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Testicular morphology and in vitro evaluation of frozen epididymal sperm of Anatolian buffalo[*]

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Summary: Epididymal sperm may be available as a source of spermatozoa for artificial insemination and in vitro embryo production in many species. The aim of the study was to investigate epididymal sperm freezing on spermatological parameters of Anatolian buffaloes and in vitro evaluation of frozen epididymal sperm for assisted reproductive biotechnologies. The testes and epididymides were collected from 10 Anatolian buffalo bulls (about 3-4 years of age) in Samsun Province. The testes and epididymides from each bull were removed immediately after slaughtering and transported to the laboratory on ice (+4 °C) within 40 min for further processing. After morphological measurements of testes and epididymides were determined, the spermatozoa were collected from cauda epididymides. The percentage of sperm progressive motility and abnormal spermatozoa of fresh and post-thaw sperm samples were evaluated. There was no significant difference between fresh and frozen-thawed sperm samples for progressive sperm motility and head-acrosome anomalies of spermatozoa. The middle part ($P<0.001$), tail ($P<0.001$) and total ($P<0.001$) abnormal spermatozoa rates of frozen-thawed sperm samples were higher than those of fresh sperm samples. In conclusion, collected spermatozoa from cauda epididymides of Anatolian buffalo bull may be used as fresh or frozen for artificial insemination, in vitro embryo production or intra cytoplasmic sperm injection.

Keywords: Anatolian buffalo, epididymal sperm, epididymis, morphometry, testis.

Anadolu mandalarında testiküler morfoloji ve dondurulmuş epididimal spermanın in vitro değerlendirilmesi

Özet: Epididimal sperma birçok türde suni tohumlama ve in vitro embriyo üretiminde spermatozoa kaynağı olarak kullanılabilir. Yapılan bu çalışmanın amacı Anadolu Mandaları'ndan alınan epididimal spermanın dondurulmasının bazı spermatolojik parametreler üzerine etkilerinin incelenmesi ve yardımcı üreme teknolojileri için dondurulan epididimal spermanın kullanılabilirliğinin in vitro değerlendirilmesidir. Samsun İlinde kesilen (yaklaşık 3-4 yaşlı) toplam 10 adet erkek Anadolu Mandasından kesim sonrasında testisler ve epididimisler hemen alınarak buz içinde (+4 °C) 40 dakika içerisinde daha sonraki işlemler için laboratuara getirildi. Testis ve epididimislerin morfolojik ölçüleri belirlendikten sonra spermatozoa cauda epididimisten toplandı. Nativ ve çözüm sonu örneklerde progresif spermatozoa motilitesi ve anormal spermatozoa yüzde oranları değerlendirildi. Nativ ve dondurulmuş çözülmüş sperma örnekleri arasında progresif spermatozoa motilitesi ve spermatozoa baş-akrozom anomalileri yönünden herhangi bir fark tespit edilmedi. Dondurulmuş çözülmüş spermadaki orta kısım ($P<0,001$), kuyruk ($P<0,001$) ve total ($P<0,001$) anormal spermatozoa oranları, nativ spermaya göre daha yüksek bulunmuştur. Sonuç olarak, Anadolu Mandasının cauda epididimisinden elde edilen spermatozoa nativ veya dondurulmuş epididimal sperma olarak suni tohumlamada, in vitro embriyo üretiminde ya da intra sitoplazmik spermatozoa enjeksiyonu gibi yardımcı üreme biyoteknolojilerinde kullanılabilir.

Anahtar sözcükler : Anadolu mandası, epididimal sperma, epididimis, morfometri, testis.

Introduction

The buffalo provide meat and milk for human consumption, and some raw materials like leather for industrial use. The buffaloes have better feed efficiency and are more resistant to natural conditions and diseases than cows.

The buffaloes are a common species especially in the developing countries of Asia and are spread throughout all the continents. In Europe, the buffaloes are largely grown in Italy and Bulgaria in which they are kept mainly as a source of milk and meat. Economic importance of buffalo production for Italian dairy industry is linked to the famous cheese which is called "Mozzerella" (5). Recently, buffalo population has markedly decreased in Turkey while there is an increase in buffalo population in the world. Nowadays buffalo

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population of the world and Turkey are estimated to be about 195 million and 84 000, respectively (9).

The buffaloes are primarily grown in Black Sea, Central Anatolia and Aegean Regions of Turkey. The buffaloes in Samsun Province are named as Anatolian buffalo and classified in the river type (29).

The buffalo is generally considered as a poor breeder with low reproductive efficiency and characterized by late attainment of puberty and maturity, poor expression of oestrus signs, low pregnancy rates, seasonal calving, long postpartum anoestrus and calving intervals (5). Therefore, artificial insemination which is one of the modern ways of breed improvement can be applied to improve of buffalo production. The frozen-thawed semen quality is one of the most important factors affecting the probability of pregnancy rates (2).

The implementations of new approaches for the management of ovulation (e.g., Ovsynch procedure), conducive reproductive techniques may assist to overcome reproductive inadequacy in the buffalo species (6). In that respect, epididymal sperm can be an available source of spermatozoa for using in assisted reproductive technologies if the spermatozoa are urgently retrieved from a donor which is severely injured or dead (11). Epididymal sperm has been successfully used for artificial insemination and in vitro embryo production in many species (6), hence there is an increasing interest in transportation and preservation of epididymal sperm. Even if elite male breeders die, epididymal sperm collection offers the possibility to enable the usage of genetic material of these animals. Furthermore, epididymal sperm may be used as fresh, frozen or stored in genetic resource bank projects. The use of fresh or frozen epididymal sperm in reproductive biotechnology has already resulted in offspring in domestic and wild animal species such as horse, cattle, goat, dog, red deer, mouflon, eland and chimpanzee (11).

Lalthazuali *et al.,* (17) and Nazir et al., (20) reported that pregnancy rates in buffaloes inseminated with frozen-thawed buffalo sperm were about 20% and 66.7%, respectively. Therefore, a pregnancy rate of higher than 50% may be a good result for inseminating with frozen-thawed sperm (31).

The aim of the study was to investigate the effects of epididymal sperm freezing on spermatological parameters of Anatolian buffalos.

Materials and Methods

Animals: The testes and epididymides were collected from 10 Anatolian buffalo bulls (about 3-4 years of age) in Samsun Province.

Collection, Processing and Microscopic Evaluation of Material: The testes and epididymides from each bull were removed immediately after slaughtering and transported on ice (+4 °C) within 40 min for further processing (18). Immediately upon arrival to the laboratory, testes-epididymis volumes were measured by a volumetric beaker. The caput, corpus and cauda epididymides were dissected free from the testes and weighted. After testes major and minor diameters were measured by manual calipers, they were weighted, and then circumferences of testes were measured.

The blood vessels were removed from the tail of each cauda epididymis for avoiding the toxic affect of blood on sperm (18). After several longitudinal incisions were performed on the distal end of the cauda to disclose the sperm from tubuli to the outer environment, and then the percentage of sperm progressive motility and abnormal spermatozoa of fresh semen were evaluated according to Tekin (30). The sperm samples were diluted with Bioxcell® extender (+ 4°C).

Freezing of sperm: The sperm, diluted by Bioxcell® extender, loaded into the 0.25 mL straws and sealed with a heat sealer. The sealed straws containing the sperm were transferred to a refrigerator (+ 4 °C) for an equilibration period of 5 h, and then they were placed in the liquid nitrogen vapors, 5 cm above the level of liquid nitrogen for 10 min (1). Thereafter, the straws were immersed directly into liquid nitrogen and transferred to a canister which was placed in a liquid nitrogen tank.

Thawing and post-thaw evaluation: Straws were thawed at 37°C for 30 sec in a water bath after 24 h storage period in liquid nitrogen (3). The percentage of sperm progressive motility (27) and abnormal spermatozoa were evaluated according to Tekin (30).

Statistical analysis: Testicular morphology (testes-epididymis volume, caput, corpus and cauda epididymis weights, testes weights, major-minor diameters and circumferences of testes) and spermological traits (sperm progressive motility and abnormal spermatozoa rate) were described with descriptive statistics. Data analyses were executed by GLM procedure (28). All traits analysed with using SAS Statiscal System (26).

Results

The mean of testes-epididymis volume, the mean weights of testes, caput, corpus and cauda epididymis, the mean of testes major-minor diameters and circumferences of Anatolian buffalo bulls (n=10) were presented in Table 1.

There was no significance difference between fresh sperm samples and frozen-thawed sperm samples for progressive sperm motility and head-acrosome anomalies of spermatozoa. There were differences between fresh sperm samples and frozen-thawed sperm samples for the middle part ($P<0.001$), tail ($P<0.001$) and total ($P<0.001$) abnormal spermatozoa rates (Table 2).

Table 1. Morphological measures of testis and epididymis of Anatolian buffalos (n=10)
Tablo 1. Anadolu mandalarında testis ve epididimisin morfolojik ölçüleri (n=10)

Measurements	Organs	$\overline{X} \pm S_{\overline{X}}$	min	max
Weight (g)	Testis	170.00±10.52	126.00	240.00
	Caput Epididymis	6.52±0.27	5.30	8.20
	Corpus Epididymis	2.51±0.13	2.00	3.48
	Cauda Epididymis	6.69±0.58	4.10	9.46
Major diameter (cm)	Testis	10.33±0.44	9.00	12.80
Minor diameter (cm)	Testis	5.16±0.21	3.80	5.80
Circumference (cm)	Testis	23.69±1.07	18.85	29.04
Volume (mL)	Testis - Epididymis	153.50±9.94	100.00	210.00

Table 2. Spermological parameters before freezing and after thawing (n=10)
Tablo 2. Dondurma öncesi ve çözdürme sonrası spermatolojik parametreler (n=10)

Spermological parameters		Fresh	After thawing	P
		$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
Progressive motility (%)		34.50 ± 5.79[b]	20.50 ± 4.31[a]	0.068
Abnormal spermatozoa (%)	Head-acrosome	2.50 ± 0.45	3.30 ± 0.61	0.309
	Middle part	14.50 ± 1.38[a]	24.60 ± 1.66[b]	0.000
	Tail	7.60 ± 1.70[a]	30.50 ± 4.97[b]	0.000
	Total	24.60 ± 2.32[a]	58.40 ±4.87 [b]	0.000

a,b : For each traits, means with the different letter in the same row were significantly different
a,b : Her özellik için, aynı satırda farklı harfle ifade edilen ortalamalar önemli derecede farklıdır

Discussion

Although spermatogenic cell divisions in testes begin about 12 months of age and active spermatogenesis may be seen from 15 months in buffalo bulls which were well nourished and have optimum body condition score, the ejaculate comprises viable spermatozoa only after 24 or 30 months of age. The male buffalo bulls mature more slowly than the cattle bulls, so they require a longer time period from the initial phase of spermatogenesis to the accomplishment of puberty (22). Therefore, the results obtained at testicular morphology and spermological traits were evaluated in about 3-4 years old Anatolian buffalo bulls in the study.

In the present study, the results for mean testicular weights, the mean major and minor diameters (cm) of the testes of buffalo bulls were compatible with those of the study (5), while mean epididymal weights were lower than those of Arrighi *et al.,*(5).

The epididymidis in which sperm maturation occurs and spermatozoa preserve is a part of the male reproductive tract and connected to the testis. Therefore, the epididymis is dissected free from the testis and epididymal spermatozoa may be harvested for using in artificial insemination (8). Collection of the spermatozoa from epididymidis has been achieved in various animal species such as primate (10), sheep (25), cattle (12), equine (21) and African buffalo (13, 15). Herold *et al.* (15) and Lessard *et al.* (19) have harvested epididymal spermatozoa from African buffaloes and North American buffaloes, respectively. Annett (4) stated that using epididymal spermatozoa for artificial insemination in domestic or wild animal species has enormous potential for retaining of valuable genetic material.

It was reported that buffalo spermatozoa were more susceptible for injuries born of freezing and thawing processes than bovine spermatozoa (23), thus dilution of sperm in an appropriate extender is important for lifespan of spermatozoa during cryopreservation (24). In this study, post-thaw percentage of progressive sperm motility in diluted Bioxcell extender was lower than that of Akhter *et al.* (1) who collected semen from five adult Nili-Ravi buffalo bulls with artificial vagina (at 42 ºC). Moreover, the results for sperm abnormalities of head, mid part and tail in diluted Bioxcell extender in the present study were higher than those of Akhter *et al.* (1). The use of epididymal sperm rather than ejaculated in the present study caused the differences in post-thaw percentage of sperm motility.

Herold *et al.* (13) stated that progressive motility of fresh epididymal sperm was 31 ± 23 %, while post-thaw progressive motility of sperm frozen with AndroMed, Trilady or Red Ovine Freezing Buffer was 13 ± 13 %, 18 ± 12 % and 28 ± 16 %, respectively. In the present study, progressive motility of fresh sperm and post-thawed sperm frozen with Bioxcell were average 34.50 ± 5.79 % and 20.50 ± 4.31 %, respectively. Herold *et al.* (15) reported that progressive motility of fresh sperm, collected from epididymides of African buffalo bulls which were more than 3 years old, was average 35 ± 21 %. Furthermore, post-thaw progressive motility of sperm frozen with Triladyl or AndroMed was 17 ± 11 % and 14 ± 13 %, respectively. The differences in the results of our study and those of Herold *et al.* (13) and Herold *et al.* (15) were attributed to different extenders used in the studies or manipulations. Herold *et al.,* (14) stated that fresh and post-thaw progressive motility of epididymal sperm from African buffalo bulls varied from 19 % to 31 % and 14 % to 29 %, respectively. Although fresh-progressive motility of the present study was higher that

of Herold *et al.,* (14), the result was in agreement with the study (14) that reported post-thaw progressive motility. However, the rate of the fresh or post-thawed epididymal spermatozoa progressive motility obtained from the study is low but this value may be in the range of the requirements for using it in assisted reproductive technologies.

Bearden and Fuquay, (7) mentioned that abnormality in epididymal sperm was distal cytoplasmic droplet which may have occurred during maturation process at cauda epididymidis. Although abnormality of epididymal sperm is generally higher than that of ejaculated sperm, in the present study, mean of total abnormal spermatozoa rate of fresh epididymal sperm was similar to that of Javed *et al.* (16) who made a total of 24 observations over a one-year period and stated that the mean of total abnormal spermatozoa rate of ejaculated sperm from buffalo bulls was 22.25 ± 1.54. Unfortunately, sperm samples may not be frozen without any damage. Therefore, total abnormalities of post-thawed sperm samples were higher than those of fresh sperm samples in the present study.

In conclusion, collected spermatozoa from cauda epididymides of Anatolian buffalo bull may be used as fresh or frozen for artificial insemination, in vitro embryo production or intra cytoplasmic sperm injection.

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