TÜBİTAK

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The effect of different extenders on the motility and morphology of ram sperm frozen or stored at 4 °C

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Abstract: This study was conducted to evaluate the effects on sperm motility and abnormality in ram semen extended with skimmed milk (M), sodium citrate (SC), Tris (T), and Bioxcell® (B) after storage in liquid form at 4 °C and in frozen form. Ejaculates were collected from 3 Akkaraman rams by artificial vagina twice a week during the non-breeding season. After pooling, each pooled ejaculate was split into 4 equal aliquots and diluted with skimmed milk (M), sodium citrate (SC), Tris (T), and Bioxcell® (B) extenders. Sperm motility was significantly higher ($P < 0.05$) in M compared with B, SC, and T on the first and second days (48 h) of storage. With regard to the percentage of total abnormal spermatozoa, the results in M were different from ($P < 0.05$) from those of SC and T on the first day (24 h) of storage but no different from B. Differences among extenders were found to be significant post-thawing for spermatozoa motility and the percentage of total abnormal spermatozoa. The total abnormality of semen diluted with M was significantly lower than that observed in the other extenders. Consequently, it was found that skimmed milk was better than the other extenders in terms of the sperm parameters evaluated during liquid storage and post-thaw.

Key words: Ram semen, extenders, liquid storage, cryopreservation, sperm parameters

Koç spermasının kısa süreli saklanması ve dondurulmasında farklı sulandırıcıların motilite ve morfoloji üzerine etkisi

Özet: Bu çalışma, koç spermasının yağsız süt tozu (M), sodyum sitrat (SC), Tris (T) ve Bioxcell® (B) sulandırıcıları ile sulandırılıp, +4 °C de kısa süreli saklanması ve dondurulması sonrası spermatozoa motilitesi ve anormalitesi üzerine etkisinin saptanması amacıyla yapıldı. Ejakulatlar 3 Akkaraman koçundan sezon dışı dönemde suni vajen yardımıyla haftada 2 kez alındı. Alınan ejakulatlar birleştirildikten sonra 4 eşit hacme bölünmüş ve yağsız süt tozu (M), sodyum sitrat (SC), Tris (T) ve Bioxcell® (B) sulandırıcıları ile sulandırılmıştır. Kısa süreli saklamanın 24 ve 48. saatlerinde elde edilen en yüksek spermatozoa motilitesi yağsız süt tozunda elde edildi. Saklamanın 24. saatinde, anormal spermatozoa oranı bakımından, yağsız süt tozu sulandırıcısı ile Tris ve sodyum sitrat sulandırıcı içeren gruplar karşılaştırıldığında istatistiksel olarak aradaki farklılıklar önemli bulunurken ($P < 0,05$), Bioxcell® ile yağsız süt tozu arasındaki farklılıklar istatistiksel açıdan önemsiz bulunmuştur. Çözdürme sonrası spermatozoa motilitesi ve total anormal spermatozoa oranı üzerine sulandırıcılar arasındaki farklılıklar önemli bulunmuştur. Süt tozu sulandırıcısında, total anormal spermatozoa oranı diğer sulandırıcılara göre önemli derecede daha düşüktür. Sonuç olarak, çözdürme ve kısa süreli saklama sonrası bazı spermatolojik parametreleri değerlendirildiğinde yağsız süt tozu sulandırıcısının diğer sulandırıcılardan daha iyi sonuçlar verdiği saptandı.

Anahtar sözcükler: Koç sperması, sulandırıcılar, kısa süreli saklama, kriyopreservasyon, sperm parametreleri

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Introduction

Akkaraman is a fat-tailed, indigenous breed constituting 45% of the sheep population in Turkey. Most lamb meat production is from this breed (1). However, this breed generally produces low yields. Therefore, studies on crossing Akkaraman sheep with high-producing sheep breeds have been widely used to improve their yield traits. Artificial insemination (AI) allows for the rapid dissemination of genetic material from a small number of superior sires to a large number of females (2). Semen from farm animals used for this purpose can be stored in liquid form at 4 °C for a short time or it may be kept for a long period in a cryopreserved state with liquid nitrogen (3).

For the effective use of artificial insemination techniques in the sheep industry, investigation on the methods of ram semen dilution and freezing is necessary. Many extenders have been used for freezing ram semen. 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, lactose solution, and commercial diluents (3-5).

The successful storage of ram semen in liquid and frozen forms depends on the composition of the extender used. The recommended maximum storage time without impaired fertility after AI has traditionally been said to be as short as 6-12 h. Storage diluents and techniques have been developed and adapted with the aim of improving the cryopreservation of ram semen, but fertility after cervical AI with frozen-thawed sperm remains significantly lower than that achieved with fresh semen. Some factors that contribute to the reduced viability of cryopreserved ram sperm include extender composition, cryoprotectant concentration, egg yolk from different avian species, and the cooling, freezing, and thawing rates, as well as the quality of semen used. Different cryoprotectants or extenders have been used to prevent cryoinjuries. The most common cryoprotectant in use for sperm cryopreservation is glycerol. A suitable diluent is a basic necessity for the successful preservation of spermatozoa and for obtaining higher conception rates in field trials using diluted semen (5-8).

Although information is available on stored ram liquid semen up to 24 h, there is a need to further investigate the short-term and long-term preservability of Akkaraman ram semen for the extensive utilization of this valuable germplasm by artificial insemination.

This study was designed to evaluate and compare the quality (subjective motility and total abnormality) of Akkaraman ram spermatozoa preserved at 4 °C for up to 48 h and at freezing temperatures using 4 different extenders.

Material and methods

Animals and semen collection

The rams were housed at the Education Research and Practice Farm in the Faculty of Veterinary Medicine at the University of Ankara. The rams were kept under natural light and maintained under a uniform and constant nutritional regime, with each ram being fed a daily diet of 1 kg of concentrate, dried grass, salt lick, and water ad libitum.

Using an artificial vagina, semen samples were obtained from 3 mature Akkaraman rams (3 and 4 years of age) with proven fertility. A total number of 21 ejaculates were collected from the animals during the non-breeding season (summer). Semen samples were pooled to eliminate individual differences. From this, 7 pooled ejaculates were included in the study.

Semen evaluation

The ejaculates were evaluated and accepted if the following criteria were met: a volume of 0.75 mL, a sperm concentration $>2.5 \times 10^9$ spermatozoa/mL; sperm motility >70%, and a total morphological abnormality frequency of <20.

Sperm motility was assessed using a phase contrast microscope (×400 magnification), with a warm stage maintained at 37 °C. A wet semen mount was made using 2 µL of semen placed directly on a microscope slide and covered by a cover slip. For each sample, at least 5 microscopic fields were examined by 2 trained observers. The mean of the 2 successive evaluations was recorded as the final motility score (9).

For the sperm morphology assessment, a small drop of semen was added to Eppendorf tubes containing 0.5 mL of Hancock's solution (10). A

single drop of this mixture was put on a microscope slide and covered with a cover slip. The percentage of abnormal sperm (detached heads, acrosomal aberrations, abnormal mid-pieces, or tail defects) was recorded by counting a total of 200 spermatozoa under phase contrast microscopy (×1000 magnification; oil immersion).

Semen extenders

We used 4 extenders (T, SC, M, and B) in the present study. As a first step, the extenders, without gylcerol, were used for short term storage and prepared as follows:

Extender T

Tris-citric acid egg yolk extender was prepared by using 3.63 g of tris-(hydroxymethyl)-aminomethane, 1.99 g of citric acid, 0.5 g of glucose, and 20% egg yolk in 80 mL of distilled water.

Extender SC

Sodium citrate extender was prepared from a 2.9% aqueous solution of trisodium citrate and 20% egg yolk in 80 mL of distilled water.

Extender M

Milk extender was prepared by using 10 g of skimmed milk powder and 0.9 g of glucose in 100 mL of distilled water, heated to 95 °C for 10 min, and then cooled to room temperature before the addition of 10% egg yolk (6).

Extender B

The Bioxcell® extender was prepared according to the manufacturer's (IMV technologies, L'Aigle, France) instructions. Bioxcell® (egg-yolk free and concentrated medium) is a commercial diluent.

In the second step, 7% glycerol was added to each extender (with the exception of Bioxcell®) for cryopreservation of the ram semen.

Semen preservation

Short-term preservation

Each ejaculate was equally transferred into 4 tubes and diluted with extenders at 1:10 (v/v) rates. The daily spermatozoa motility and abnormal spermatozoa rates during storage at 4 °C were determined in each of the different extenders.

Semen freezing

The pooled semen was diluted with 4 different extenders, namely the T, SC, and M extenders, which included egg yolk, and the egg yolk-free diluent B. Each pooled ejaculate was split into 4 equal aliquots and diluted with the 4 different extenders for a total of 4 experimental semen groups (37 °C) at a final concentration of 800×10^6 spermatozoa per milliliter. Following dilution, the sperm was drawn into 0.25 mL plastic straws (IMV, Laigle, F-61300, France) and sealed with polyvinyl alcohol (PVA). Straws were equilibrated at 4 °C for 2 h and spermatozoa motility and total abnormal spermatozoa rates were assessed at the end of this period to determine differences in extenders in a water bath at 37 °C for 30 s for microscopic evaluation. After equilibration, the straws were suspended on a styrofoam rack 4 cm above the liquid nitrogen (vapor) for 15 min. The straws were then plunged into the liquid nitrogen, where they were stored until thawing. After storage for a period of 4 weeks, the semen straws were thawed in a water bath (37 °C for 30 s) for microscopic semen evaluation immediately after thawing.

Statistical analysis

Experiments were replicated at least 7 times. Data were expressed as the mean ± standard error of the mean (SEM). Data were subjected to analysis of variance (ANOVA) using mixed-model procedures. All analyses were carried out using the SPSS 12 for Windows.

Results

The data on motility and abnormal spermatozoa preserved in T, SC, M, and B extenders for 2 days at 4 °C are presented in Table 1. Spermatozoa motility was significantly higher ($P < 0.05$) in M (68.5, 47.5%) compared with B (55, 40%), SC (51.2, 25.5%), and T (48.7, 36.5%) at the first and second days of storage. The percentage of total abnormal spermatozoa in M (18.7%) was different from ($P < 0.05$) that of SC (27.3%) and T (26.5%) on the first day of storage but the rate was not different from that of B (23.8%). On the second day of storage, however, the total abnormality was not found to be different in any of the extenders. The percentage of abnormal spermatozoa increased with the number of days in storage for all of the extenders.

Table1. The mean percentage of motility and total abnormality of ram spermatozoa preserved for 2 days at 4 °C in 4 different extenders.

	0 h				24 h				48 h			
	T	M	SC	B	T	M	SC	B	T	M	SC	B
Motility (%)	82.5 ± 0.1[a]	84.0 ± 0.1[a]	80.0 ± 0.2[a]	84.5 ± 0.1[a]	48.7 ± 0.3[a]	68.5 ± 0.3[b]	51.2 ± 0.5[a]	55.0 ± 0.6[a]	36.5 ± 0.2[a]	47.5 ± 0.4[b]	25.5 ± 0.5[a]	40.0 ± 0.4[a]
Total abnormality (%)	10.2 ± 0.3[a]	8.5 ± 0.2[a]	11.0 ± 0.2[a]	9.5 ± 0.3[a]	26.5 ± 0.4[a]	18.7 ± 0.2[b]	27.3 ± 0.3[a]	23.8 ± 0.2[ab]	37.3 ± 0.3[a]	45.5 ± 0.6[a]	45.1 ± 0.5[a]	37.5 ± 0.7[a]

*Groups with different letters (a, b) in the same row are significantly different ($P < 0.05$).

T: Tris

M: Skimmed milk

SC: Sodium citrate

B: Bioxcell®

Differences among the extenders in terms of spermatozoa motility and total abnormality were found to be significant post-thawing. The data on motility and abnormality post-thawing in the T, SC, M, and B extenders are presented in Table 2. Spermatoza motility was significantly higher ($P < 0.05$) in M (21.2%) compared with B (7.0%), SC (11.5%), and T (16.2%) after being thawed. The total abnormality of semen diluted in M (50%) was lower than that observed in the other extenders.

Table 2. The mean percentage of motility and total abnormality of ram spermatozoa after freeze-thawing in 4 different extenders.

Extenders	Motility (%)	Total abnormality (%)
T	16.2 ± 0.2^{a}	55.4 ± 0.5^{ab}
M	21.2 ± 0.1^{b}	50.6 ± 0.8^{a}
B	7.0 ± 0.1^{c}	65.0 ± 0.5^{c}
SC	11.5 ± 0.2^{c}	60.0 ± 0.4^{bc}

Groups with different letters (a, b, c) in the same column are significantly different ($P < 0.05$).
T: Tris
M: Skimmed milk
B: Bioxcell®
SC: Sodium citrate

Discussion

From the present results it was observed that the type of extender used in diluting Akkaraman ram semen is an important factor in the successful preservation (at 4 °C in the refrigerator) and cryopreservation (at −196 °C in liquid nitrogen) of ram spermatozoa.

Many extenders have been used for freezing ram semen and reports show that the type of extender used plays an important role in the successful preservation of ram semen (11-13). Skimmed milk extenders preserve sperm motility better than other extenders. These findings were in agreement with the results of Lopez-Saez et al. (13) and Kulaksız et al. (14) on ram semen. Contrary to our results, however, Paulenz et al. (15) and Gündoğan (16) reported better spermatozoa motility and membrane integrity rates in a Tris-based extender than in the sodium citrate and skimmed milk extenders. Lopez et al. (17) observed no differences between sodium citrate-, Tris-, and milk-based extenders when subjected to liquid storage at 4 °C. The post-thawing sperm motility (16.2%) determined for the Tris extender used in our study was close to the findings of Tekin et al. (18) but Soylu et al. (19) reported better spermatozoa motility and membrane integrity in Tris extender than that found in our study.

To our knowledge, no previous study is available on the liquid preservation of ram semen in prepared extenders containing soy lecithin (Bioxcell) as an egg yolk substitute. In the present study, sperm motility showed higher values for Bioxcell compared to Tris and sodium citrate extenders as the liquid of storage. However, the parameters used for semen quality in this study showed significantly lower values for Bioxcell post-thawing in comparison with the other extenders. However, Gil et al. (20) found that Bioxcell was not different from a skimmed milk extender for the freezing of ram semen.

It is most likely that the cryogenic damages are due to the irreversible destruction of single components of the structural organization of sperm cells. Sperm are subjected to major changes in osmotic pressure during freezing and thawing. The stress on sperm membranes is dependent upon the basic extender used and the concentration of cryoprotectant as they interact with the freezing and thawing rates (21-23). Moreover, in the present study, the portion of ejaculates collected during a non-mating season (summer) had lower motility values and a higher percentage of total abnormal spermatozoa in all of the extenders after freezing.

In conclusion, the freezability of Akkaraman ram semen was adversely affected by the summer term in which the samples were collected. The skimmed milk extender showed the best liquid storage and post-thaw motility compared to the other extenders. Regardless of the extender type, however, semen freezing increased the rate of sperm morphological abnormalities.

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