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**RESEARCH ARTICLE**

# Freezing of Washed Angora Goat Semen with Extenders Added Bull or Ram Seminal Plasma [1]

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## Summary

The aim of this study was to investigate necessity of separation of Angora goat seminal plasma and beneficial of supplementation of bovine or ovine seminal plasma (SP) to extender prior to semen cryopreservation. Ejaculates from 4 Angora Goats were used in this study. Four experimental groups were designed: T (Angora Goat Semen (AGS) washed with Tyrod's solution with hepes (TALP), extended with TRIS containing egg yolk (TEG); C (Control, AGS not washed, only extended with TEG); TO (AGS washed with TALP, extended with TEG containing ovine seminal plasma (SP); TB (AGS washed with TALP, extended with TEG containing bovine SP). When post-thaw AGS parameters of four groups were analyzed, it was attained that supplementation of bovine or ovine seminal plasma to extender improved post-thaw AGS parameters compared with C and T groups ($P<0.05$). In conclusion, the results of the current study suggest that AGS should be washed. Moreover, the results of this study point out that supplementation of bovine or ovine SP instead of Angora goat SP separated from semen can be used prior to freezing.

**Keywords:** *Angora goat, Semen, Seminal plasma, Washing, Cryopreservation*

# Yıkanmış Ankara Tekesi Spermasının Boğa veya Koç Seminal Plazması Eklenmiş Sulandırıcılarla Dondurulması

## Özet

Bu çalışmanın amacı dondurma öncesi Ankara tekesi spermasının seminal plazmasından ayrılmasının gerekliliği ile boğa veya koç seminal plazmasının sulandırıcıya eklenmesinin yararlılığının araştırılmasıdır. Bu çalışmada dört Ankara tekesinden alınan sperma kullanıldı. 4 deney grubu tasarlandı: T (Hepesli Tyrod's solüsyonuyla yıkanmış; yumurta sarısı içeren TRIS solüsyonu ile sulandırılmış Ankara Tekesi Sperması (AGS), C (Kontrol, Yıkanmamış AGS; yalnızca TEG ile sulandırılmış), TB (TALP ile yıkanmış; boğa seminal plazması (SP) içeren sulandırıcı ile sulandırılmış AGS), TO (TALP ile yıkanmış; koç SP içeren sulandırıcı ile sulandırılmış AGS). Dört gruba ait çözüm sonu spermatolojik parametreler analiz edildiğinde; boğa veya koç seminal plazmasının dondurma öncesi sulandırıcıya eklenmesinin, C ve T grupları ile karşılaştırıldığında, çözüm sonu spermatolojik parametreleri olumlu yönde etkilediği saptanmıştır ($P<0.05$). Sonuç olarak, bu çalışmadan elde edilen veriler AGS'nın dondurma öncesi yıkanması gerektiğini ortaya koymaktadır. Ayrıca, bu çalışmanın sonuçları, Ankara Tekesi spermasından ayrılan, Ankara Tekesi seminal plazması yerine, boğa veya koç seminal plazmasının kullanılabileceğini göstermektedir.

**Anahtar sözcükler:** *Ankara keçisi, Semen, Seminal plazma, Yıkama, Kryopreservasyon*

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## INTRODUCTION

Angora (Ankara) goats have been reared for its mohair and meat. However, it is currently considered a breed at risk of extinction. In this context, the cryopreservation of gametes is important because it would allow us to support a genome resource bank for this breed for an indefinite period of time. The ability to cryopreserve spermatozoa from all of the domestic species is challenging. Although all of the cells should endure similar physical stresses associated with the cryo-preservation processes, sperm from the different species very different in size, shape and lipid composition, all of them have effect on cryosurvival. Therefore, when a cryopreservation protocol has been optimized for sperm of one species, it could not be good for sperm of other species. Bovine and goat or ovine sperm-freezing extenders, for instance, include similar or same ingredients, however, interaction between goat seminal plasma and egg yolk (or skim milk) are deleterious to the sperm, a condition not observed with bovine or ovine seminal plasma and egg yolk [1-3].

The most common cryopreservation diluents for goat semen contain either egg yolk or skim milk. But the dilution of goat semen into extenders with egg yolk or skim milk can be detrimental to spermatozoa. The harmful interactions between seminal plasma and egg yolk were first documented by Roy [4] and with milk by Nunes et al.[5]. Roy [4] determined that sperm cells maintained their motility in egg yolk diluents if the seminal plasma was separated.

Lebouef et al.[6] determined that EYCE and SBUIII were likely the same molecule or enzyme interacting egg yolk and skim milk ingredients. The EYCE was identified as phospholipase A, and SBU III as a 60 kDa glycoprotein lipase from the goat bulbourethral gland (BUSgp60) [2,7,8]. EYCE catalyzes hydrolyzes egg yolk lecithin into fatty acids and lysolecithin. This reaction causes the sperm membranes to be more fusogenic so inducing the acrosome reaction [9], and chromatin decondensation [10], which is toxic to the sperm [11]. BUSgp60 lipase has structural homology to porcine pancreatic lipases and, similar to EYCE, BUSgp60 is responsible for hydrolysis of plasma membrane triglycerides and triglycerides in the skim milk that result in fatty acid production (lysolecithin production with egg yolk, and oleic acid with milk triglycerides) that is toxic to sperm [2,12,13]. It is certain that EYCE and BUSgp60 (same or not) have detrimental effects to the quality of the sperm cells during cooling and cryopreservation, if goat semen is extended with media containing egg yolk or skim milk [2,13]. Moreover, individual changes of testosterone levels may be affecting concentration of EYCE and BUSgp60 in male Angora goats [14].

The conventional method of overcoming the harmful interactions of seminal plasma and egg yolk or milk proteins is to dilute the goat semen sample in buffered diluents and then separate the seminal plasma from the sperm by centrifugation. Spermatozoa are washed either once or twice, each for 10-15 min at 550-950 x g [1,5,15]. Results of alot of research have indicated that removal of seminal plasma is necessary for maximizing post-thaw motility and acrosomal integrity in goat semen [1,13,16,17]. Recently, Ustuner et al.[17] have revealed that the presence of buck seminal plasma has a detrimental effect on motility and dead spermatozoa. However, some other researches indicated positive results for semen cryo-preservation without washing [15,16,18,19]. Pellicer-Rubio and Combarnous [13] used egg yolk (lecithin) or triglyceride to freeze goat semen without any detrimental effect. However, it is well known that cryoprotective feature of egg yolk and skim milk takes root from lecithin and triglyceride composition.

When seminal plasma is separated with washing process, EYCE or SBUIII is not only separated but also other beneficial component and compound in seminal plasma that sperm cells need to survive after ejaculation. Goat seminal plasma as other species seminal plasma contains inorganic components (such as sodium, potassium, calcium, magnesium, clor, inorganic phosphor) organic components (such as glycriel-phosphoryl-choline, protein especially seminal plasma proteins, sugar especially fructose) and some components called decapacitors preventing early capacitating and acrosome reaction [20,21].

In this study we tested whether or not seminal plasma separation was necessary to attain successful cryopreservation. Finally the aim of this study was to investigate whether there were any beneficial effects of bovine or ovine seminal plasma added to extenders instead of Angora goat seminal plasma.

## MATERIAL and METHODS

### *Animals*

In this study 4 male Angora goat, varying in age between 2 and 4 years, were used during the breeding season (October-December). The animals were housed at the Ankara University, Faculty of Veterinary Medicine, Education Research and Practice Farm (40°11' N - 32°39' E; altitude of 1040 m above sea level; Ankara, Turkey) and maintained under uniform feeding, housing and lighting conditions. The Angora goats were fed 0.91 kg of concentrate daily and good quality hay and water were supplied ad libitum.

***Semen Collection and Pooling***

Semen samples were collected by means of artificial vagina from four Angora goats with estrual goat were pooled to eliminate individual differences, if mass activity (3+) and motility (75%) were normal. Sperm concentration was assessed using haematocytometer [22] .

***Seminal Plasma Separation (Semen Washing) and Groups***

One aliquot of pooling semen was diluted with TALP (Tyrod's solution with hepes; 1.168 g NaCl; 0.0462 KCl; 0.4200 $NaHCO_3$; 0.807 ml Lactic acid; 0.0070 g $NaH_2PO_4$; 0.0617 g $CaCl_22H_2O$; 3 ml from 100 mM $MgCl_26H_2O$; 0.4766 g Hepes (acid) add 200 ml Bi-distilled water) solution (1:10), except Control (C) group. Then, Semen diluted with sperm Talp was divided three centrifuge tubes and centrifuged in order to removal of seminal plasma at 600 x g in 15 min. After centrifugation, supernatants (including seminal plasma) were removed and semen pellets were used for extension and cryopreservation. In this way, briefly four experimental groups, one of which was control, were designed:

*T:* Angora Goat Semen (AGS) washed with TALP (600 x g in 15 min) and extended with TRIS (Tris 3.63%, citric acid 1.82%, glucose 0.5%, penicillin 100 000 IU, streptomycin 1.0 g $ml^{-1}$), containing 20% egg yolk (v/v) extender, 7% glycerol (TEG modified from Evans and Maxwell [23]).

*TO:* AGS washed with TALP (600 x g in 15 min) and extended with TEG containing 5% ovine seminal plasma (SP) (v/v).

*TB:* AGS washed with TALP (600 x g in 15 min) and extended with TEG containing 5% bovine SP (v/v).

*C (Control):* AGS not washed, only extended with TEG not containing neither bovine nor ovine SP.

***Semen Cryopreservation***

Each 0.25 ml French straws were contained $50x10^6$ motile spermatozoa. Straws were sealed with hot compress. Then straws were equilibrated at 4°C in 2 hours. After equilibration, straws were frozen over nitrogen vapour, 4 cm above the nitrogen level, plunged, and stored in liquid nitrogen for at least 2 weeks before analysis. The straws were thawed in a water bath at 40°C for 20 s.

***Post-thaw Semen Analyses***

***Motility:*** Sperm motility was assessed subjectively using a phase-contrast microscope (400x) with a warm slide.

***Morphology:*** For morphological analysis, one drop sample from groups was diluted in 1 ml Hancock's solution (prepared with 62.5 ml formalin, 150 ml sodium saline solution, 150 ml buffer solution, and 500 ml distilled water [24]), placed under a coverslip and evaluated by differential interference phase-contrast microscopy under immersion. The morphological alternations were classified as described by Ax et al.[25].

***Membrane integrity:*** To evaluate membrane integrity, 100 µl of semen sample was diluted with 900 µl of 100 mM hypotonic solution (composed of 9 g fructose plus 4.9 g sodium citrate per liter of distilled water). After 5 min, smear was prepared and evaluated considering sperm tail curling (%) (Hypo-osmotic swelling Test/HOST) [19].

***Dead spermatozoa:*** Percentage of dead spermatozoa after thawing was evaluated with eosin staining (eosin-Y 1.67 g and sodium citrate 2.9 g dissolved in 100 ml distilled water) as described by Ax et al.[25].

***Statistical Analysis***

The mean post-thaw semen parameters of motility, dead spermatozoa, abnormal spermatozoa and hypo-osmotic swelling test (curling tail spermatozoa) for the 10 trial carried out during this study were subjected to analysis of variance (one way ANOVA), and differences among means were tested for significance by the Fisher's PLSD. The SPSS 10.0 software was used for all statistical analyses. Differences with values of $P<0.05$ were considered to be statistically significant.

## RESULTS

In this study, AGS fraction washed with TALP (T; 34.5%) presented the highest motility means after thawing as compared with control (C; 25.5%) ($P<0.05$). Same situation (advantage of washing) was observed for other parameters but there did not have statistic significant (i.e. dead spermatozoa ratio, abnormal spermatozoa ratio and Hyposmotic swelling test) *(Table 1).*

Bovine or ovine seminal plasma addition to washed semen fractions before freezing significantly improved post-thaw semen parameters compared with T and C groups ($P<0.05$) *(Table1).* Percentage of dead and abnormal spermatozoa was the highest in group T and C as compared with TB and TO groups ($P<0.05$) *(Table 1).* Percentage of acrosome abnormality in group T and C was the highest compared other groups ($P<0.05$) *(Table 1).* It was determined that membrane integrity of spermatozoa after thawing was improved with washing as compared with control ($P<0.05$) *(Table 1).*

***Table 1.*** *Post-thaw semen parameters of AGS washed with Talp and extended with TEG added bovine* ***(TB)*** *or ovine* ***(TO)*** *seminal plasma or not added any seminal plasma* ***(T)***

***Tablo 1.*** *Boğa* ***(TB)*** *veya koç* ***(TO)*** *seminal plazması eklenmiş veya eklenmemiş* ***(T)*** *TEG ile sulandırılmış, yıkanmış Ankara Tekesi spermasının çözüm sonu parametreleri*

| Groups | Motility (%) | Dead Spermatozoa (%) | Abnormal Spermatozoa (%) | Acrosome Abnormality (%) | HOS Test (%) |
|---|---|---|---|---|---|
| **T (n=10)** | 34.5±2.5 $^{b}$ | 54±3.8 $^{bc}$ | 55±2.9 $^{bc}$ | 36±3.7 $^{bc}$ | 38±3.4 $^{bc}$ |
| **TO (n=10)** | 49.5±3 $^{c}$ | 36±1.8 $^{a}$ | 32±4.2 $^{a}$ | 15±2.3 $^{a}$ | 49±3.5 $^{a}$ |
| **TB (n=10)** | 37±2.1 $^{b}$ | 52±2.7 $^{b}$ | 47±4 $^{b}$ | 27±2.3 $^{b}$ | 42±2.7 $^{b}$ |
| **C (n=10)** | 25.5±2.9 $^{a}$ | 61±4.4 $^{c}$ | 65±3.4 $^{c}$ | 41±4.7 $^{c}$ | 30±3.4 $^{c}$ |

***a, b, c:*** *Within columns means with different letters differ significantly P<0.05*
***a, b, c:*** *Aynı sütunda farklı harfler gruplar arası farkı göstermektedir P<0.05*

## DISCUSSION

Removal of seminal plasma improved the percentage of motile spermatozoa in frozen-thawed samples (P<0.05). These findings about with separation of seminal plasma are in disagreement with those of Ritar and Salamon [26], Chauhan and Anand [27] and Tuli and Holtz [28] and Azerado et al.[19] that verified a higher percentage of motility when the seminal plasma was present than when it was removed. On the other hand, also these findings for sperm TALP were similar with those of Ritar and Salamon [26] who obtained higher rates of live spermatozoa after thawing when the seminal plasma had been removed. Also, Ustuner et al.[17] obtained similar findings with current study, and pointed out that the presence of buck seminal plasma had a detrimeantal effect on post-thaw motility. Hence, the solution used for seminal plasma separation may have been important. The seminal plasma during the non-breeding season under temperate climate conditions is capable of stimulating motility of ejaculated and epidiymal sperm [29].

Acrosome abnormality after thawing was higher in C group compared with T, TO and TB groups (P<0.05). According to Pellicer-Rubio and Combarnous [13], the physiological function of BUSgp60 in goat SP could be related to the acrosome reaction because it has been showed a high degree of similarity with lipases of the PL-RP2 subfamily, which present lipase and phospholipase activities, for participation in the energy metabolism of spermatozoa. On the other hand, Manjuah and Therien [30] observed that some proteins called bovine seminal plasma proteins (BSP 1,2,3,4) had important roles stabilization of acrosome membrane. So removal of goat SP containing BUSgp60 (called EYCE) and addition of ovine or bovine SP containing BSP 1,2,3,4 or BSP like proteins may have decreased acrosome abnormality in T, TO and TB groups.

Membrane integrities assessed with Hypo-osmotic swelling test (%) in TO and TB groups were higher as compared with other groups. Difference between TO and other groups was statically significant (P<0.05). Hence it may be pointed out that washing and addition of ovine or bovine seminal plasma positively affected membrane integrity. Nevertheless Azerado et al.[19] did not find any difference between washing and not washing goat semen after thawing for membrane integrity. Different results may have rooted from different washing solutions. As they used Krebs-Ringer phosphate plus sodium citrate, we used TALP as a washing solution.

Percentage of abnormal spermatozoa was higher in C and T groups, compared with the TO and TB groups in our study. Percentage of low abnormal spermatozoa in TO and TB groups may have been associated with beneficial effects of bovine or ovine seminal plasma on spermatozoa during cryopreservation and thawing process. Manjunath and Therien [30] pointed out that there are components (called BSP or GSP) in bovine and ovine seminal plasmas, respectively and these components have important roles in stabilization and protection of spermatozoa membrane. Addition of bovine or ovine seminal plasma to extenders must have protected spermatozoa membrane during cryopreservation and thawing process.

Addition of ovine seminal plasma to extender was more beneficial than bovine seminal plasma in the current study. This effect may be associated with high glyceril phosphoryl colin (GPC) concentration of ovine seminal plasma, together with BSP like proteins. GPC is catalyzed by phosphates to gylyceril and colin in seminal plasma. Colin can be used by spermatozoa as a energy source and also glyceril has some cryoprotective effects [21]. Therefore, GPC in ovine SP may have improved post-thawing sperm parameters, especially motility in TO group.

In conclusion, the results of investigation indicate that (1) post-thaw sperm parameters of Angora goat improve when seminal plasma separated prior to cryopreservation process; (2) the supplementation of bovine or ovine seminal plasma to extender prior to freezing of washed AGS has beneficial effects on post-thaw sperm parameters. Furthermore, the addition of ovine seminal plasma to extenders used for AGS is more suitable than bovine seminal plasma as compared post-thaw sperm parameters.

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