

Short Term Preservation of Ram Semen with Different Extenders

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Summary

The objective of the present study was to evaluate the changes in daily spermatozoa motility, hypoosmotic resistance test, dead and abnormal spermatozoa rates of ram semen was diluted and stored at 4°C. A total of 25 ejaculates were collected from five rams via an artificial vagina to evaluate the spermatological features of ram semen. Thereafter, an additional 25 ejaculates were collected for the dilution process. The ejaculates were pooled and diluted with five different extenders namely Tris (T), sodium citrate (SC), milk-powder (M), glucose phosphate (GP) extenders of which include egg-yolk, and AndroMed® (A) egg-yolk free diluent. Being preserved at 4°C, the spermatological properties were daily evaluated until no motile spermatozoon was observed. It was observed that certain spermatological properties of ram semen were in optimal range. After dilution process, all spermatological parameters were influenced by the storage time and extenders ($P<0.01$). However the extenders had no effect on primary spermatological parameters of ram semen. Contrary to the gradual decrease in spermatozoa motility and hypoosmotic swelling test (HOST) value, the dead and abnormal spermatozoa rates increased gradually during the storage period. In addition, significant correlations were found between the spermatozoa motility and HOST and dead and abnormal spermatozoa rates ($r: 0.8$, $r: -0.8$, and $r: -0.9$, respectively, $P<0.01$). In conclusion, the spermatological features of ram semen diluted with T and SC and preserved at 4°C for a short term was found to be better preserved in a longer period than that of diluted by M, A and GP.

Keywords: *Extender, Ram semen, Short term storage, Spermatological features*

Koç Spermasının Farklı Sulandırıcılar ile Kısa Süre Saklanması

Özet

Sulandırılarak 4°C'de saklanan koç spermasındaki günlük spermatozoa motilitesi, hypoosmotik rezistans test, ölü ve anormal spermatozoa oranlarındaki değişimleri araştırmak amacıyla yapılan bu çalışmada kullanılan 5 koçtan sun'i vagina yardımıyla toplam 25 ejakulat spermatolojik özellikleri değerlendirmek, 25 ejakulat da sulandırma işlemleri için toplandı. Ejakulatlar 5 farklı sulandırıcı, yumurta sarısı içeren Tris- (T), sodium citrate (SC), süt tozu (M) ve glucose phosphate (GP) ve yumurta sarısı içermeyen AndroMed® (A) ile sulandırıldı. Daha sonra 4°C'de saklanarak spermatolojik özellikler motil spermatozoon görülmeyene kadar günlük olarak değerlendirildi. Koçların bazı spermatolojik özelliklerinin normal sınırlar içerisinde olduğu gözlemlendi. Sulandırma işlemi sonrasında ise bütün spermatolojik parametrelerin saklama süresinden ve sulandırıcılardan etkilendiği ($P<0.01$) tespit edildi. Ancak sulandırıcıların sulandırma sırasındaki spermatolojik özelliklere etkisi gözlemlenmedi. Saklama süresince spermatozoa motilitesi ve hypoosmotic swelling test (HOST) değerleri kademeli olarak azalırken ölü ve anormal spermatozoa oranlarının yine kademeli olarak arttığı belirlendi. Ayrıca spermatozoa motilitesi ile HOST ve ölü ve anormal spermatozoa oranları arasında önemli ilişkiler bulundu (sırasıyla $r: 0.8$, $r: -0.8$, $r: -0.9$, $P<0.01$). Sonuç olarak, 4°C'de sulandırılarak kısa dönem saklanan koç spermasının spermatolojik özelliklerinin T ve SC ile sulandırılanlarda M, A ve GP ile sulandırılanlardan belirgin olarak daha iyi ve daha uzun süre korunduğu sonucuna varılmıştır.

Anahtar sözcükler: *Sulandırıcı, Koç sperması, Kısa süre saklama, Spermatolojik özellik*

INTRODUCTION

Artificial insemination enables the genetic material of elite rams to spread from one farm to another. Inseminating a large number of ewes requires preservation of semen under artificial conditions. Since, artificial insemination with frozen ram semen has not been widely adopted, relatively poor semen

quality is being obtained when frozen semen is used for intra-cervical insemination. The process of freezing and thawing of semen causes injuries to ram spermatozoa and reduce fertility ¹⁻⁵. Diluted and cooled ram semen is an alternative choice to frozen semen if insemination is done within a short period



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of time after collection. Comparing with fresh semen, cooled ram semen suffers from a decrease in motility and morphological integrity, accompanied by a decline in the survival in the female reproductive tract, reduction in fertility and increased embryonic loss. These damages are less pronounced in diluted and chilled semen than in frozen-thawed ram semen⁶⁻⁹.

Inseminating at an optimal time is essential in heat to achieve an acceptable fertility result. Moreover, it is very important to preserve and store the semen under optimal conditions. The recommended maximum storage time after insemination is reported as 6-12 h¹⁰⁻¹⁴. Such a short storage time for transportation of the semen doses from the central station to the farms makes it difficult to inseminate the ewes at an optimal time in the heat. The practical use of liquid semen under farm conditions may be facilitated in case the semen is diluted and stored.

Extenders are in different compositions depending on the species, use, temperature and the duration of storage desired¹⁵⁻²⁰. Semen has been usually diluted with Tris plus egg yolk, glucose phosphate solution, egg yolk-citrate solution, homogenized whole milk, fresh and dried skim milk, coconut milk, lactose solution and the commercial diluents²¹⁻²⁴. However, all new methods for processing ram semen need to be tested before their practical application in the field. Therefore, to test the effectiveness of these methods in vitro assay is needed. Among these assays, those focusing on spermatological properties, such as motility parameters, osmotic resistance, live-dead and abnormal rates are considered to be reliable.

Few studies^{13,25,26} have been carried out to examine the properties of ram semen preserved in liquid and determine the effects of extenders on ram semen quality stored at 4-5°C. It would be interesting to evaluate the changes in the structural, functional and motility parameters of sperm during the cooled storage period in ram. Although motility changes during cooled storage has been studied, no study has been conducted to the changes in osmotic resistance and the live-dead and abnormal spermatozoa rates along with motility parameters in ram semen diluted with different extenders and stored at 4°C. Previous studies have been carried out on different ram breeds to investigate the semen quality^{14,25-28}. However none of these studies considered spermatological parameters of Pirlak ram semen. Moreover, no study was conducted using 5 different extenders together in the same experiment.

The objective of the present study was to evaluate the changes in daily spermatozoa motility, hypo-osmotic resistance test, live-dead and abnormal spermatozoa rates of ram semen that was diluted in five different extenders and stored at 4°C over a period of 14 days.

MATERIAL and METHODS

Animals and Management

This study was performed at the Animal Research Center of the Faculty of Veterinary Medicine, Afyon Kocatepe University during breeding season. The study was conducted on 5 sexually mature Pirlak (Daglic x Kivircik) rams with an average weight of 64.4 (S.E.M. 2.48) kg. All animals were around 2 years of age and kept outdoors during the daytime and housed in a semiopen barn at nights. Animals were fed by roughage and concentrate supplement and also received 500 g/day/head of concentrate mixture and 1.0 kg/day of dry alfalfa.

Extenders

Five extenders (T, SC, M, GP and A) were used in the present study. All chemicals were purchased from Sigma Chemical Co. The extenders were prepared as follows:

Extender T: Tris-based extender supplemented with 15% egg yolk (v/v) consisted of Tris (3.63 g), fructose (0.5 g), citric acid (1.99 g) and distilled water (100 ml).

Extender SC: Sodium citrate-based extender was prepared from a 2.9% aqueous solution of trisodium citrate and then supplemented with 20% egg yolk (v/v).

Extender M: Milk-powder extender was prepared out of non-fatty milk powder (11% [w/v]) and distilled water, heated to 95°C for 10 min, and then cooled to room temperature before addition of egg yolk (5% [v/v]).

Extender GP: Glucose phosphate extender was prepared from 1.54% Na₂HPO₄, 0.32% KH₂PO₄, 0.62% glucose and supplemented with 2% egg yolk (v/v).

Extender A: AndroMed® (egg-yolk free and concentrated medium) was a commercial diluent (Minitub, Tiefenbach, Germany).

Semen Collection and Processing

Five ejaculates were collected every other day from each of the five rams using an artificial vagina

and a total of 25 ejaculates were collected to evaluate the spermatological features such as semen volume, spermatozoa motility, spermatozoa concentration, HOST value, live-dead and abnormal spermatozoa rates of ram semen. After collection, the ejaculates were immediately immersed in a warm water bath at 37°C until their assessment in the laboratory. The volume of ejaculates was measured in a graduated conical tube at 0.1 ml intervals. The spermatozoa concentration was determined by means of a haemocytometer²⁹, and spermatozoa motility was estimated using a phase contrast microscope (400×).

Since the semen characteristics of Pirlak rams were in optimal range and in accordance generally reported findings in rams, additional 25 ejaculates were collected for dilution process. The ejaculates containing a semen volume varying between 1 and 2 ml, spermatozoa with >80% forward motility and concentrations higher than 3.0×10^9 spermatozoa/ml were used in dilution. Each ejaculate was equally transferred into 5 tubes and diluted with extenders at 1:1 (v/v) rates. Prior to storage at 4°C the first spermatological parameters estimation was realized and findings were saved as primary parameters. Daily spermatozoa motility, HOST value, dead and abnormal spermatozoa rates during storage at 4°C in different extenders were determined.

Stored Semen Evaluation

Spermatozoa motility estimations of the ram semen kept at 4°C were repeated once every 24 h for 4-14 days changing according to extenders. Spermatozoa motility was subjectively estimated using a phase contrast microscope (Olympus CX31, Olympus Optical Co., Ltd., Japan) with warm stage at magnification 400× according to the standard method²⁹. Each sample was examined from five different microscopic fields in at 37°C. The mean of the five estimations was considered as the final motility score.

Osmotic resistance test was performed according to Buckett et al.³² and Revell and Mrode³³, and it was evaluated according to Jeyendran et al.³⁴. The hypoosmotic swelling test (HOST) was used as a complementary test the viability assessment protocol. The assay was performed by mixing 20 µl of semen with 200 µl hypoosmotic solution (9 g fructose, 4.9 g trisodium citrate, distilled water to 1000 ml, 100 mOsm). This mixture was incubated at 37°C for 60 minutes. After incubation, 0.2 ml of the mixture was spread on a warm slide with a cover slip. A total of

400 spermatozoa were counted with a phase contrast microscope (1000× magnification, oil immersion). The percentages of spermatozoa with swollen and coiled tails were determined.

The dead spermatozoa rates were estimated via means of a stain of nigrosin-eosin mixture³⁰. The stain was prepared with 1.67 g eosin-Y, 10 g nigrosin and 2.9 g sodium citrate dissolved in 100 ml distilled water. The spermatozoa suspension smears were prepared by mixing a semen sample drop with two drops of the stain on a warm slide. Then, the stain was spread with a second slide immediately. The dead spermatozoa rates were assessed by counting 400 spermatozoa under phase-contrast microscope (400× magnification). While live spermatozoa were excluded, the dead spermatozoa were colored by red with eosin against the dark nigrosin.

The abnormal spermatozoa rates were assessed according to liquid fixation method in which at least three drops of each sample added to Eppendorf tubes containing 1 ml of Hancock solution. Hancock solution: sodium saline solution: 9.01 g NaCl in 500 ml double-distilled water. buffer solution: (A) 21.682 g $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ in 500 ml double-distilled water; (B) 22.254 g KH_2PO_4 in 500 ml double-distilled water. A total amount of 200 ml of (A) and 80 ml of (B) were mixed to obtain 280 ml of buffer solution. The final Hancock solution consisted of 62.5 ml formalin (37%), 150 ml sodium saline solution, 150 ml buffer solution, and 500 ml double-distilled water³¹. A drop of this mixture was placed on a slide and covered with a cover slip. The percentage of total spermatozoa abnormality (acrosomal abnormality, detached heads, abnormal mid-pieces and tail defects) was determined by counting a total of 400 spermatozoa under the phase contrast microscope (magnification 1000×, oil immersion).

Statistical Analysis

The obtained values were expressed as mean $\pm$ standard error of the mean (S.E.M.). Statistical analyses were performed using the Statistica® package program, version 6.0 (Statsoft Inc., Tulsa, OK, USA). Mean values were examined by analysis of variance (ANOVA). Tukey's post hoc test was used to compare the significance of the differences among five extender groups in terms of the effects on the spermatological features based on the storage period. Pearson's correlation coefficients were used to evaluate the correlations among spermatological parameters. Significance was considered at the $P < 0.05$ level³⁵.

RESULTS

The overall mean values of semen volume, spermatozoa motility, spermatozoa concentration, HOST value, dead and abnormal spermatozoa rates of Pirlak rams were found as 1.52 ± 0.14 ml, $82.4 \pm 2.0\%$, $4.24 \pm 0.22 \times 10^9$ spermatozoa/ml, $69.25 \pm 3.81\%$, $7.05 \pm 0.60\%$ and $4.65 \pm 0.32\%$ respectively, in which no significant difference was observed among the rams ($P > 0.05$).

Afterwards, ejaculates were diluted with different extenders and the first evaluations were realized. Prior to storage at 4°C , the mean values of the spermatozoa motility, HOST values, dead and abnormal spermatozoa rates were found as $81.6 \pm 2.45\%$, $68.7 \pm 1.04\%$, $6.9 \pm 1.16\%$ and $4.9 \pm 0.46\%$ respectively, and weren't affected by the extenders ($P > 0.05$). The average spermatological features of the first and the following days are presented in Tables. Differences between just after dilution and first day parameters were found to be significant in SC, M, A and GP for spermatozoa motility, in M and A for HOST values, in A and GP for dead spermatozoa rates and in SC and GP for abnormal spermatozoa rates ($P < 0.05$).

Subjective Spermatozoa Motility

The spermatozoa motility in all diluters was higher than 50% on day 2, whereas only those diluted with T and SC were over 50% on day 4 and all were lower

Table 1. Mean ($\pm$ S.E.M.) values of daily spermatozoa motility of ram semen during preservation at 4°C in different extenders (n: 25)

Tablo 1. Farklı sulandırıcılarda 4°C 'de saklanan koç spermasındaki ortalama ($\pm$ S.E.M.) spermatozoa motiliteleri (n: 25)

Days	Spermatozoa motility (%)				
	T	SC	M	GP	A
1	$78.0 \pm 3.7^{\text{Aa}}$	$74.0 \pm 2.4^{\text{Aa}}$	$72.0 \pm 3.7^{\text{ABa}}$	$68.0 \pm 2.0^{\text{Ba}}$	$72.0 \pm 2.0^{\text{ABa}}$
2	$69.0 \pm 4.7^{\text{Aa}}$	$70.0 \pm 0.2^{\text{Aab}}$	$66.0 \pm 4.2^{\text{ABab}}$	$52.0 \pm 2.0^{\text{Bb}}$	$58.0 \pm 4.9^{\text{Bb}}$
3	$59.0 \pm 3.7^{\text{Ab}}$	$62.0 \pm 3.7^{\text{Abc}}$	$58.0 \pm 3.8^{\text{Abc}}$	$12.0 \pm 8.0^{\text{Cc}}$	$48.0 \pm 3.7^{\text{Bc}}$
4	$56.0 \pm 2.0^{\text{Abc}}$	$56.0 \pm 5.1^{\text{Ac}}$	$46.0 \pm 5.1^{\text{Bcd}}$	0^{Cd}	$38.0 \pm 2.0^{\text{Bd}}$
5	$47.0 \pm 2.4^{\text{Acd}}$	$42.0 \pm 3.7^{\text{Ad}}$	$28.0 \pm 3.6^{\text{Bde}}$		$28.0 \pm 2.0^{\text{Be}}$
6	$42.0 \pm 2.0^{\text{Ad}}$	$26.0 \pm 5.1^{\text{Be}}$	$16.0 \pm 4.1^{\text{Cef}}$		$18.0 \pm 2.0^{\text{BCf}}$
7	$38.0 \pm 2.0^{\text{Ad}}$	$14.0 \pm 5.1^{\text{Bf}}$	$8.0 \pm 4.4^{\text{Bfg}}$		$8.0 \pm 2.0^{\text{Bg}}$
8	$34.0 \pm 2.0^{\text{Ade}}$	$6.0 \pm 4.0^{\text{Bfg}}$	$6.0 \pm 4.2^{\text{Bg}}$		$2.0 \pm 2.0^{\text{Bg}}$
9	$26.0 \pm 2.4^{\text{Aef}}$	$4.0 \pm 2.4^{\text{Bg}}$	$2.0 \pm 2.0^{\text{Bg}}$		0^{Ch}
10	$24.0 \pm 2.4^{\text{Af}}$	$2.0 \pm 2.0^{\text{Bg}}$	0^{Ch}		
11	$14.0 \pm 2.4^{\text{Ag}}$	0^{Bh}			
12	$12.5 \pm 2.5^{\text{Ag}}$				
13	$10.0 \pm 10.0^{\text{Ag}}$				
14	0^{Ah}				

T: Tris-based extender, SC: Sodium citrate-based extender, M: Milk-powder extender, GP: Glucose phosphate extender, A: AndroMed® diluent.

A-C: The different superscript uppercase letters in each row are statistically different among extenders ($P < 0.05$)

a-h: The different superscript lowercase letters in each column are statistically different among days ($P < 0.05$)

than 50% on day 5 (Table 1). The motility on the 3rd day was dramatically decreased ($P < 0.01$) only in GP. Same trend was observed in semen diluted with A and the gradual decrease on same day was significant ($P < 0.05$). Likewise, the decrease in spermatozoa motility of semen diluted with T, SC and M extenders was significant ($P < 0.05$). The spermatozoa motility was observed up to 13th day, but it was not observed on the 14th day in the semen diluted with T extender.

Osmotic Resistance

Decreases were found within all extenders at 4°C for the osmotic resistance test (Table 2). In relation to the time of storage, semen diluted with T showed the spermatozoa curling up to day 13, but these values were 10, 9, 3 and 8 days for SC, M, GP and A, respectively.

Table 2. Mean ($\pm$ S.E.M.) values of HOST of ram spermatozoa during storage at 4°C in different extenders (n: 25)

Tablo 2. Farklı sulandırıcılarda 4°C 'de saklanan koç spermatozoasındaki ortalama ($\pm$ S.E.M.) HOST değerleri (n: 25)

Days	HOST (%)				
	T	SC	M	GP	A
1	$68.8 \pm 0.6^{\text{Aa}}$	$68.5 \pm 8.4^{\text{Aa}}$	$62.4 \pm 1.8^{\text{ABa}}$	$66.1 \pm 3.8^{\text{Aa}}$	$53.0 \pm 3.9^{\text{Ba}}$
2	$63.7 \pm 0.8^{\text{Aab}}$	$65.1 \pm 0.9^{\text{Aab}}$	$51.6 \pm 3.1^{\text{ABb}}$	$56.7 \pm 7.2^{\text{ABa}}$	$43.8 \pm 2.6^{\text{Bab}}$
3	$61.1 \pm 1.2^{\text{Aab}}$	$60.1 \pm 2.5^{\text{Aabc}}$	$33.9 \pm 3.6^{\text{Bc}}$	$13.8 \pm 6.9^{\text{Cb}}$	$34.0 \pm 2.7^{\text{Bbc}}$
4	$57.3 \pm 1.6^{\text{Ab}}$	$58.3 \pm 2.9^{\text{Abc}}$	$26.4 \pm 2.9^{\text{Bcd}}$		$32.8 \pm 2.3^{\text{Bcd}}$
5	$54.2 \pm 1.9^{\text{Abc}}$	$51.7 \pm 2.7^{\text{Acd}}$	$22.6 \pm 3.4^{\text{Bd}}$		$28.8 \pm 1.2^{\text{Bcd}}$
6	$46.9 \pm 2.6^{\text{Acd}}$	$43.3 \pm 6.5^{\text{Ad}}$	$17.8 \pm 4.8^{\text{Bd}}$		$23.9 \pm 2.9^{\text{Bde}}$
7	$38.4 \pm 1.2^{\text{Ade}}$	$30.2 \pm 9.1^{\text{Ae}}$	$4.8 \pm 4.8^{\text{E}}$		$16.9 \pm 5.7^{\text{Bef}}$
8	$34.1 \pm 1.5^{\text{Aef}}$	$17.7 \pm 7.2^{\text{Bf}}$	$4.2 \pm 4.2^{\text{E}}$		$13.8 \pm 3.8^{\text{Bcf}}$
9	$33.4 \pm 2.2^{\text{Aef}}$	$7.4 \pm 4.6^{\text{Bg}}$	$3.4 \pm 3.4^{\text{E}}$		
10	$26.4 \pm 4.6^{\text{Af}}$	$2.6 \pm 2.6^{\text{Bg}}$			
11	$26.1 \pm 4.1^{\text{Af}}$				
12	$24.7 \pm 2.2^{\text{Af}}$				
13	$13.6 \pm 2.1^{\text{Ag}}$				

T: Tris-based extender, SC: Sodium citrate-based extender, M: Milk-powder extender, GP: Glucose phosphate extender, A: AndroMed® diluent.

A-C: The different superscript uppercase letters in each row are statistically different among extenders ($P < 0.05$)

a-g: The different superscript lowercase letters in each column are statistically different among days ($P < 0.05$)

Dead Spermatozoa Rate

The dead spermatozoa rate was significantly affected ($P < 0.01$) in semen diluted with M, A and GP compared with T and SC (Table 3). Differences between semen diluted with T and SC extenders were found non-significant ($P > 0.05$) for 3 days. The longest living spermatozoa were observed in T extender up to 13 days.

Abnormal Spermatozoa Rate

Regarding the percentage of abnormal spermatozoa, there were significant effects of extenders ($P < 0.05$) and storage times ($P < 0.01$). The abnormal spermatozoa

rates gradually increased during storage period (Table 4). The minimal increase was obtained daily as $1.04 \pm 0.19\%$ in semen diluted with T extender.

Table 3. Mean ($\pm$ S.E.M.) values of dead spermatozoa rate of ram semen during storage at 4°C in different extenders (n: 25)

Tablo 3. Farklı sulandırıcılarda 4°C 'de saklanan koç spermasındaki ortalama ($\pm$ S.E.M.) ölü spermatozoa oranları (n : 25)

Days	Dead spermatozoa rate (%)				
	T	SC	M	GP	A
1	7.7 $\pm$ 0.3 ^{Bk}	7.7 $\pm$ 1.0 ^{Bf}	7.3 $\pm$ 0.4 ^{Bi}	9.3 $\pm$ 1.4 ^{Ac}	9.6 $\pm$ 0.8 ^{Ag}
2	8.4 $\pm$ 0.4 ^{Gk}	8.2 $\pm$ 1.2 ^{Cf}	12.6 $\pm$ 1.2 ^{Ah}	11.1 $\pm$ 1.5 ^{Bb}	12.8 $\pm$ 1.4 ^{Af}
3	9.3 $\pm$ 0.5 ^{Cj}	9.4 $\pm$ 1.5 ^{Ce}	14.8 $\pm$ 1.4 ^{Ag}	15.0 $\pm$ 1.1 ^{Aa}	13.9 $\pm$ 1.4 ^{Be}
4	11.9 $\pm$ 1.3 ^{Ci}	10.1 $\pm$ 1.1 ^{De}	16.9 $\pm$ 1.7 ^{Af}		14.9 $\pm$ 1.0 ^{Bde}
5	12.7 $\pm$ 1.2 ^{Chi}	13.5 $\pm$ 1.1 ^{Cd}	18.6 $\pm$ 1.9 ^{Ae}		15.7 $\pm$ 1.2 ^{Bd}
6	13.7 $\pm$ 0.9 ^{Ggh}	14.4 $\pm$ 1.2 ^{Ccd}	21.8 $\pm$ 2.2 ^{Ad}		17.0 $\pm$ 1.1 ^{Bc}
7	14.0 $\pm$ 1.2 ^{Cfg}	14.8 $\pm$ 1.6 ^{Cc}	24.3 $\pm$ 4.6 ^{Ac}		18.9 $\pm$ 1.1 ^{Bb}
8	14.9 $\pm$ 1.1 ^{Cef}	15.1 $\pm$ 0.2 ^{Cc}	26.1 $\pm$ 5.2 ^{Ab}		21.1 $\pm$ 1.0 ^{Ba}
9	15.5 $\pm$ 1.1 ^{Ce}	17.4 $\pm$ 0.6 ^{Bb}	32.4 $\pm$ 6.3 ^{Aa}		
10	18.4 $\pm$ 1.2 ^{Ad}	18.6 $\pm$ 1.6 ^{Aa}			
11	23.2 $\pm$ 1.9 ^{Ac}				
12	25.3 $\pm$ 2.9 ^{Ab}				
13	36.0 $\pm$ 0.0 ^{Aa}				

T: Tris-based extender, SC: Sodium citrate-based extender, M: Milk-powder extender, GP: Glucose phosphate extender, A: AndroMed® diluent. A-D: The different superscript uppercase letters in each row are statistically different among extenders ($P < 0.05$)

a-k: The different superscript lowercase letters in each column are statistically different among days ($P < 0.05$)

Table 4. Mean ($\pm$ S.E.M.) values of abnormal spermatozoa rate of ram semen during storage at 4°C in different extenders (n: 25)

Tablo 4. Farklı sulandırıcılarda 4°C 'de saklanan koç spermasındaki ortalama ($\pm$ S.E.M.) anormal spermatozoa oranları (n: 25)

Days	Abnormal spermatozoa rate (%)				
	T	SC	M	GP	A
1	5.4 $\pm$ 0.5 ^{Ak}	5.7 $\pm$ 0.8 ^{Ae}	5.5 $\pm$ 0.8 ^{Ah}	5.8 $\pm$ 0.5 ^{Aa}	5.3 $\pm$ 0.7 ^{Ae}
2	5.9 $\pm$ 0.4 ^{Bjk}	5.9 $\pm$ 0.9 ^{Be}	7.9 $\pm$ 0.9 ^{Ag}	6.3 $\pm$ 0.8 ^{Ba}	5.6 $\pm$ 0.8 ^{Be}
3	6.1 $\pm$ 0.5 ^{Bjk}	6.2 $\pm$ 0.8 ^{Bde}	9.2 $\pm$ 0.8 ^{Af}	6.7 $\pm$ 0.9 ^{Ba}	5.9 $\pm$ 0.9 ^{Bde}
4	6.8 $\pm$ 0.4 ^{BCij}	7.2 $\pm$ 0.7 ^{Bcd}	11.6 $\pm$ 2.1 ^{Ae}		6.1 $\pm$ 0.8 ^{Cde}
5	7.5 $\pm$ 0.4 ^{BChi}	8.3 $\pm$ 1.9 ^{Bbc}	12.1 $\pm$ 2.6 ^{Ae}		6.7 $\pm$ 1.1 ^{Ccd}
6	8.1 $\pm$ 0.3 ^{Bgh}	8.5 $\pm$ 1.6 ^{Bb}	14.3 $\pm$ 1.9 ^{Ad}		7.7 $\pm$ 1.4 ^{Bc}
7	8.7 $\pm$ 0.3 ^{Dg}	13.7 $\pm$ 3.2 ^{Ba}	15.6 $\pm$ 2.3 ^{Ac}		10.9 $\pm$ 2.4 ^{Cb}
8	8.9 $\pm$ 0.4 ^{Dfg}	14.8 $\pm$ 5.2 ^{Ca}	16.7 $\pm$ 3.1 ^{Ab}		16.1 $\pm$ 4.6 ^{Ba}
9	10.2 $\pm$ 0.6 ^{Ce}	15.1 $\pm$ 5.1 ^{Ba}	18.8 $\pm$ 3.6 ^{Aa}		
10	12.1 $\pm$ 0.8 ^{Bd}	15.6 $\pm$ 6.6 ^{Aa}			
11	14.3 $\pm$ 0.4 ^{Ac}				
12	17.1 $\pm$ 0.9 ^{Ab}				
13	18.5 $\pm$ 2.6 ^{Aa}				

T: Tris-based extender, SC: Sodium citrate-based extender, M: Milk-powder extender, GP: Glucose phosphate extender, A: AndroMed® diluent. A-D: The different superscript uppercase letters in each row are statistically different among extenders ($P < 0.05$)

a-k: The different superscript lowercase letters in each column are statistically different among days ($P < 0.05$)

Correlations

Correlation coefficients between spermatological features revealed positive relationships between spermatozoa motility and HOST values ($r=0.837$, $P < 0.001$), but negative results both dead and abnormal

spermatozoa rates ($r=-0.871$, $r=-0.909$, respectively, $P < 0.001$). In the same time, the HOST values were negatively correlated with dead and abnormal spermatozoa rates ($r=-0.675$ and $r=-0.677$, respectively, $P < 0.01$). In addition, positive correlations were observed between dead and abnormal spermatozoa rates ($r=0.974$, $P < 0.001$).

DISCUSSION

The spermatological features are considered as valuable and reliable measures of semen quality. The axosome and associated dense fibers of the middle pieces in spermatozoa are covered by mitochondria that generate energy from intracellular stores of ATP. They are responsible for spermatozoa motility. Motility is essential for healthy spermatozoa. The spermatozoon plasma membrane is rich in polyunsaturated fatty acids and susceptible to peroxidative damage with consequent loss of membrane integrity, decreased spermatozoa motility^{24,36,37}. In a recent study in human, it was concluded that plasma membrane phosphatidylserine translocation and loss of mitochondrial membrane potential were suggestive of an early apoptosis phenotype, as typically observed in somatic cells³⁸. The spermatozoa motility in diluted ram semen with different extenders was gradually decreased during the storage period in this study (Table 1), and confirms the results of the other references^{21,25,39}.

Liquid storage of ram semen at room temperature does not maintain the fertilizing ability of spermatozoa for a long time. When the temperature $4-5^\circ\text{C}$ is used for the liquid storage, ram spermatozoa can be able to preserve their motility and fertilizing ability for a longer period. However, Holt and North⁴⁰ observed that changes in plasma membrane in ram semen maintained at 5°C which was induced by cold. A decrease in the hypoosmotic resistance of spermatozoa has also been observed after storage in this study (Table 2).

Although Jones and Martin⁴² found that increase the percentage of egg yolk enhanced the toxicity of dead spermatozoa in semen stored at room temperature, egg yolk was found to reduce the rate of peroxidation in ram spermatozoa⁴¹. Furthermore, within an aerobic or even a partially aerobic medium, the production of reactive oxygen is inevitable³⁷. Storage at 4°C does not completely arrest spermatozoa metabolism; therefore, the accumulation of the toxic products, including free radicals, might be involved in

the damage suffered by spermatozoa. The effects of peroxidation on ram spermatozoa include irreversible loss of motility, inhibition of fructolysis and respiration, and structural damage to the plasma membrane⁴³. During the present study, semen samples were stored in closed tubes, but the space between the upper part of the tube and semen surface might have contained enough oxygen to maintain the aerobic metabolism of the spermatozoa between assessments. However, the study surprisingly showed that dead spermatozoa rates were lower in extenders T (including 15% egg yolk) and SC (including 20% egg yolk) than in M (including 5% egg yolk), GP (including 2% egg yolk) and A (egg yolk free) (Table 3).

Considering the cellular metabolism during liquid storage, there is a gradual decrease in motility and morphological integrity^{3,4,18}. It has been reported that organic peroxides are produced when ram semen is held at 5°C and their accumulation is related to the quality loss of spermatozoa^{14,26,44,45}. Maxwell and Stojanov¹⁹ added some antioxidants to diluent Tris-glucose-egg yolk 20% (v/v) for liquid storage, and these antioxidants improved semen quality during liquid storage at 5°C, however the beneficial effects of the antioxidants were not maintained throughout the 12 days of storage. The abnormal spermatozoa rate was gradually increased during the storage period in this work (Table 4).

Extenders containing the main components have also been used for liquid storage of ram semen with differing success. Paulenz et al.¹⁴ reported a better spermatozoa motility and membrane integrity in tris-based extender than the sodium citrate- and milk-based extenders. Contradictory results were reported for frozen-thawed semen. Gil et al.⁸ found significantly higher percentages of uncapacitated spermatozoa in milk extender than in tris-citrate-fructose extender. In addition, Lopez et al.⁴⁴ observed that sodium citrate-based extenders maintained spermatozoa motility longer than milk-based extenders. In the present study, the tris-based extender preserved semen quality parameters longer over time than the sodium citrate- and milk-based, AndroMed® and glucose phosphate extenders. However, the results raise doubt about the usefulness of AndroMed® for dilution of ram semen. Kosum²² found that AndroMed® wasn't different from the other extenders for dilution and freezing of ram semen.

Comparison between extenders and daily values of spermatological features were tested to determine

the changes during the storage period. This revealed that there were significant changes from first day onwards. The differences between both storage period and extenders might be due to contents of diluters. Especially the glucose and egg yolk lipoproteins may have positive effects on the spermatozoa. The energy source of spermatozoa run off and the decrease in quality of spermatozoon may originate from the metabolic activity at 4°C. Similar findings were reported in rams^{45,46,47}, in boars^{16,17,48}, in bulls^{11,18,49}.

Results in this study showed that the semen quality parameters dramatically declined during the days of storage. In the ram semen diluted with T and SC extenders and stored at 4°C, spermatozoa motility could be maintained at greater rate than 50% for only 4 days. This interval decreased to 3 days in M and 2 days in A and GP. It was concluded that tris-, sodium citrate- and milk-power extenders were more suitable as diluters than AndroMed® and glucose phosphate extenders for ram semen. Nevertheless, AndroMed® and glucose phosphate may be used alternatively as an extender alone in dilution for short periods in farm conditions, if necessary.

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