCL, WAP and ALH of skim milk based extender (E3) were higher than tris based extender groups (E1 and E2) (P<0.05). Sperm velocity-related parameters are an indirect indicator of mitochondrial function [50] and fertility [48]. It was observed that there was a decreasing tendency in sperm velocity parameters of all Equex-supplemented groups.

As a result of our study, it was concluded that there is an interaction between Equex and extender ingredient. The addition of Equex to sperm extenders positively affected post-thaw sperm quality in all groups, but this positive effect was more evident in skim milk-based extender than in tris-based extender. For the tris-based extender groups, the positive effect of Equex addition was less evident in the cystein-containing group (E2E).

Declarations

Availability of Data and Materials: The data underlying this study are available from the corresponding author upon reasonable request (E. Şengül).

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Conflict of Interest: The authors have no conflict of interest to report.

Author Contributions: EŞ, CD: Experimental design, spermatological analyses, writing manuscript; BAK: Spermatological analyses; İÇ: Semen collection; BB, BÜ: Writing and editing manuscript; ZN: Experimental design, writing & editing manuscript, visualization, supervision. All authors read, revised, and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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