


Effects of Semen Extender Supplemented with Bovine Serum Albumin (BSA) on Spermatological Traits of Saanen Buck Semen Stored at +4°C

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Abstract

The aim of this study was to investigate the effects of two modified semen diluents on the spermatological parameters of Saanen goat sperm stored at 4°C. Ejaculates were obtained from five Saanen bucks by means of an electro-ejaculator and divided into five aliquots. Then, aliquots of ejaculates were used to create five experimental groups as follows: Group 1 (control), group 2A containing seminal plasma (SP) and bovine serum albumin (BSA; 10 mg/mL⁻¹), group 2B containing BSA without SP, group 3A containing egg yolk (EY; 17.00%) and SP and finally group 3B containing EY without SP. All aliquots of semen were extended with Tris-based extender. Spermatological parameters of experimental groups were observed at 0, 6, 12 and 24 h periods during storage at 4°C. Motility of semen in groups 2A and 2B was found to be best values compared to other groups. However, the motility of group 3A at 24 h was 0.00%. In conclusion, it was concluded that the addition of 10 mg/mL⁻¹ of BSA to Tris-based extender could be useful in the storage of Saanen goat semen regardless of SP at 4°C.

Keywords: Bovine serum albumin, BSA, Extender, Goat semen, Short term storage

Sığır Serum Albumin (BSA) Katkılı Sulandırıcının +4°C'de Saklanan Saanen Teke Spermasının Spermatolojik Özellikleri Üzerine Etkileri

Öz

Bu çalışmanın amacı, modifiye edilmiş farklı iki sperma sulandırıcısının, 4°C'de saklanan Saanen ırkı teke spermalarının spermatolojik özellikleri üzerindeki etkilerini araştırmaktır. Ejakülatlar beş adet tekeden elektrojakülatör yardımıyla toplanmıştır ve beş eşit parçaya bölünmüştür. Daha sonra beş deney grubu oluşturmak üzere ejakülatlar şu şekilde porsiyonlanmıştır: grup 1 (kontrol), grup 2A seminal plazma (SP) ve bovine serum albumin içeren (BSA; 10 mg/mL⁻¹), grup 2B BSA içeren seminal plazma içermeyen, grup 3A yumurta sarısı (YS; %17.00) ve SP içeren ve son olarak grup 3B YS içeren SP içermeyen. Tüm semen porsiyonları Tris bazlı sulandırıcılar ile sulandırılmışlardır. 4°C'ye soğutulma süresi boyunca deney gruplarının spermatolojik parametre gözlemleri 0, 6, 12. ve 24. saatlerde yapılmıştır. Grup 2A ve 2B'nin motilite değerlerinin diğer gruplarla karşılaştırıldığında en iyi sonuçları verdiği bulunmuştur. Fakat grup 3A'nın 24. saatteki motilitesi %0.00 olarak saptanmıştır. Sonuç olarak, Tris-bazlı sulandırıcıya ilave edilen 10 mg/mL⁻¹ BSA'nın Saanen ırkı keçi spermasının soğutulmasında (4°C) seminal plazma farkı gözlemlenmesinin kullanılabileceği sonucuna varılmıştır.

Anahtar sözcükler: Sığır serum albumini, BSA, Sulandırıcı, Keçi semen, Kısa süreli saklama

INTRODUCTION

The demand for goat milk has increased since processed goat milk form can be used as an alternative to cow's milk in terms of high levels of small fat spheres, calcium, iron, vitamin B12, vitamin C and vitamin D contents in the absence of breast milk ^[1]. Artificial insemination (AI) technique has an important place in goat breeding. It is

one of the most frequently preferred biotechnological methods, especially in intensive cultivation production systems, to control reproduction and creation of high yield (meat, milk and wool) elite herds ^[2]. The AI has several advantages in terms of goat breeding. For example, transfer of the genes of an animal with high milk yield to another low-yielding animal with AI, transfer of genes between different farms and short-term storage options are a few



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of these advantages. By storing semen in the cold (+4°C) or transport of chilled semen from one place to another in suitable semen extenders requires less equipment and labor than cryopreservation and allows the transfer of good quality genes over long distances [2,3]. At the same time, since the metabolism of cooled spermatozoa is reduced to the basal level, sperms can maintain their viability for up to 24 h or more [4]. To support this, one of the studies reporting higher the pregnancy rate obtained using semen stored at room temperature or cooled to +4°C than that of frozen semen can be given as an example [4]. For example, Kulaksız et al. [5], have shown that domestic or exotic goat breeds' semen samples may have different sensitivities against cryopreservation. In addition, in a study conducted in Gurcu bucks, fresh and post-thawing spermatological parameters have been reported to vary depending on the season [6]. Of course, in chilled semen, problems like decreased motility, deterioration of structural integrity, decreased fertility and increased embryonic losses can occur [7]. However, all these complications due to spermatozoa damage are less than frozen semen. The most common method of protection against harmful effects that may occur during sperm chilling is to add egg yolk to the extenders [8]. However, egg yolk has a disadvantage; each prepared extender with egg yolk may not have the same structure. That is, it is difficult to standardize it. Because eggs may contain various pathogens and also quality differences may occur depending on the time period after laying and storage conditions [9,10]. The presence of the egg yolk coagulation enzyme (EYCE), which is secreted from the bulbourethral gland and named as phospholipase A2, reduces the survival rate of goat spermatozoa [11]. The EYCE shows this effect by hydrolyzing egg yolk lecithin to fatty acids and lysolecithin [12]. Toxic substances released after hydrolysis cause damage to spermatozoa membrane and DNA by stimulating chromatin decondensation as a result of acrosome reaction. Considering all of these reasons, separation of seminal plasma may be a good choice with different solutions or it may be necessary to develop a different type of extender that does not contain egg yolk [13,14]. There are studies mentioning the use of bovine serum albumin (BSA) as an alternative protein source in goat semen extenders instead of egg yolk and it has been reported that it can be used not only for buck semen but also for ram semen extenders [10,15]. The BSA is a protein molecule with a large structure which is available in reproductive system fluids. Furthermore, in many research, it has been reported that BSA increases the viability and motility of spermatozoa after cryopreservation [16]. This study was conducted to investigate the usability of Tris-based semen extenders supported by BSA instead of egg yolk as a cryoprotectant in the cooling of buck semen.

MATERIAL and METHODS

Ethical Approval

All procedures on animals were carried out according

to approval by the Local Ethics Committee for Animal Experiments of Istanbul University with the number of 2016/24 during this study.

Bucks and Semen Collection

In breeding season (September-December), semen samples were collected from five Saanen bucks (3-4 years of age) housed for this aim. The bucks belonged to the Reproduction and Artificial Insemination Department, Faculty of Veterinary Medicine, Istanbul University, Turkey and kept under regular nutrition and care conditions (roughage = 2.000 g, concentrated feed = 500 g; crude protein = 20.00%; crude cellulose = 6.80%; crude ash = 7.40%; crude fat = 3.20%; Calcium = 1.00%; Phosphorus = 0.60% and Sodium = 0.40%). Semen was collected from each buck by electro-ejaculation method and during this time, the bucks were sedated with 0.22 mg/kg⁻¹ intramuscular injections of xylazine (Alfazyne, Ege Vet, Izmir, Turkey) and 1.10 mg/kg⁻¹ flunixin meglumine (Finadyne, Intervet, Istanbul, Turkey). Semen was obtained with the aid of small electrical currents using an electro-ejaculator device (Ruakura, MK IV Ram Probe; Alfred Cox, Surrey, UK). This process was repeated 10 times, twice a week. During the semen collection procedure, the pre-lubricated rectal probe was placed in about 10 cm into the rectum of the sedated buck, laying on its side. Semen was collected into sterile tubes heated to 35°C at specific time intervals and in sets, as a result of a total of 10 electrical stimulations.

Evaluation of Semen

Immediately after ejaculates were obtained from each buck, they were stored in the water bath at 26°C and samples were taken for spermatological examinations from each ejaculate. Sperm volume, motility, and concentration were determined for ejaculates. Volume was measured with the help of an automatic pipette. Subjective motility was evaluated in a phase-contrast microscope and spermatozoa concentration was assessed with a Thoma cell counting chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) using Hancock solution [17]. As a result of all these examinations, only ejaculates with ≥80% motility rate and 2×10⁹ per mL sperm concentration were used for dilution, cooling, and storage stages in the current study. All selected ejaculates were pooled to minimize individual differences among bucks before the study procedures.

Dilution and Cooling

Pooled ejaculates were divided into five aliquots and processed as follows: 1) Aliquot was diluted with Tris-based extender (TBE; Tris 273.70 mM, Fructose 55.50 mM, Citric acid 72.87 mM, Penicillin 1000 IU/mL⁻¹ and Streptomycin 1 mg/mL⁻¹) with seminal plasma as control (group 1) [18]. All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, Missouri, USA); 2) An aliquot was diluted with TBE containing BSA (10 mg/mL⁻¹) with seminal plasma

in group 2A; 3) An aliquot was diluted with TBE containing BSA (10 mg/mL⁻¹) and without seminal plasma in group 2B; 4) An aliquot was diluted with TBE added with egg yolk (17.00%) with seminal plasma in group 3A; 5) An aliquot was diluted with TBE added with egg yolk (17.00%) and without seminal plasma in group 3B^[17]. Aliquots from pooled ejaculate were centrifuged for 5 min at 1.500 g in group 2B and group 3B in order to separate seminal plasma. Instead of removed supernatants, extenders of their own group were added, as much as the volume they were removed. All semen samples were diluted, to reach final concentration of 800×10⁶ per mL spermatozoa. Cooling was performed with Bio Cool® (BC-III-40; SP Industries, New York, USA) at a rate of 0.20-0.30°C per min. Motility, progressive motility, viability and morphology (percentage of abnormal spermatozoa) examinations were recorded at 0th, 6th, 12th and 24th h in semen cooled from 26°C to 4°C^[2,19]. Post-cooling motility and progressive motility control were performed with a computer-assisted sperm analyzer (CASA, HTM-IVOS, version 12.3; Hamilton Thorne Biosciences, Beverly, USA). The ratio of live spermatozoa was determined using eosin-nigrosin staining method^[19]. Dead/alive examination was evaluated under a light microscope at 400×^[17,19]. Morphological examinations (acrosome, head, mid-piece and tail abnormalities) were performed on samples taken from the mixture prepared with Hancock solution with immersion oil technique under a phase-contrast microscope (1000×). At least 200 spermatozoa were evaluated for each sample in the calculation of morphological examination and viability percentage^[17,19].

Statistical Analysis

All analyses were performed using IBM SPSS Statistics for Windows, Version 21.0. (Armonk, NY: IBM Corp.). All data were expressed as mean ± standard deviation. Two-way mixed ANOVA method was performed for motility, live spermatozoa and abnormal spermatozoa rates. Observations at 0th, 6th, 12th and 24th h were taken as within-subject variables, groups (1, 2A, 2B, 3A and 3B) were taken as between-subject factor and followed by Games-Howell post hoc tests. Shapiro-Wilk Normality Test was used to test the normality of distribution assumption, Levene's Test for homogeneity of variance assumption and Mauchly's Test of Sphericity for testing the sphericity assumption. All statistics were two-tailed and a P value of less than 0.05 was considered significant.

RESULTS

Motility Assessment

A two-way mixed ANOVA was conducted to examine the effect of experimental groups (1, 2A, 2B, 3A and 3B) and time (0th, 6th, 12th and 24th h) on motility rate (%). The analysis revealed a main effect of time (F (2.7, 121.56) = 479.72, P<0.001) and an interaction between time and groups (F (10, 8, 121.56) = 33.26, P<0.001).

Post hoc tests showed that motility rate was significantly higher in Group 1 compared to Group 3A (Mdiff = 21.4, P<0.01), in Group 2A compared to Group 3A (Mdiff = 28.4, P<0.001), in Group 2B compared to Group 3A (Mdiff = 25.5, P<0.001) and in Group 3B compared to Group 3A (Mdiff = 15.4, P<0.05).

Also, there was no statistical significance between Group 1-Group 2A, Group 1-Group 2B, Group 1-Group 3B, Group 2A-Group 2B, Group 2A-Group 3B, Group 2B-Group 3B (P>0.05). The worst results were found in Group 3A compared to all groups in terms of motility (Table 1).

Viability Assessment

A two-way mixed ANOVA was conducted to examine the effect of experimental groups (1, 2A, 2B, 3A and 3B) and time (0th, 6th, 12th and 24th h) on live spermatozoa rates (%). The analysis revealed a main effect of time (F (3, 135) = 401.7, P<0.001) and an interaction between time and groups (F (12, 135) = 13.9, P<0.001).

Post hoc tests showed that live spermatozoa rates were significantly higher in Group 1 compared to Group 3A (Mdiff = 26.1, P<0.001), in Group 2A compared to Group 3A (Mdiff = 27.8, P<0.001) and in Group 2B compared to Group 3A (Mdiff = 32.7, P<0.001).

Also, there was no statistical significance between Group 1-Group 2A, Group 1-Group 2B, Group 1-Group 3B, Group 2A-Group 2B, Group 2A-Group 3B, Group 2B-Group 3B, Group 3A-Group 3B (P>0.05). The worst results were found in Group 3A compared to all groups in terms of live spermatozoa rates (Table 2).

Morphological Assessment

A two-way mixed ANOVA was conducted to examine the

Table 1. 0, 6, 12 and 24 h motility results (%)

Time	Group 1 Control	Group 2A BSA & SP+	Group 2B BSA & SP-	Group 3A EY & SP+	Group 3B EY & SP-
0 h	50.00±7.61	71.80±7.75	71.50±8.51	70.80±11.24	74.00±6.99
6 h	66.50±12.70	64.50±10.91	61.00±11.49	29.00±20.78	50.00±16.49
12 h	55.50±14.99	47.00±14.94	42.00±20.02	8.50±5.29	32.50±15.32
24 h	22.00±4.83	38.50±11.55	36.00±16.46	0.00±0.00	13.50±14.91

BSA: Bovine Serum Albumin; SP: Seminal Plasma; EY: Egg Yolk; +/-: with/without

Table 2. 0, 6, 12 and 24 h live spermatozoa rates (%)

Time	Group 1 Control	Group 2A BSA & SP+	Group 2B BSA & SP-	Group 3A EY & SP+	Group 3B EY & SP-
0 h	73.60±10.47	68.00±11.82	76.60±10.69	67.60±14.51	71.20±9.97
6 h	56.90±14.11	58.10±12.87	68.80±15.61	24.80±20.16	50.60±22.18
12 h	54.50±16.16	53.30±15.83	49.50±10.12	12.50±3.27	41.10±21.58
24 h	24.60±8.69	36.90±7.27	40.90±18.42	0.00±0.00	13.80±15.31

BSA: Bovine Serum Albumin; SP: Seminal Plasma; EY: Egg Yolk; +/-: with/without

Table 3. 0, 6, 12 and 24 h abnormal spermatozoa rates (%)

Time	Group 1 Control	Group 2A BSA & SP+	Group 2B BSA & SP-	Group 3A EY & SP+	Group 3B EY & SP-
0 h	9.50±3.50	10.60±4.40	10.90±5.25	12.40±5.68	11.50±4.42
6 h	12.00±3.43	14.00±4.39	15.20±7.34	18.50±13.31	14.80±4.61
12 h	34.50±3.43	17.70±3.83	21.20±7.68	42.60±7.98	34.40±2.50
24 h	52.40±9.29	29.00±6.61	35.60±7.54	77.50±7.01	48.10±5.06

BSA: Bovine Serum Albumin; SP: Seminal Plasma; EY: Egg Yolk; +/-: with/without

effect of experimental groups (1, 2A, 2B, 3A and 3B) and time (0th, 6th, 12th and 24th h) on abnormal spermatozoa rates (%). The analysis revealed a main effect of time ($F(2.5, 114.7) = 423.6, P < 0.001$) and an interaction between time and groups ($F(10.1, 114.7) = 22.2, P < 0.001$).

Post hoc tests showed that abnormal spermatozoa rates were significantly higher in Group 1 compared to Group 2A (Mdiff = 9.2, $P < 0.001$), in Group 1 compared to Group 2B (Mdiff = 6.3, $P < 0.05$), in Group 3A compared to Group 1 (Mdiff = 10.6, $P < 0.001$), in Group 3A compared to Group 2A (Mdiff = 19.9, $P < 0.05$), in Group 3A compared to Group 2B (Mdiff = 17, $P < 0.001$), in Group 3A compared to Group 3B (Mdiff = 10.5, $P < 0.001$), in Group 3B compared to Group 2A (Mdiff = 9.3, $P < 0.001$) and in Group 3B compared to Group 2B (Mdiff = 6.4, $P < 0.05$).

Also, there was no statistical significance between Group 1-Group 3B, Group 2A-Group 2B, Group 2A-Group 3B, Group 2B-Group 3B ($P > 0.05$). The worst results were found in Group 3A compared to all groups in terms of abnormal spermatozoa rates (Table 3).

DISCUSSION

In recent years, proteins that reduce cell damage and cell loss, such as BSA, have been added to freezing solutions as membrane stabilizers to optimize cryopreservation of cells. It has been reported that the use of 10% or 15% of BSA can replace egg yolk in ram semen diluents and can be used to increase sperm motility and viability after thawing [20]. Similar to this research, our study showed that the addition of BSA (10%) to two groups' extenders used in cooling activities of buck semen has a positive effect on motility, viability, and abnormal spermatozoa rates. It is thought that BSA can show this positive effect due to its protein

structure, cryoprotective and cold protection properties. In other words, BSA, used instead of egg yolk, was found to be beneficial in terms of spermatological traits in the short-term storage of buck semen with or without seminal plasma. In the present study, motility and viability rates (%) were found to be 38.50 ± 11.55 & 36.90 ± 7.27 for group 2A and 0.00 ± 0.00 & 0.00 ± 0.00 for group 3A at 24th h, respectively. These results support the motility data of the study of Najjjan et al. [15], in which they frozen and thawed buck semen using 10.00% BSA supplemented Tris-based extender. It is well known that egg yolk-based extenders are often used to protect spermatozoa against cold shock during short-term storage and it is recommended by many researchers that low-density lipoproteins (LDL) may be required for spermatozoa protection [21,22]. Some scientists also claim that LDL adheres to the cell membrane during cooling and freezing [23,24]. In this study, we aimed to investigate a substitute for egg yolk by considering different thoughts about egg yolk based extenders. Besides all these effects, there are a few important points to remember about egg yolk. That is since the egg yolk is a biological substance, it is unlikely that each egg will have the same characteristics. In such a case, the percentage of protein contained in each semen extender prepared with egg yolk will not be the same. In addition to this, it should be taken into account that individual egg yolk quality may vary depending on the number of days after laying and storage as well as the fact that egg yolk may be the carrier of various pathogens [9,10]. Moreover, the presence of EYCE, also known as phospholipase A, originating from the bulbourethral gland, is reported to reduce the survival rates of goat spermatozoa [11]. This function of EYCE is known due to the hydrolysis of egg yolk lecithins to fatty acids and lysolecithins [12]. This final component is harmful for the buck spermatozoa. For these reasons, it may be

necessary to develop a synthetic extender without egg yolk. The sudden drop in the motility rate (%) in group 3A (8.50 ± 5.29) at the 12th h supports this idea. Additionally, the abnormal spermatozoa ratios given in *Table 3* were quite high in group 3A (77.50 ± 7.01), although this ratio was lower in group 2A at 24th h (29.00 ± 6.61) compared to group 1 (52.40 ± 9.29). These results strongly support the harmful effect of EYCE and the positive effect of BSA on spermatological traits. At the 12th h, although there was no distinction in motility between the group 1 (55.50 ± 14.99) and group 2A (47.00 ± 14.94) or group 2B (42.00 ± 20.02), a higher value was observed in the control group in terms of morphological disorder. This shows that the cryoprotective effect of BSA in seminal plasma or non-seminal plasma medium positively affects spermatozoon morphological integrity in cooling and storage of goat semen. BSA maintains membrane integrity by regulating the cholesterol flow from the plasma membrane of the mammalian spermatozoa to the outside of the cell to prevent fat accumulation on the cell membrane [25]. Cryoprotective effect of BSA is still continuing as the prolonged period of cold storage of sperm (by the 24th h) and synergistic effect especially in seminal plasma environment. In the group 3A there was a reduction more than 80% in motility within 12 h, whereas in group 2A there was no dramatic decrease like in group 3A. Similarly, when the motility ratio was evaluated in the seminal plasma groups [group 2A (47.00 ± 14.94) and group 3A (8.50 ± 5.29)] at the 12th h, there was a strong distinction between them in terms of motility. When the same groups were examined at 24th h, it was observed that the motility continued in group 2A but stopped completely in group 3A. As expected in live spermatozoa rates at 24th h, the best percentage values were found in groups 2A (36.90 ± 7.27) and 2B (40.90 ± 18.42) compared to group 3A (0.00 ± 0.00). When the abnormal spermatozoa rates were examined at 24th h, the best value was found in group 2A (29.00 ± 6.61) and the worst value was found in group 3A (77.50 ± 7.01). Further, the BSA is a special and critical source of protein for the reproductive system. It has been preferred in many experiments related to *in vitro* embryo production of different species and successful results have been obtained [26-28]. Moreover, it has been reported by researchers that BSA has beneficial effects on sperm motility and viability in many experiments [10,15,16]. Many studies have supported that BSA-supplemented extenders reduce contact between buck seminal plasma proteins and seminal plasma components like spermatozoa membrane [28]. In a study by Beltran et al. [29], the use of 10 mg of BSA resulted in 32.91% motility after thawing. In the same study, they used different amounts of egg yolk to the diluent and found that the best motility result after thawing was found in the 5% egg yolk group. This result suggests that the low egg yolk ratio may cause less coagulation and may be higher for post-thaw motility. In our study, the best results in terms of motility, viability and abnormal spermatozoon after cooling in group 2A were similar to Beltran et al. [29]. As a result, the

known disadvantages of EYCE on spermatozoa are tried to be overcome with the use of BSA. Positive results such as enabling the transfer of Ca^{2+} ions to the plasma membrane, cholesterol and phospholipid ratio reduction in the external acrosomal membrane and sperm hyperactivity stimulation appear to be the evidence of the beneficial effect of using BSA as a supplement in semen extenders [30]. According to many works, it has been reported that BSA protects the integrity of the preserved membrane of sperm cells against unexpected temperature changes and oxidative stress [10,30]. For all these reasons, this study suggests the use of BSA instead of egg yolk in extenders used for cooling of buck semen. In conclusion, the best values found in the BSA-supplemented groups (groups 2A and 2B) in terms of motility, morphology, and viability. The harmful effects of egg yolk supplemented Tris-based extender on motility, morphology, and viability were observed intensely between 6 h and 24 h. In the cooling process up to the 6th h, when the groups 2A, 2B and 3B were examined, no sudden and rapid reduction was found in the motility rates, but it was observed that the motility decreased rapidly in group 3A. The reason for this is thought to be denaturation of proteins, agglutination and initiation of ROS activity as a result of the reaction of EYCE with egg yolk. If seminal plasma is removed by centrifugation, egg yolk-supplemented Tris-based extender can be used. However, since this method has a negative effect on spermatozoa viability and consequently causes time and material losses, it can be said that the use of BSA instead of egg yolk may be more beneficial in short-term storage of buck semen.

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