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SEASONAL CHANGES IN TESTES SIZE, FRESH AND POST-THAWING SEMEN CHARACTERISTICS, SERUM TESTOSTERONE LEVEL, AND PHOSPHOLIPASE A₂ ACTIVITY IN GURCU MALE GOATS

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ABSTRACT

The aim of current investigation was to evaluate seasonal changes in the testicular measurements, fresh and post-thawing spermatological characteristics, serum testosterone (T) levels in Gurcu goats. Moreover, seminal plasma phospholipase A₂ activity levels were determined. Four adult Gurcu male goats (2-3 years old) were used as semen donor and ejaculates (n=113) from these goats were weekly collected with artificial vagina in the presence of natural estrus or estrogenized female fantom during breeding season (BS, September-February) and non breeding season (NBS, March-August), respectively. Moreover, jugular blood was collected and serums were separated to determine Testosterone level during BS and NBS. All andrologic parameters, except testes width (P<0.05), were not changed statistically during BS and NBS in Gurcu bucks. It was determined that ejaculate volume (1.27±0.05 vs 0.76±0.04mL; P=0.001), fresh progressive sperm motility (80.39±1.00 vs 74.43±2.26%; P=0.019) were significantly higher in BS compared to NBS, respectively. However, sperm concentration (3.93x10⁹ vs 2.48x10⁹ spermatozoa/mL; P=0.001) was interestingly higher during NBS compared with BS, although total number of sperms per ejaculate were similar during BS (3.03x10⁹ spermatozoa/ejaculate) and NBS (2.93x10⁹ spermatozoa/ejaculate), P>0.05. Also there was no differences in post-thaw progressive sperm motility between BS and NBS (25.0±2.86 vs 19.32±2.55%; P=0.145). T did not any show significant change between BS and NBS (13.44 ±2.37 vs. 13.06±1.18 ng/mL). Although seasonal changes were observed in andrologic parameters, fresh and post-thawing spermatozoa in Gurcu bucks, this effect was limited.

Keywords: Gurcu goats, phospholipase A₂, seasonal variation, semen characteristics, testes, testosterone.

INTRODUCTION

The Gurcu breed of goat, which is native to Turkey and adapted to Caucasian environmental conditions, is currently threatened by extinction. Protecting Gurcu goats, one of the general sources of Turkish native goat breeds, is extremely important because these breeds may have an important beneficial effect on small ruminant breeding in the future as a genetic source (Sezgin *et al.*, 2010). Biotechnological methods (cryopreservation of sperm) have been preferred in studies carried out on the protection of genetic resources.

Semen quality and quantity, as indicators of reproductive efficiency in male goats, are influenced by factors such as breed, season, and frequency of ejaculation as well as techniques of breeding (Greyling and Grobbelaar 1983). Although a number of common points exist among cryopreservation of buck semen and other domestic animals' semen, such as semen extenders, cryoprotectants, and speed of freezing and thawing, a number of applications are needed to obtain more successful cryopreservation results in this species. For example, secretions of bulbourethral gland

(phospholipase A₂, etc.) is deleterious for the survival of spermatozoa when goat semen is chilled or frozen with egg yolk (Lebouf *et al.*, 2000). Meanwhile, differences among goat breeds are another important factor affecting the success of cryopreservation. For instance, Kulaksız *et al.* (2013) have shown that semen from native and exotic goat breeds may have different sensitivity against cryopreservation.

A good number of studies on the testis size, spermatological indices, and testosterone levels in different goat breeds have been conducted in Turkey and other parts of the world (Ahmad and Noakes 1996; Kamal *et al.*, 2005; Zamiri and Heidari 2006; Talebi *et al.*, 2009). However, seasonal changes studies on the freezability of buck semen are limited (Tuli and Holtz 1995; Wang *et al.*, 2015; Gallego-Calvo *et al.*, 2015). As far as we are aware, there has not yet been any study about Gurcu goats in these issues and our study is the first to assess these parameters in this breed.

Storage of Gurcu bucks semen, well adapted to harsh environment of the Caucasian region, is important for animal husbandry and breeding of goats in Turkey. This study has focused on the following area: (a) determination of testis size and semen characteristics of

Gurcu goats (b) detection of changes in testicular, spermatological parameters values, and testosterone levels, as well as seminal plasma phospholipase A₂ levels during breeding and nonbreeding seasons.

MATERIALS AND METHODS

Location and climatic conditions: The current study was conducted on the experimental farm of the Kafkas University in the northeast of Turkey, (latitude of 40°34'23"N, longitude of 43°02'27"E, and 1751 m above the sea level.). This harsh environment is characterized by cold, hard and long winters and cool summers. This investigation considered the months including September through February as the breeding season, and the another half as the nonbreeding season. These seasons were determined by considering the descriptions of the mating period that Kuru *et al.* (2016) reported in their study conducted in Kars conditions, Turkey. This study was done after obtaining the approval from the Kafkas University Animal Experiments Local Ethics Committee, Kars, Turkey (KAÜ-HADYEK-2012/012).

Animals, feeding, and management: Four 14-month healthy male Gurcu goat (40–45 kg live weight) were caged in the Kafkas University Goat Breeding Unit, Kars, Turkey. The animals were maintained under feeding conditions with natural photoperiod, environmental humidity, and temperature. All bucks, housed in a covered shelter, were allowed 1 kg hay per day. They were also fed a 12% protein supplement each morning. Clean and safe water was made available at all times. Free access was provided to vitamin/mineral blocks. A general management schedule for de-worming, disease prevention, and hoof trimming was followed throughout.

Determination of body weight and scrotal and testicular measurements: Each buck was weighed once a month for a year with concomitant measurement of scrotum and testis (April 4, 2013, to March 31, 2014). The scrotal circumference was measured with a flexible metric tape (Tape, Scrotal Metric, A Neogen Company, MI, USA). Testes width, testes length, and scrotal thickness were determined by using a caliper (Digital caliper 0–20 mm/0–8, Insize Co., Ltd., GA, USA). The testicular volume was measured using the water volume dislodged on the cylinder (Demirci, 2002).

Semen collection, evaluation, and cryopreservation: Semen was collected from each buck once a week throughout 1 year (October 1, 2013, to September 30, 2014), with the aid of an artificial vagina. At cases when the bucks failed to mount or ejaculate, some ejaculates were not available. During the research period, a total of 113 ejaculates were collected from 4 Gurcu bucks for 12 months. Semen samples were evaluated for volume (mL), mass activity (0-5), sperm concentration ($\times 10^9$),

progressive sperm motility (%), and total spermatozoa number ($\times 10^9$). The percentage of progressive sperm motility was estimated by subjective microscopic examination using a phase-contrast microscope supplied with a heated stage at 37°C and magnification 400× after dilution with extender (dilution rate 1:10). Sperm concentration was determined using the hemocytometric method after diluting semen with Hayem solution (dilution rate 1/400) (Tekin, 1994). The skimmed milk-based extender was prepared within a period of one week and kept at 5°C. The composition of skimmed milk-based solution was 10 g skimmed milk powder and 0.9 g glucose, formulated on the basis of 100 mL; 10% (v/v) egg yolk and 5% (v/v) glycerol were added to skimmed milk-based solution. Diluted semen was loaded into 0.25-mL French straws at doses of 100×10^6 spermatozoa per straw. The plastic straws were sealed with polyvinyl alcohol powder. The straws were placed in a refrigerator at 4°C for 2 h before freezing. After equilibration, the straws were frozen horizontally on a rack about 4 cm above liquid nitrogen (LN₂) held in an insulated container. The nitrogen vapor reduced the temperature within the straws to -120°C in approximately 15 min. Then, the straws were transferred rapidly to LN₂ containers at -196°C. The straws were stored in LN₂ until the evaluation time. After 1-month storage, three straws from each buck were thawed in a warm bath (37°C). After 1 min, the contents of the straw were monitored microscopically. After thawing of straws, a 3- μ L aliquot of each sample was placed on a warm (37°C) slide and covered with a cover slip before examination under a phase-contrast microscope (Nikon Eclipse E400, Nikon Corp., Japan) at 400× magnification. After observing four or five different fields, the percentage of progressive motile sperm cells was recorded for each sample. Throughout the experiment, two blind, without knowing trial groups, technicians evaluated all the samples, and their mean values were recorded as a percentage (Kulaksız and Daşkın 2010).

Serum testosterone level: Blood samples were taken weekly from the jugular vein of each animal into 10-mL vacutainer tubes for the entire observation period. Serum were separated by centrifugation at 3500 rpm for 10 min at room temperature and stored at -20°C until analysis for serum testosterone using coated enzyme-linked immunosorbent assay (ELISA) kits (Goat testosterone: MBS702733, MI, USA). The detection limit of the assay was 0.1–20 ng/mL.

Seminal plasma extraction and phospholipase A₂ activity: The ejaculates were centrifuged (10,000 rpm for 10 min), and seminal fluids were separated for chemical analysis. Phospholipase A₂ activity in the seminal fluid was determined using the sPLA₂ assay kit (Cayman chemical item no. 765001, MI, USA). The measurement

range of this assay was 0.02–0.2 $\mu\text{mol}/(\text{min} \cdot \text{mL})$ sPLA₂ activity.

Statistical analysis: The data were statistically analyzed using the SPSS® software program (SPSS 18.0, IL, USA). The distributions of the data were evaluated by the Shapiro–Wilk test. The *t*-test was used to compare all the data both in breeding and nonbreeding seasons. The results were presented as mean \pm standard error of the mean (SEM). $P < 0.05$ was considered statistically significant in evaluating the results.

RESULTS

When the seasonal and off-seasonal testicular characteristics of Gurcu bucks were evaluated, except testes width ($P < 0.05$), the differences in scrotum circumference excluding testes length, testicular volume and scrotal skin thickness were observed to be statistically insignificant ($P > 0.05$) (Table 1).

The effect of breeding and nonbreeding seasons, the semen volume was higher in the breeding season than nonbreeding season ($P < 0.001$) (Table 2). Concomitant with a significant difference found between the breeding and nonbreeding season in terms of fresh progressive sperm motility ($P < 0.001$) (Table 2). However, sperm concentration per mL was interestingly higher during nonbreeding season compared with breeding season, although total number of spermatozoa per ejaculate were similar ($P > 0.05$) during breeding season and nonbreeding season (Table 2). Also there was no differences ($P > 0.05$) in post-thaw progressive sperm motility between BS and NBS (Table 2).

A statistically significant difference was not found between the breeding and nonbreeding seasons in testosterone levels ($P > 0.05$) (Table 3). Phospholipase A₂ activities of Gurcu bucks in individual months and between November and December are given in Table 4. Phospholipase activities varied individually, but monthly changes (between November and December) were not found to be statistically significant ($P > 0.05$).

Table 1. Body weight and testicular measurements in Gurcu bucks in nonbreeding and breeding seasons.

Parameters	Nonbreeding season	Breeding season	P
Body weight (kg)	54.30 \pm 1.02	53.93 \pm 0.96	0.624
Skrotal circumference (cm)	24.50 \pm 0.47	24.40 \pm 0.46	0.821
Testes length (cm) right	10.23 \pm 0.19	10.18 \pm 0.18	0.859
Testes length (cm) left	9.79 \pm 0.20	9.78 \pm 0.20	0.969
Testes width (cm) right	6.11 \pm 0.14*	6.07 \pm 0.13*	0.746
Testes width (cm) left	5.72 \pm 0.10*	5.71 \pm 0.09*	0.882
Testicular volume (mL)	329.00 \pm 9.44	326.21 \pm 9.01	0.776
Scrotal skin thickness (cm)	0.65 \pm 0.02	0.65 \pm 0.02	0.919

* Show statistical differences in the same column, $P < 0.05$; Nonbreeding season: March-August; Breeding season: September-February

Table 2. Semen characteristics of Gurcu bucks during the breeding and nonbreeding seasons.

Traits	n	Breeding season	n	Non-breeding season	P
Semen volume (mL)	64	1.27 \pm 0.05	49	0.76 \pm 0.04	<0.001
Mass activity	64	4.73 \pm 0.06	49	4.67 \pm 0.12	0.624
Sperm concentration ($\times 10^9$ mL)	64	2.48 \pm 0.10	49	3.93 \pm 0.18	<0.001
Total Spermatozoa ($\times 10^9$ mL)	64	3.03 \pm 0.21	49	2.94 \pm 0.26	0.789
Progressive sperm motility (%)	64	80.39 \pm 1.00	49	74.43 \pm 2.26	0.019
Post-thaw progressive sperm motility (%)	50	25.00 \pm 2.86	37	19.32 \pm 2.55	0.145

Breeding season: September-February, Non-breeding season: March-August

n: No. of ejaculates examined

Table 3. Seasonal variations in testosterone level in Gurcu bucks in the breeding and nonbreeding seasons.

	Testosterone (ng/mL)	
Season	n	Mean \pm SEM
Breeding season	8	13.44 \pm 2.37
Nonbreeding season	12	13.06 \pm 1.18
P value	-	0.894

Breeding season: September-February, Nonbreeding season: March-August

n: Number of testosterone hormone samples

Table 4. Monthly variation in phospholipase activity individual bucks.

Months	Phospholipase A2 activity ($\mu\text{mol}/\text{min}/\text{mL}$)				
	n	Mean \pm SEM	Buck no	n	Mean \pm SEM
November	15	101.69 \pm 9.74	31	6	64.44 \pm 6.19a
			35	6	80.11 \pm 22.35ab
December	14	79.99 \pm 12.19	40	6	75.63 \pm 13.39ab
			80	6	121.65 \pm 1.82b
			81	6	112.53 \pm 2.63ab
P value	-	0.173	P value	-	0.022

n: Number of phospholipase enzyme samples

DISCUSSION

Although many studies have been conducted to examine the semen characteristics in different breeds, studies on the semen characteristics of regional/domestic genotypes are limited. As far as we are aware, no information or scientific study is available about the reproductive characteristics of Gurcu bucks in Turkey, particularly regarding the seasonal variation in semen quality and quantity. This is novel study recorded relevant data regarding the reproductive characteristics of Gurcu bucks.

A paucity of literature showed that semen could be taken from bucks once or twice a week throughout the year (Roca *et al.*, 1992; Karagiannidis *et al.*, 2000; Barkawi *et al.*, 2006; Chentouf *et al.*, 2011). The present study predicted to obtain 208 ejaculates from 4 bucks once a week but obtained only 113 ejaculates. Although the Gurcu bucks have continued sperm production throughout the year, getting regular semen ejaculate from the Gurcu bucks throughout the year is not possible; also the sustainability of such studies is extremely difficult. It should also be considered that libido is reduced in size outside the breeding season, which may cause some problems in taking semen using an artificial vagina.

Discrepant studies have been reported regarding the seasonal variations and scrotum measurements. For instance, Webb *et al.* (2004) reported that the scrotum measurements of the Gorno Altai and South African domestic bucks changed seasonally. Chentouf *et al.* (2011) detected the highest and lowest scrotum circumference measurements as 27.7 cm and 24 cm in summer and winter, respectively, testis measurements were significantly affected by the season. These values were close to the measurements obtained from the goat breed used in the present study. Souri and Mirmahmoudi (2014) found the highest values of the Markhoz bucks scrotum circumference (35.2 cm), testis length (14.7 cm), and testis width (6.1 cm) in autumn. These values were higher than the values as obtained in the present study. This difference might be due to the influence of co-factors such as breed, age, care and nutrition of goats, and individual measurement methods used in the study. Moreover, Şogorescu *et al.* (2011) found the lowest and

highest values of testicle size of the Carpathian goats in May (221 mL) and August (394 mL), respectively. These values were in agreement with the values of testicles size obtained from the Gurcu bucks used in the present study.

This study showed that the semen volume was significantly affected breeding and nonbreeding seasons ($P < 0.001$). These results were consistent with the findings of Greyling and Grobbelar (1983), Ahmad and Noakes (1996), and Zamiri and Heidari (2006) who reported seasonal variations in the amount of semen. Similarly, Barkawi *et al.*, (2006) found that the amount of semen changed depending on the season, and the highest amount of semen was detected in the breeding season.

Some researchers reported that sperm motility alterations were significantly affected by seasons (Kridli *et al.*, 2007; Talebi *et al.*, 2009; Wang *et al.*, 2015), while some other researchers showed that the change in fresh sperm motility was not affected by seasons (Chentouf *et al.*, 2011; Dorado *et al.*, 2012). In addition, the fresh sperm motility in this study was higher than the year-round motility values detected by some researchers (Kamal *et al.*, 2005; Qureshi *et al.*, 2013). The reasons for the differences between the motility values obtained in this study and those obtained in other published studies included breed, evaluator and methods used in the evaluation. However, the motility values in this study were similar to those reported by Roca *et al.* (1992) and Farshad *et al.* (2012).

It was reported that the sperm concentration changed according to seasons and this change was statistically significant (Karagiannidis *et al.*, 2000; Barkawi *et al.*, 2006; Zamiri and Heidari 2006; Talebi *et al.*, 2009). Inconsistent with our observations, no statistically significant difference was found between the seasons in terms of sperm concentration by Choe *et al.* (2006) in Korean domestic bucks and by Kamal *et al.* (2005) in Saanen and Nubian bucks. In this study, the sperm concentration of Gurcu bucks was found to be higher than the values reported in four seasons by Talebi *et al.* (2009), Souri and Mirmahmoudi (2014), Qureshi *et al.* (2013), and Wang *et al.* (2015), lower than the values reported by Ahmad and Noakes (1996) and Barkawi *et al.* (2006, and similar to the values reported by Roca *et al.*

(1992), Kamal *et al.* (2005), Kridli *et al.* (2007), and Chenteouf *et al.* (2011).

There is a limited number of studies on seasonal effects on the freezing of goat semen in the literature. Tuli and Holtz (1995) conducted a study in Germany and found that the freezability of Boer buck semen was influenced by seasons. They detected the best post-thawing sperm motility and viability percentages during winter. In this regard, the present study had similarities with the study by Tuli and Holtz (1995). On the contrary, Tuli and Holtz (1995) did not find any significant difference in the summer and autumn seasons in terms of post-thawing spermatological parameters. Ustuner *et al.* (2009) reported that breeding season and removal of seminal plasma had beneficial effects on the freezability of Saanen goat semen in Turkey. Wang *et al.* (2015), in their study in China, reported that the best seasons for the freezing of Xinong Saanen buck semen were the summer and autumn seasons. They also detected that post-thawing sperm motility were adversely affected by the spring and winter seasons. The present study found a no statistical difference between the seasons, regarding the post-thawing progressive sperm motility levels of Gurcu bucks. However, numerically, the study determined that the post-thawing spermatological parameters were better in breeding season than in nonbreeding season.

Perez and Mateos (1995), Todini *et al.* (2007) and Amrane *et al.* (2013), found significant seasonal differences in testosterone levels in their studies conducted on different breeds of goats in different environments. They also observed the lowest testosterone values in spring and winter seasons and the highest values in summer and autumn seasons. In our study, there was no statistically significant difference between the testosterone values in the breeding season and the nonbreeding season. Compared with the values given in the literature, the higher or lower blood testosterone values in this study were attributed to the factors including time and place of taking blood, the method used for evaluation (different from other studies, this study used the ELISA testosterone kit specific to goats), and serum or plasma assay.

Although the adverse effect of phospholipase in bucks was well established, studies measuring the phospholipase activity are limited. The present study tried to determine the phospholipase A₂ activity of the Gurcu bucks. However, Aguiar *et al.* (2013) conducted a study in Brazil without mentioning the breed and found the phospholipase A₂ activity as 8.1 ± 0.1 U/mL and 6.2 ± 0.5 U/mL in dry and rainy seasons, respectively. The values found by them were different from the phospholipase A₂ activity levels detected in the present study in the Gurcu bucks. Our study could detect the phospholipase activity only in November and December.

In conclusion, the testicular and spermatological characteristics of Gurcu bucks were defined and the

seasonal change was revealed for the first time. Although seasonal changes were observed in andrologic parameters, fresh and post-thawing spermatozoa in Gurcu bucks, seasonal change was limited. Another result of this working was that taking semen from the Gurcu bucks was extremely difficult and it was also impossible to maintain this process throughout the year.

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