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DESERT CLIMATIC EFFECTS ON FREEZABILITY AND SOME BIOCHEMICAL CONSTITUENTS OF BARKI RAM SEMEN

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ABSTRACT

This study utilized 12 mature fertile Barki rams located in the Maryout Research Station. Semen was collected during June – August (summer season, 1999) and during December – March (winter season, 1998). Semen was collected using an artificial vagina with 0.5 ml Tris buffer in the collection tubes (1:1 dilution). Semen samples were diluted and packed in straws (0.25ml) and frozen in liquid nitrogen (-196°C). Data on physical characteristics of semen were recorded (volume, motility, % acrosome integrity, % dead and live sperm, pH, concentration and % abnormality). In addition, seminal plasma of both seasons were harvested and Na^+, K^+, free amino acids and total protein were determined. Also, SDS-PAGE was conducted to characterize the peptide fractions of seminal plasma of both seasons. Results indicated higher ($p<0.05$) post-thaw (0h) motility in winter (44.1%) than in summer (17.2%) ejaculates, whereas at 4 hrs. post-thaw the percent intact acrosome approached 72.3% and 65.9% for summer and winter ejaculates, respectively. Moreover, percent of dead and abnormal spermatozoa were higher ($p<0.05$) in post-thaw spermatozoa of summer than winter ejaculates. Sodium concentration was not different between summer and winter ejaculates, however K^+ concentration was higher ($p<0.05$) in winter (71.7 ppm) than in summer (47.3 ppm) ejaculates. This resulted in different K^+/Na^+ ratio between the two seasons. Approximately, total protein was found to be as much twice (14.0g/dL) in summer as in winter (7.7g/dL). The glutamic acid and glycine were higher in winter than summer season. The SDS-PAGE exhibited two more peptide fractions (330 and 24 kDa) in winter than summer seminal plasma. The total number of peptide fractions was 14 in winter and 12 in summer.

Keywords : Climate, Ram, Semen, SDS-PAGE protein, Amino acids, minerals.

INTRODUCTION

Sheep and goats are known as seasonal breeder animals (Evans and Maxwell, 1987). They are called "short-day breeders" since their breeding begins in autumn when daylight is shortening. Breeding activity may also be related with other climatic factors such as feedstuffs, temperature or rainfall. El-Wishy *et al.* (1976) in their study of the seasonal variation on sexual desire and semen characteristics of young (1.5-2.5 years) and mature (4-5 years) Ossimi rams, reported that the complex of the environmental factors controlling sexual activity, exhibited different seasonal effects on different parts of the male reproductive system and on semen quality. Moreover, El-Sherbiny *et al.* (1982) found that season affected semen pH, methylene blue reduction time and percentage of motile sperm of Ossimi and

Merino rams. Under desert condition, Ibrahim (1997) found that the best quality semen of rams occurred in winter, although the sexual activity of rams was not affected by the ambient temperature of hot summer. Few studies have been conducted on the seminal plasma composition as affected by season or ambient temperature. Therefore, the objective of the present study aimed at investigating some biochemical components in ram's seminal plasma and their relations to sperm physical characteristics under different climatic conditions.

MATERIALS AND METHODS

Animals and Management : This study was carried out in Maryout Research Station Desert Research Center, El-Amrya, Alexandria. Twelve mature Barki rams (3-4 years and 60 kg body weight) were used for semen collection. The animals were kept together in one pen yard under equal management conditions. They were fed pelleted concentrate ration containing 14% crude protein. The concentrates were supplemented with barley as a source of energy. Berseem hay was offered *ad lib.*, water was offered twice daily and animals were allowed for free grazing for 3-4 hours a day. The study was carried out during December– March, 1998 (Rainy cold) and June – August 1999 (Hot dry).

Semen collection and evaluation : Semen was collected twice a week for a total of 12 weeks/season using an artificial vagina. The semen ejaculates were collected in clean collection tubes contained 0.5 ml extender (Tris buffer). Samples were directly transferred to the adjacent laboratory and placed in a water bath adjusted at 37°C. Volume was estimated by substracting 0.5ml of the total ejaculate volume. Percentage of progressive motility was assessed by a warm stage phase-contrast stereomicroscope (X400).

Percent of dead and live sperms was examined by a mixture of eosin-aniline (0.1%, Saacke, 1970). Using a pH paper indicator, the pH values were determined with an accuracy of 0.1 unit. Sperm concentration was estimated by a calibrated spectrophotometer (Amerus, USA). Abnormalities and acrosome integrity were determined (X800) by the glutraldhyde method (Johnson *et al.*, 1976).

Thawing procedure and post-thaw evaluation : Diluted samples were deep frozen (-196°C) in liquid nitrogen. Frozen samples were thawed at 65°C for 7 seconds for each. Thawed samples were incubated at 37°C and evaluated at 0, 2 and 4 hours.

Seminal plasma preparation and evaluation : Three ejaculates were collected consecutively of the same ram. These ejaculates were pooled and centrifuged (1800 rpm/30 minutes). The supernatant (Seminal plasma) was aspirated in a clean tube and frozen (-70°C) until used for assays. The seminal plasma was used for determination of sodium, and potassium elements, total protein and free amino acids and subjected to the SDS-PAGE for peptide characterization.

Sodium and potassium were assessed in twenty-two seminal plasma samples of 11 rams (one ejaculate of the hot and the other of the cold season) using flamephotometer (Corning 400, USA). Pure sodium chloride and potassium chloride solutions were used for the preparation of the two standard curves (0-100 ppm) for Na^+ and K^+, respectively. The eleven ram's ejaculates seminal plasma were pooled for each season. An aliquot of 250 µl seminal plasma was diluted in 25 ml deionized distilled water (1:100) for biochemical and mineral determinations.

Total protein in seminal plasma was determined by a Unicam spectrophotometer at a wavelength of 750 nm (Lowry *et al.*, 1951) in the 22 seminal plasma samples.

Free amino acids in samples were determined by the method of Hamilton (1962). A sample of 1 ml seminal plasma was mixed with 50 mg of sulfosalicylic acid and centrifuged at 3500Xg for 5 minutes. The supernatant was decanted and diluted with the sodium citrate buffer (2.9%) in a ratio of 1 sample: 4 buffer. Concentration of amino acid was determined by the formula:

$$\text{g A.A./100 g sample (\%)} = \frac{\text{Sample area} \times \text{Std. conc.} \times \text{Dilution}}{\text{Std. area} \times 10^{-4} \times \text{Weight of sample (g/ml)}}$$

Peptide pattern : Sodium dodecyle sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinuous buffer system described by Laemmli (1970), and modified by Hames and Rickwood (1990).

The seminal plasma sample was mixed with an equal volume of sample buffer (2% SDS, 10% glycerol, 0.002 bromophenol blue, 5% 2-mercaptoethanol) and submitted to heat treatment for 5 min in a boiling water bath prior to be applied to the gel. Samples were allowed to cool to room temperature, finally centrifuged at 1000Xg for 5 min to remove any insoluble material causing streaking during electrophoresis.

Preparation of the buffers and gels and loading samples were done according to El-Agamy (1990). Staining peptides was done by the coomassie blue stain (Hames and Rickwood, 1990). A standard low and high molecular weight marker was used to determine the peptide molecular weight according to the method of Weber and Osborn (1969).

Statistical analysis : Data were statistically analyzed using the method of Least Squares analysis of variance using General Linear Models (GLM) Procedure (SAS, 1995). The complete randomized block design was established. Duncan's Multiple Range Test (DMRT) was used to compare between means of season for semen characteristics (Steel and Torrie, 1980). Means of total protein, sodium and potassium concentration in seminal plasma were compared between seasons using simple comparisons " t " test.

RESULTS AND DISCUSSION

Semen physical characteristics : As shown in Table (1) that percentage of post-thaw motility significantly ($p<0.05$) declined as time of incubation progressed. Meanwhile, percentage of progressive motility was higher ($p<0.05$) for the cold rainy (44.1, 34.0 and 22.2%) than for the hot dry season (17.2, 11.3 and 7.2%) at 0, 2 and 4h of post-thaw incubation, respectively. On the other hand, percent of intact acrosome (Table 2) or abnormal spermatozoa (Table 3) was not different ($p>0.10$) between summer and winter ejaculates when checked at 0 h post-thawing. Subsequently a significant decline in percent of intact acrosome was found at 2 & 4h of incubation. Additionally, percentage of secondary sperm abnormality was higher in the summer than in winter (12.4 vs 11.1 for hot vs cold season). These findings are in agreement with that of Fiser and Fairfull (1983) on ram's spermatzoa who reported that vigour of frozen-thawed spermatozoa assessed as progressive motility was significantly lower during the longer photoperiod. Also, post-thaw motility for buffalo and bulls was higher in cold rainy than in hot dry season (Bahga and Khokar, 1991). Similar findings were obtained with goat's spermatozoa (Ahmed *et al.*, 1997).

Table 1: Effect of season on post-thaw ram' sperm motlity (mean±SEM).

Season	% Motility Incubation (hr. / 37°C)		
	0	2	4
Hot dry	$17.2 \pm 2.2^{a**}$	11.3 ± 1.9^{a}	7.2 ± 1.4^{a}
Cold rainy	44.1 ± 2.2^{b}	34.0 ± 1.9^{b}	22.2 ± 1.5^{b}

* Mean within a column with different superscripts are significantly different ($p<0.05$).
** Values are Overall mean of 48 ejaculates per season.

Table 2: Effect of season on post-thaw ram' sperm intact acrosome (Mean±SEM).

Season	% Motility Incubation (hr. / 37°C)		
	0	2	4
Hot dry	$80.9 \pm 0.5^{**}$	75.7 ± 0.6^{a}	72.3 ± 0.9^{a}
Cold rainy	81.5 ± 0.5	72.7 ± 0.6^{b}	65.9 ± 0.8^{b}

* Mean within a column with different superscripts are significantly different ($p<0.05$).
** Values are Overall mean of 48 ejaculates per season.

Table 3: Effect of season on post-thaw ram' sperm primary and secondary abnormality (Mean±SEM).

Season	% Abnormality	
	Primary	Secondary
Hot dry	6.3 ± 0.2	12.4 ± 0.5[a]
Cold rainy	6.6 ± 0.3	11.1 ± 0.4[b]

* Mean within a column with different superscripts are significantly different (p<0.05).

Although the ejaculate volume and initial motility in raw semen in the present study were higher in cold (1.1ml and 90.3%) than in hot season (0.9ml and 88.2%) (Table 4), there were no significant differences in sperm concentration per ml or percent intact acrosome due to climatic season. The decline in the post-thaw motility (at 0h) in summer (17%) than winter (44.1%) ejaculates has drawn our attention to monitor the composition of seminal plasma in both seasons. Therefore, pools of seminal plasma of summer and winter ejaculates were harvested, stored and analyzed for sodium, potassium, total protein, free amino acids and electrophoretic peptide pattern.

Table 4: Effect of season on post-thaw ram' semen characteristics in raw ejaculates (Mean±SEM).

Parameter	Hot dry	Cold rainy
Volume (ml)	0.9 ± 0.3[a**]	1.1 ± 0.03[b]
Motility (%)	88.2 ± 0.9[a]	90.3 ± 0.5[b]
Concentration (10^9)	3.6 ± 0.1	3.8 ± 0.5
Intact acrosome (%)	85.9 ± 0.5	86.3 ± 0.4
Alive sperm (%)	92.1 ± 0.4[a]	94.3 ± 0.3[b]
Primary abnormality (%)	5.1 ± 0.2[a]	5.8 ± 0.2[b]
Secondary abnormality (%)	10.3 ± 0.4	10.8 ± 0.3

* Mean within a column with different superscripts are significantly different (p<0.05).
** Values are Overall mean of 48 ejaculates per season.

Table (5) exhibits concentration of Na^+ and K^+ in ram' seminal plasma during hot and cold months. There was no significant difference in Na^+ concentration between hot and cold season, however there existed a significant (p<0.05) increase in K^+ concentration in seminal plasma of cold season (71.7 ppm) as compared to that of hot season (47.3 ppm). It has been found earlier (Mann, 1964) that sodium and potassium concentrations were 103 and 71mg/100ml ram seminal plasma, respectively. The increase in K^+ in the extracellular fluid (seminal plasma) caused a decreas in Na^+/K^+ ratio. Similar finding was noted in winter seminal plasma of buffalo bulls ejaculates (Singh *et al.* , 1969). Moreover, Mann (1964) stated that K^+ might be an

important factor in initiating the motility and metabolism of spermatozoa. In invertebrates, Mann (1964) found a vital role of K^+ ions on flagella movement of spermatozoa, which depends on ion gradient around sperm cell membrane and this pumped into the tail by head-piece machinery. Furthermore, Kononov *et al.* (1993) found a positive relation between bull's fertility and K^+ concentration in seminal plasma. Also, the K^+/Na^+ ratio was found to have a strong relationship with the maintenance of a constant osmotic pressure within and outside the sperm cell (Patel, 1985). The change in this ratio between seasons in the present study could explain the change of freezability and post-thaw sperm survival. Recently, Al-Ali *et al.* (1997) suggested that maintaining the ionic equilibrium around ram sperm cell might be a vital factor for maintaining sperm survival after processing and storage.

Table 5: Effect of season on post-thaw ram' seminal plasma Na^+ and K^+ concentrations (Mean±SEM).

Season	Concentration (ppm)	
	Na^+	K^+
Hot dry	138.0 ± 7.5^a	47.3 ± 1.9^a
Cold rainy	143.3 ± 9.5^a	71.7 ± 7.0^b

* Mean within a column with different superscripts are significantly different ($p<0.05$).
** Values are means of 11 ejaculate' seminal plasma per season.

Seminal plasma total protein in hot summer ejaculates was found to be nearly as much two-folds (14.1g/100 ml) as that in cold winter (7.79/100 ml). This significant ($p<0.01$) increase could be ascribed to the integrity of spermatozoal cell membrane which is less effective in hot than in cold seasons (Mann, 1964). Furthermore, there is a possibility that proteins and enzymes found in seminal plasma are not in a soluble form but occur as particulate matter originating from disrupted epithelial cells of the accessory glands or from the spermatozoa. Karcheva and Bewlov (1989) found a decrease of total protein and activities of acid phosphatase (AcP) and asparatate and alanine aminotransferase (AST & ALT) in seminal plasma during the breeding season of Danube finewool rams.

It has been found that free glutamic acid and glycine were higher in seminal plasma of winter than in summer ejaculates (Table 6). It has been found that the excessive dilution of semen exerts deleterious effect on sperm survival, which could be alleviated by the inclusion in the media of some amino acids such as glycine, alanine, valine, leucine, lysine and glutamic acid (Mann, 1964). Glutamic acid has been found to be the predominant of not less than 17 amino acids in both fresh testicular tissues and in seminal plasma (Sexton *et al.*, 1970). Also, Ibrahim *et al.* (1984a&b) found that glutamic acid constitutes about 35-45% of the total free amion acids and the highest concentrations in bull seminal plasma was found in cold winter and the lowest in hot summer ejaculates. This finding agrees with the present

result in which glutamic acid constitutes 30% of total free amino acids in cold winter and 21% in hot summer ram' seminal plasma.

Table 6: Effect of season on free amino acids (mg/100ml) in ram' seminal plasma (Mean±SEM).

Amino acid	Hot dry	Cold rainy
Aspratic acid	19.4	12.8
Threonine	85 8	87.9
Serine	69.3	70.9
Glutamic acid	97.4	140.1
Proline	8.01	4.9
Glycine	8.62	14.87
Alanine	14.23	15 39
Cystine	2 63	1.26
Valine	8 12	7.31
Methionine	6.24	6.54
Isoleucine	10.86	6.51
Leucine	27.26	14.89
Tyrosine	5.29	4.19
Phenylalanine	3.95	3.95
Histidne	22.27	24.55
Lysine	30.91	26.49
Arginine	22 40	22.25
(Amonia)	5.94	6.96

* Values are derived of one pool representing 11 ejaculates within a season.

The electrophoretic peptide pattern (Figure 1) of ram seminal plasma exhibits 14 different fractions of peptides in winter semen with a range of molecular weight of 15 to 330 kDa. However, summer seminal plasma contained 12 peptide fractions with molecular weights ranging between 15 to 130kDa. The first column of the photo represents the standard protein markers, while columns 1, 3 and 5 represent seminal plasma of winter ejaculates with increasing protein concentrations of 21, 42 and 63 mg/lane, respectively. However, columns 2, 4 and 6 represent seminal plasma of summer ejaculates with the same protein concentrations. Obviously, the top of lanes of the winter samples exhibits protein aggregates which is represented by one band (M.Wt ≈ 330kDa). Moreover, a small molecular weight – peptide fraction (≈24kDa) exists in winter but disappeared in summer ram' semen. These two fractions could contribute to the higher post-thaw sperm motility in winter than summer ejaculates. Early publications on the electrophoretic behaviour and protein concentrations in human and animal seminal plasma have been extensively reviewed (Mann, 1964). In the last two decades more attention has been paid to the identification and characterization of such peptides and their roles in sperm characteristics. Al-Hanak and Spasova (1983) noted that accumulation of more proteins of

higher electrophoretic mobility developed more favorable condition in preservation of ram spermatozoa. Furthermore, Amann *et al.* (1987) noted that out of 27 protein fractions (range ≈13 to 120kDa) there found only 2 peptides (23 and 26 kDa) of positive significance on sperm motility. Recently, Killian *et al.* (1993) and Cancel *et al.* (1997) have indicated that two proteins of 25 and 55 kDa were predominant in higher fertility bulls, while the existence of 16 kDa – protein was predominant in lower fertility bulls.

It has been concluded that under desert condition, sheep could be raised for breeding purposes with emphasis on semen preservation during cold winter months for the purpose of semen preservation and artificial insemination all around the year. Moreover, a multidisplinary study is necessary to investigate the chemical, physiological and immunological role (s) of each of the peptide fraction in ram seminal plasma.

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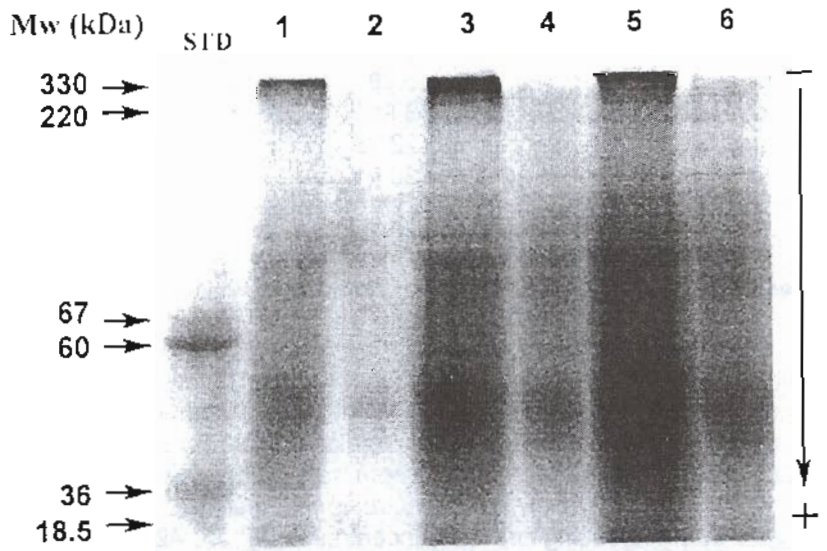

Fig 1: Electrophoretic patterns for dry hot summer and cold rainy seasons seminal plasma.

- SDS-PAGE (10%T) of seminal plasma proteins of sheep prepared in cold rainy and hot dry seasons. STD: Standard protein marker. (Anode is toward the bottom or photo).
- Lanes 1, 2 had 21mg protein, while 3, 4 had 42mg protein and lanes 5&6 had 63mg protein, respectively.
- Lanes 1, 3 and 5, represents the cold rainy season, while, lanes 2, 4 and 4, represents the hot dry season.

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تأثير المناخ الصحراوي علي تحملّ الحفظ بالتجميد وبعض المركبات الكيموحيوية للسائل المنوي لكباش البرقي

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أجريت هذه الدراسة بمحطة بحوث مريوط التابعة لمركز بحوث الصحراء - بوزارة الزراعة حيث كان الهدف منها دراسة أثر شهور الحرارة المرتفعة (يونيو-يوليو-أغسطس) مقابل شهور الحرارة الباردة (ديسمبر-يناير-فبراير) علي المقدرة التجميدية والحفظية للسائل المنوي لكباش الأغنام البرقي تحت الظروف الصحراوية حيث تم استخدام ١٢ كبش بالغ وتم جمع السائل المنوي بمعدل مرتين أسبوعيا بالمهبل الصناعي في أنابيب جمع تحتوي كل منها علي ٠,١مل مجفف الترس (معدل تخفيف المبدئي ١:١) وتم التخفيف بنفس المخفف بعد الفحص المبدئي والحكم علي صلاحية القذفة (معدل التخفيف النهائي ١ :١٠) وتم التعبئة في قصيبات حفظ السائل المنوي (حجم ٠,١ مل) ثم جُمدت وحفظت هذه القصيبات في النتروجين السائل (-١٩٦°م).

وتم دراسة الخصائص الفيزيائية للسائل المنوي من حيث حجم القذفة ونسبة الحركة التقدمية وتكامل الأكروسوم ونسبة الحي والميت والمشوه ودرجة الحموضة وتركيز الحيوانات المنوية. وبالإضافة لذلك تم تقدير الصوديوم والبوتاسيوم والأحماض الأمينية الحرة والبروتين الكلي في بلازما السائل المنوي. وأجريت الهجرة الكهربية SDS-PAGE علي بلازما السائل المنوي من كلا فصلي السنة.

وأظهرت النتائج ارتفاعاً معنوياً في نسبة الحركة التقدمية للحيوان المنوي عند تسييل القصيبات والتحضين عند ٣٧°م والفحص عند ٠، ٢، ٤ ساعات وكان هذا الارتفاع في عينات السائل المنوي لفصل البرودة عنه في عينات فصل الحرارة (٤٤,١% للشتاء و١٧,٢% للصيف عند oh). أما الأكروسوم السليم فتناقص عند ٤ ساعات من التحضين في عينات فصل الصيف (٦٥,٩%) عنه في عينات فصل الشتاء (٧٢,٣%). وأيضاً نسبة الحيوانات المنوية المشوهة والميتة زادت ($p<0.05$) في فصل الصيف عنها في فصل الشتاء.

لم يختلف تركيز أيونات الصوديوم في بلازما السائل المنوي الصيفي عنه في الشتوي في حين ازداد ($p<0.05$) تركيز أيونات البوتاسيوم في الشتاء (٧١,٧ جزء في المليون) عنه في الصيف (٤٧,٣ جزء في المليون) ونتج عن ذلك اختلاف في نسبة البوتاسيوم/الصوديوم K^+/Na^+ بين الموسمين.

وعلي الجانب الآخر ازداد تركيز البروتين في عينات الصيف (١٤جم/١٠٠مل) عنه في عينات الشتاء (٧,٧جم/١٠٠مل) وأيضاً ارتفع تركيز حمض الجلوتاميك والجليسين في عينات الشتاء عن عينات الصيف. في حين ظهر ٢ حزمة ببتيدية (٢٤، ٣٣٠ كيلو دالتون) أكثر في عينات الشتاء (١٤ ببتيد) عنه في عينات الصيف (١٢ ببتيد).

ومن هذه النتائج نخلص إلي أنه يمكن عمل برامج تربية باستخدام التلقيح الصناعي للأغنام في الصحراء مع الوضع في الاعتبار بأن يتم جمع السائل المنوي من الكباش في فترة البرودة (فصل الشتاء) للحصول علي جودة سائل منوي عالية وحفظها لاستخدامها في التلقيح علي مدار العام في أثناء موسم التربية والتزاوج وبالإضافة لذلك توصي هذه الدراسة بإجراء مزيد من الدراسات علي كيفية التعرف علي نوعيات الجزيئات الببتيدية والنشاط المناعي أو الانزيمي لتلك الببتيدات الموجودة في السائل المنوي المفرز شتاءً.