

First Commercial Semen Cryopreservation and Main Spermatological Features of Anatolian Buffalo

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Abstract

Conventional buffalo semen freezing studies are limited in Anatolian buffaloes, which are overly sensitive to exogenous stimulation. The present study's object was to determine the main features of Anatolian Buffalo semen obtained by artificial vagina method for the first time. A total number of 150 ejaculates were collected from three Anatolian Buffalo bulls (app. 4 years of age). The mean pH, volume and concentration of semen were found 6.63 ± 0.15 , 1.61 ± 0.5 ml, $1629 \pm 222.67 \times 10^6$ spermatozoa/ml, respectively. The sperm motion characteristics were determined by using a computer-assisted sperm analysis system (CASA); the total and progressively motile sperm values were $57.12 \pm 5.63\%$, $23.22 \pm 4.47\%$ and other kinetic parameters such VAP, VSL, VCL, ALH, BCF, STR, LIN were found $94.71 \pm 8.48 \mu\text{m/s}$, $72.6 \pm 7.08 \mu\text{m/s}$, $160.9 \pm 15.66 \mu\text{m/s}$, $7.8 \pm 3.75 \mu\text{m}$, 29.15 ± 1.56 Hz, $76.91 \pm 3.87\%$, $46.21 \pm 2.61\%$, respectively after thawing. Among buffalo bulls, differences in semen pH values were statistically significant ($P < 0.05$), while differences in ejaculate volume, semen concentration, total motility, progressive motility, VAP, VSL, VCL, ALH, BCF, STR, and LIN were not ($P > 0.05$). As a result, frozen Anatolian buffalo semen can be obtained economically and can be used for animal breeding in assisted reproductive biotechnology such as artificial insemination or in vitro embryo production commercially.

Introduction

Domestic water buffaloes are studied under two classes as river (*Bubalus bubalis*) and swamp buffaloes (*Bubalus carabensis*). Despite of, one breed comprises swamp buffaloes, there are many breeds such as Murrah, Nilli-Ravi, Kündi, Surti, Jafarabadi, Nagpuri, Pandharpuri and Mediterranean buffalo in river buffaloes (Kelgokmen, 2015). Anatolian water buffalo (as shown in Figure 1) located in Turkey originated from the Mediterranean subgroup of river buffaloes (Soysal *et al.*, 2007). Although Turkey had a high population of Anatolia buffalo earlier in this century, the buffalo population had fallen below 100 thousand head in 2010 (Atasever *et al.*, 2008; Aköz *et al.*, 2017; Çolak *et al.*, 2017). Thus, genetic materials (DNA, somatic cell) of Anatolian buffalo against the danger of extinction; have

been stored in the national gene bank of Turkey (Arat, 2011).

Sperm cryopreservation forms the basis of gene banks with its ease of method, success in its transfer to the field, economical storage and alternative fertilization application options. Besides, the use of frozen semen by artificial insemination is the most common biotechnological practice that enables rapid and inexpensive genetic progress in many animal species for increasing yield and product quality (Morrell, 2011).

The first buffalo sperm cryopreservation in the world was carried out in the 1950s (Roy *et al.*, 1956). In this sense, conventional cryopreservation of sperm with different buffalo species is a method that has been used for a long time (Andrabi, 2009). Until recently, artificial inseminations in Anatolian buffaloes were generally performed with Mediterranean buffalo semen that

imported from Italy. Because of there was no frozen Anatolian buffalo semen for commercial purposes (Aköz *et al.*, 2017). Due to the high prices of imported frozen buffalo semen, breeders in Turkey prefer natural breeding rather than artificial insemination (Isik, 2015; Yılmaz, 2013; Okuyucu *et al.*, 2018). For these reasons, the National Anatolian Buffalo Breeding Project, coordinated and supported by the General Directorate of Agricultural Research and Policies (TAGEM) was initiated in 2010. As an outcome of this leap sperm production from Anatolian buffalo bulls, with its superior genomic characterization and acceleration of breeding activities by artificial insemination, is one of TAGEM's priority strategic plans (Soysal *et al.*, 2020). For these reasons, the first commercial Anatolian buffalo sperm production was started at the Lalahan International Livestock Research and Training Center, Ankara.

Cryopreservation of epididymal Anatolian buffalo sperm has been studied, and valuable data have obtained (Selcuk *et al.*, 2015; Yeni *et al.*, 2017). However, collection process was not performed with artificial vagina. It is revealed that it is not suitable for commercial production and it is necessary to conduct new studies with common methods due to its effects on ejaculate's quality.

In the present study, unlike the studies mentioned above, it was the first time to use a controlled semen collection system (Lalahan Model) in terms of occupational safety and animal welfare for semen collection from Anatolian buffaloes which are overly sensitive to exogenous stimulation (Korkmaz *et al.*, 2019; Baştan, 2020). For this purpose, a novel collection system was used and semen characteristics of frozen Anatolian buffalo semen collected by artificial vagina were investigated after freezing.

Materials and Methods

Animals

In this study, three Anatolian Buffalo bulls (apr. 4 years of age) were used at the Lalahan International Center for Livestock Research and Training (Ankara,



Figure 1. Anatolian buffalo bull.

Turkey), and maintained under uniform feeding and housing conditions that individually in pens.

Preparation of Artificial Vagina

Semen was collected using a bovine artificial vagina with soft neoprene liner (AV; 30 cm long; 5 cm internal diameter; IMV, France). The internal temperature of the artificial vagina was maintained 40 °C. In order to lubricant, the contact of bull penis with the soft neoprene liner of the AV, the inner surface of the liner was covered with a thin layer of sterile petroleum jelly (Vaseline®). Sterile glass conical cylindrical 15-ml collection tube, which used to collect semen and transporting it to the laboratory was placed at the end of artificial vagina. The protective felt cover was used to keep the artificial vagina and collection tube at the correct temperature during the collection process and prevent semen thermal shock after the bull ejaculation (Ansari *et al.*, 2017; Baştan 2018).

Semen Collection

In terms of occupational safety and animal welfare, a controlled semen collection model (Lalahan model) was used to collect semen from Anatolian buffalo bulls (as shown in Figure 2). In this way, each bull came to the semen collection arena from its individual pen without a bull handling person. A female Anatolian buffalo was used as a teaser animal to steer male buffaloes. A total number of 150 ejaculates were collected by using the Lalahan Model with the aid of an artificial vagina twice a week during 30 weeks (Korkmaz *et al.*, 2019; Baştan, 2020).

Determination of Semen Volume, Concentration and pH

The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals, and sperm concentration was determined by using the Accucell photometer (IMV, L'Aigle, France). The pH of semen samples was determined by indicator paper strips and digital pH meter simultaneously (Hanna-Hi 221, Smithfield, RI) (Khawaskar *et al.*, 2012).

Semen Freezing Processing

A soybean-based semen extender (Andromed, Minitüb, Germany) was used for diluting ejaculates with >80 total motile sperm to a final concentration of 100×10^6 spermatozoa/ml. Samples were cooled at +4°C for 3 hours. Afterwards, they were packaged in 0.25 ml French straws (IMV, L'Aigle, France) by using an automatic straw filling and sealing machine (MX4, IMV, L'Aigle, France). The straws were frozen to -140°C (-3°C/min from +4 to -10 °C; -40 °C/min from 10 to 100 °C; -20 °C/min from -100 to -140 °C) by using an automatic freezing machine (Digital cool 5300ZB 250, IMV, L'Aigle, France), plunged into liquid nitrogen and

stored at -196°C (Tuncer *et al.*, 2010; Ansari *et al.*, 2017).

Thawing and Post-Thaw Evaluation

After one month of storage period at -196°C , the straws were thawed in a water bath (37°C , 30 s) for post-thaw kinetics analysis. Afterwards, a $3\mu\text{l}$ sample of semen was put onto a prewarmed four chamber slide ($20\mu\text{m}$, Leja slides, IMV, L'Aigle, France) and sperm kinetics parameters were determined by using computer-assisted sperm analysis system, as shown in Figure 3 (CASA; IVOS I, Hamilton Thorne Inc., Beverly, USA). CASA was set up as follows: frame rate 60 Hz; minimum contrast 80; low and high intensity gates 0.30–1.70; low and high static size gates 0.10–3.40; low and high elongation gates 8–97; default cell size 5 pixels; default cell intensity 70. In the analysis settings, spermatozoa with VSL 70% and VAP $50\mu\text{m/s}$ were evaluated as progressively motile. The motility parameters were expressed in percentage units. Other kinematics average path velocity (VAP, $\mu\text{m s}^{-1}$), straight line velocity (VSL, $\mu\text{m s}^{-1}$), curvilinear velocity (VCL, $\mu\text{m s}^{-1}$), straightness ($\text{STR} = [\text{VSL}/\text{VAP}] \times 100$), linearity ($\text{LIN} = [\text{VSL}/\text{VCL}] \times 100$), beat-cross frequency (BCF, Hz), amplitude of lateral head displacement (ALH, μm) were also evaluated and expressed with their own units (Tuncer *et al.*, 2010; Sahin *et al.*, 2020).

Statistical Analysis

For statistical analyses, data were examined with Shapiro-Wilk test for normality and with Levene test for homogeneity of variances as parametric test assumptions. The statistical control of the difference between the variables was done with ANOVA. The Tukey test used to evaluation of differences between the buffalo bulls. Descriptive statistics for each variable were calculated and presented as mean \pm standard error (Mean \pm SE). All statistical analyzes were examined using the SPSS® 22.0 package program and $P < 0.05$ level was considered significant.



Figure 2. Semen collection by Lalahan Model in Anatolian buffalo bull.

Results

The present study aimed to describe some sperm characteristics and spermatological parameters of Anatolian Buffalo sperm. In particular, the data important for AI stations and parameters that routinely used by andrology laboratories were prioritized. The data pH, volume, concentration of semen, total motility and progressive motility were given Table 1. The mean pH, volume, concentration of semen, total motility and progressive motility were found 6.63 ± 0.15 , $1.61 \pm 0.5\text{ ml}$, $1629 \pm 222.67 \times 10^6$ spermatozoa/ml, $57.12 \pm 5.63\%$, $23.22 \pm 4.47\%$ respectively after thawing.

The sperm kinetic values (VAP, VSL, VCL, ALH, BCF, STR, LIN) were given Table 2. The mean VAP, VSL, VCL, ALH, BCF, STR and LIN were found $94.71 \pm 8.48\mu\text{m/s}$, $72.6 \pm 7.08\mu\text{m/s}$, $160.9 \pm 15.66\mu\text{m/s}$, $7.8 \pm 3.75\mu\text{m}$, $29.15 \pm 1.56\text{ Hz}$, $76.91 \pm 3.87\%$, $46.21 \pm 2.61\%$, respectively after thawing. While the pH values are statistically significant among the bulls ($P < 0.05$), differences in terms of other spermatological and CASA parameters (ejaculate volume, sperm concentration, total motility, progressive motility, VAP, VSL, VCL, ALH, BCF, STR, LIN) were not significant ($P > 0.05$).

Discussion

According to this study, the mean semen volume of the Anatolian buffalo was determined as $1.61 \pm 0.5\text{ ml}$. When compared to domestic buffalo breeds such as Murrah, Surti, Jafarabadi, Nili Ravi, Kundihi and swamp buffaloes (2.58 ml , $3.16 \pm 0.76\text{ ml}$, $4.72 \pm 0.24\text{ ml}$, $2.80 \pm 1.62\text{ ml}$, $2.25 \pm 0.01\text{ ml}$, 2.9 ml , respectively), it can be evaluated that the volume of semen obtained in Anatolian buffalo ejaculation is lower than other breeds (Malik *et al.*, 1974; Jainudeen *et al.*, 1982; Dhami *et al.*, 2005; Bhakat *et al.*, 2011; Kaka *et al.*, 2012; Khawaskar *et al.*, 2012).

The sperm concentration of the Anatolian buffalo, another parameter obtained in the study, was

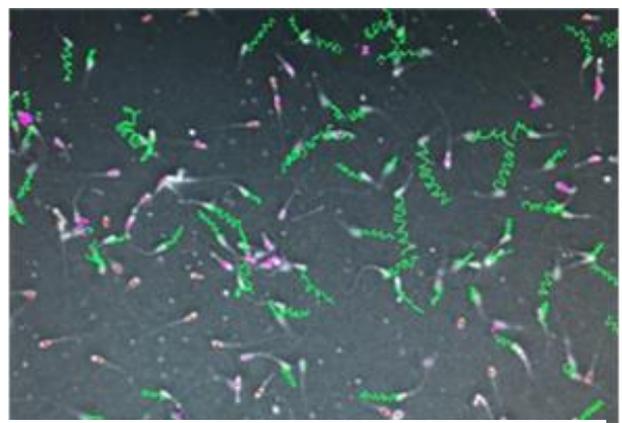


Figure 3. Assessment of motion and kinematic characteristics of semen using computer-assisted semen analysis (CASA).

Yellow color: Progressive motile sperm, Pink color: Motile sperm, Red color: Immotile sperm.

determined as $1629 \pm 222.67 \times 10^6$ sperm/ml. This value is in other domestic buffalo breeds; $990.65 \pm 143.9 \times 10^6$ sperm/ml in Mediterranean buffaloes, 998.91×10^6 sperm/ml in Murrah breed, $885.42 \pm 24.9 \times 10^6$ sperm/ml in Surti breed, $838.30 \pm 25.74 \times 10^6$ sperm/ml in Jafarabadi buffaloes. It has been reported that $1000 \pm 50.0 \times 10^6$ sperm/ml in Nili ravi, $1542 \pm 9.20 \times 10^6$ sperm/ml in Kundhi, and for Swamp buffalo 1006×10^6 sperm/ml. As a matter of fact, although Anatolian buffalo semen volume was found low compared to other domestic buffalo breeds, it is observed that the concentration of spermatozoa is higher (Malik *et al.*, 1974; Jainudeen *et al.*, 1982; Galli *et al.*, 1993; Bhakat *et al.*, 2011; Ghodasara *et al.*, 2018; Kaka *et al.*, 2012; Khawaskar *et al.*, 2012).

In the study, the semen pH value was determined as 6.63 ± 0.15 . This value varies between 6.10 and 6.92 (Murrah; 6.92, Surti; 6.89 ± 0.16 , Nili-Ravi 6.55 ± 0.50 , Kundhi; 6.10 ± 0.007) in some domestic buffalo breeds. The semen characteristics of a bull in frozen semen production are important for extender optimization before freezing. In this process, post-thaw sperm concentration and production efficiency are evaluated according to the variables specified. Nevertheless, sperm characteristics such as semen volume, concentration, and pH may in a large variation specific to the breed, as well as they may vary depending on factors such as age, climate, number of false mounting and ejaculation frequency (Khawaskar *et al.*, 2012;

Murphy *et al.*, 2018; Sankhi *et al.*, 2019; Şahin *et al.*, 2020).

Computer aided sperm analysis systems (CASA), which reveal in vitro spermatozoon morphology and kinetic parameters in a detailed and systematic manner, are an analysis technique widely used in andrology laboratories. For validating the commercial license of frozen buffalo semen in Turkey, it must have at least 40% total motility and 15 million motile spermatozoa after thawing basically, according to Republic of Turkey Ministry of Agriculture and Forestry. Hence, the percentages of total motile and progressive motile spermatozoa are important among the sperm kinematic parameters in the evaluation of sperm quality. There are many studies indicating the correlation of these parameters with conception rates (Mahmoud *et al.*, 2013; İncan *et al.*, 2018; Vincent *et al.*, 2018). In this context, kinematic parameters determined by CASA analysis are presented in Table 1 and 2. Considering the parameters obtained from samples that were frozen with a soy lecithin-based plant-derived extender (AndroMed®), it would be more accurate to compare the results with studies using similar extenders. Ansari *et al.* (2017) and Singh *et al.* (2018) determined the total motility values in the sperm cryopreservation studies performed with the same extender in Nili Ravi and Murrah buffaloes as $49.2 \pm 1.7\%$ and $38.3 \pm 2.3\%$ at post-thaw evaluation. The progressive motility value in Murrah buffaloes with the same extender was determined as $22.3 \pm 1.8\%$ at post-thaw. Compared with the total motility and progressive

Table 1. The Anatolian Buffalo semen characteristic and post-thaw motility parameters.

Parameters	Buffalo Bull No: 1 (n=40)	Buffalo Bull No: 2 (n=50)	Buffalo Bull No:3 (n=60)	Mean \pm SE	Range	P-Value
Ejaculate Volume (ml)	1.75 \pm 0.52	1.60 \pm 0.55	1.55 \pm 0.44	1.61 \pm 0.5	0.2 – 4.1	P=0.545
Concentration ($\times 10^6$ sperm/ml)	1589 \pm 254.65	1622 \pm 227.84	1683 \pm 189.86	1629 \pm 222.67	625 - 2678	P=0.352
pH	6.65 \pm 0.17 ^a	6.62 \pm 0.13 ^{ab}	6.61 \pm 0.11 ^b	6.63 \pm 0.15	6.45 – 6.72	P<0.05
Total Motility (%)	56.74 \pm 6.45	56.66 \pm 5.11	57.81 \pm 5.29	57.12 \pm 5.63	48 - 76	P=0.808
Progressive Motility (%)	23.25 \pm 4.24	23.49 \pm 4.8	22.92 \pm 4.25	23.22 \pm 4.47	16 - 35	P=0.931

a,b: Different letters on the same line for each parameter represent a statistically significant difference (P < 0.05).

Table 2. The Anatolian Buffalo sperm kinetics parameters.

Parameters	Buffalo Bull No: 1 (n=40)	Buffalo Bull No: 2 (n=50)	Buffalo Bull No: No: 3 (n=60)	Mean \pm SE	Range	P-Value
VAP ($\mu\text{m}/\text{sec}$)	94.94 \pm 9	94.25 \pm 8.5	94.97 \pm 7.97	94.71 \pm 8.48	79.9 – 137.8	P=0.963
VSL ($\mu\text{m}/\text{sec}$)	73.58 \pm 6.44	71.49 \pm 8.35	72.91 \pm 6.03	72.6 \pm 7.08	60.5 – 103.6	P=0.706
VCL ($\mu\text{m}/\text{sec}$)	161.74 \pm 15.21	158.44 \pm 17.66	162.57 \pm 13.59	160.9 \pm 15.66	125.8 – 207.8	P=0.723
ALH (μm)	7.24 \pm 0.44	8.81 \pm 6.22	7.24 \pm 0.38	7.8 \pm 3.75	5.9 – 8.6	P=0.397
BCF (Hz)	29.36 \pm 1.3	29.45 \pm 1.93	28.71 \pm 1.26	29.15 \pm 1.56	25.7 – 32.6	P=0.330
STR (%)	77.87 \pm 2.4	76.16 \pm 5.61	76.9 \pm 2.36	76.91 \pm 3.87	68 - 84	P=0.489
LIN (%)	46.82 \pm 2.15	45.82 \pm 3.35	46.13 \pm 2	46.21 \pm 2.61	43 - 55	P=0.578

motility ($57.12 \pm 5.63\%$ and $23.22 \pm 4.47\%$) values obtained in this study, it can be expressed that the first Anatolian buffalo sperm cryopreservation trial was successful and its cryotolerance was higher when compared with other breeds. To clarify, in some domestic buffalo breeds were frozen with different extenders, the total motility values were between 37.92% and 67.84% ($37.92 \pm 1.12\%$, $49.3 \pm 12.8\%$, $43.25 \pm 3.40\%$, $57.41 \pm 0.92\%$), and the progressive motility values vary between 20.4% and 30.64% (Kaka *et al.*, 2012; Gaviraghi *et al.*, 2013; Kumar *et al.*, 2016; Singh *et al.*, 2017; Ahmed *et al.*, 2020; Pathak *et al.*, 2020).

Singh *et al.* (2017), studied on Murrah buffaloes, and stated that VAP, VSL, VCL, kinematic values and ALH values were higher in buffaloes showing high fertility characteristics than buffaloes showing low fertility characteristics. He also stated that BCF, STR and LIN values were lower in buffaloes with high fertility characteristics. It has been reported that high motility does not only play a role in determining fertility, but also other sperm kinematics such as swimming pattern and sperm head movements play crucial roles also (Singh *et al.*, 2017). The data obtained from this study are similar to the values stated in previous studies conducted on different domestic river buffalo breeds (Gaviraghi *et al.*, 2013; Kumar *et al.*, 2016; Singh *et al.*, 2017; Singh *et al.*, 2018). Preliminary field studies (not statistically sufficient at the moment) were also carried out which pregnancy was obtained with Anatolian buffalo semen.

In conclusion, Anatolian buffalo semen collected by artificial vagina and frozen commercially with the evaluation of some spermatologically parameters (pre-freeze and after thawing), non-return rates for the first time with this study. However, further studies are needed in order to determine Anatolian Buffalo sperm characteristics in a large sample count and field studies.

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