**Breeding soundness examination, semen freezing and evaluation of frozen-thawed semen in native Omani bulls – preliminary study**

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## Abstract

The present study was undertaken as part of a project on 'Characterization, Evaluation and Conservation of Indigenous Animal Genetic Resources in the Sultanate of Oman'. The objective of this study was to conduct breeding soundness examinations (BSE) and determine the characteristics of frozen-thawed semen of native Omani bulls. Breeding soundness examinations were carried out in twelve bulls age three to four years; six bulls were found to be satisfactory, while the remaining six were unsatisfactory as potential breeders. Semen was collected using an electroejaculator (EE) in the six bulls once per week for two consecutive weeks. The volume of the ejaculates and the initial sperm concentration were measured. Nine ejaculates were qualified for freezing. Semen was diluted in tris egg yolk extender and frozen in liquid nitrogen vapor. Frozen semen straws were thawed at 37°C for at least 30 seconds in a water bath for assessment of post-thaw semen quality. The mean volume of semen and sperm concentration was 9.08 ± 0.9 ml and 284.6 ± 24.8 X $10^6$/ml, respectively. The post-thaw total sperm motility was 64.6 ± 5.2% and progressive motility was 34.2 ± 4.1%, as evaluated by computer assisted sperm analysis. The hypo-osmatic swelling test positive spermatozoa was 27.1 ± 3.5%, intact acrosome was 90.7 ± 1.41% and sperm abnormalities were 8.3 ± 1.0% in post-thawed semen. Overall, the quality of the post-thawed semen in the present study was similar to that obtained from crossbred and exotic bulls. In conclusion, BSE was beneficial in bull selection, semen was collected successfully using an EE and frozen in native Omani bulls. The frozen-thawed semen was suitable for artificial insemination procedures.

**Keywords**: Native Omani bull, breeding soundness examination, semen evaluation, semen freezing, computer assisted semen analysis, hypo-osmotic swelling test.

## Introduction

The importance of animal biodiversity was recognized by the United Nations Convention on Biological Diversity for the integrity and sustainability of the biosphere and human society.[1] Conservation of indigenous genetic resources is vital for the development of new animal species and improvement of existing animal species. Oman has noticeable biodiversity in local animals that are adapted to the unique and diverse topographic and climatic conditions of the Sultanate. Limited work has been carried out to identify or conserve these breeds, which are well-adapted to Omani conditions. At present, native Omani breeding bulls are selected on the basis of subjective evaluation of physical appearance and visual observation of the testes. However, no semen collection and evaluation is usually performed before animals are used for breeding purposes. Accurate and reliable information on Omani breeds is essential in order to make optimal use of breeding programs.

The current study was a part of a project entitled: 'Characterization, Evaluation and Conservation of Indigenous Animal Genetic Resources in the Sultanate of Oman'. In the wider context of the project, the current study aimed to evaluate the reproductive efficiency of Omani breeding animals and establish a semen bank for future genetic improvement programs.

The cattle population in the Sultanate of Oman was estimated at 326,240 head. Based on the distribution and phenotypic appearance of Omani cattle, they are classified into north Omani cattle and south Omani cattle (Dofari) breed.[2] North Omani cattle are small in size with short horns, brown to dark brown color, and are found in Batina coastal planes, interior Sharqiyah and Dhahira regions of Oman.

They are highly aggressive and disease resistant and used mainly for meat production and traditional bull fighting.

Collection and cryopreservation of semen is an effective means of conservation of dwindling native Omani cattle breeds. The most commonly used methods for successful semen collection in cattle are the use of an artificial vagina and an EE. Considering the aggressive nature of the Omani bulls, semen collection with an EE was utilized. To our knowledge, there are no reports available on semen collection and cryopreservation in native Omani bulls. The objective of the present study was to conduct BSEs, freeze semen, and determine the characteristics of frozen-thawed semen of native Omani bulls.

## Materials and methods

### Breeding soundness evaluation

Breeding soundness examinations were carried out in twelve bulls age three to four years, according to the methods described by Barth.[3] Bulls were evaluated for their body condition score (BCS) on a scale of 1 to 5 (1= thin, 5 = fat)[4] and weighed on an electronic balance. Scrotal circumference (SC) was measured by a standard scrotal tape at the widest mid-scrotal point. Testes were palpated, accessory sex glands were evaluated per rectum, and semen was collected by an EE. Initial semen quality was evaluated by assessing volume, mass activity, sperm motility and sperm concentration. Based upon the established BSE standards,[3] the bulls were classified as satisfactory or unsatisfactory potential breeders.

### Animals and semen collection

Six native Omani bulls, age three to four years, qualified as satisfactory potential breeders[3] were selected for this study. The study was conducted at the Agricultural Research Centre, Rumais Station, Ministry of Agriculture and Fisheries, Sultanate of Oman (latitude 23° 36′ N: longitude 58° 37′ E). The bulls were maintained with an average body condition score of 3.0 through intensive feeding and management conditions and received 3 kg of concentrates daily, with green fodder and water ad libitum as well as mineral supplementation. These bulls were aggressive and not trained to donate semen by artificial vagina. Therefore, the bulls were adequately restrained in a cattle chute and semen collection was performed using an EE (Electro Ejaculator e320, Minitube, Tiefenbach, Germany). Electrical pulses of increasing intensity were started slowly untill a response was observed. The frequency and duration of electrical stimuli and the voltage level were similar for all bulls. Penile protrusion was followed by ejaculation of semen, which was collected in a hand-held collection device. Semen was immediately transferred to a 37°C water bath. Semen was collected once per week from each bull for two consecutive weeks.

### Semen processing and cryopreservation

The volume of the ejaculates was measured in a conical graduated tube and the sperm concentration was measured using a photometer (Spermacue, Minitube). Initial sperm motility of the fresh semen was evaluated subjectively by phase contrast microscope, as well as objectively using computer assisted semen analysis (CASA). Only ejaculates with > 70% initial total motility (> 30% progressive motile sperm) were used in the study (n = 9). Semen was diluted in an egg yolk extender containing tris, fructose, citric acid, egg yolk, streptomycin, penicillin and glycerol to give a concentration of $50 \times 10^6$ sperm/ml. Diluted semen was cooled to 4°C over 2 h, equilibrated for 4 h at 4°C, and placed in 0.5 ml straws with a suction pump at 4°C in a cold cabinet unit (Minitube, Germany). The equilibrated straws were subsequently frozen using a computerized programmable freezer (Ice Cube 1810, Sy-Lab, Neupurkersdorf, Austria) by reducing the temperature initially at a rate of 5°C/min from + 4 to -12°C, then 40°C/min from -12 to -100°C and finally at a rate of 20°C/min from -100 to -140°C. Straws were then plunged into and stored in liquid nitrogen (-196°C).

Assessment of post-thawed semen

Frozen semen straws were thawed at 37°C for at least 30 sec in a water bath for assessment of post-thaw semen quality.

*Assessment of sperm motility by computer assisted sperm analysis.* The sperm motion characteristics were evaluated using CASA (Animal Version 12.3, CEROS, Hamilton Thorne Biosciences, MA). A 3µl drop of semen was placed in a 20µm standard count analysis chamber (Leja, Nieuw-Vennep, The Netherlands). The loaded chamber was placed on the thermal plate of the microscope (37°C) for 3 min before being analyzed according to the methods of Anzar et al.[5] The instrument settings for the Hamilton Thorne CEROS animal software (version, 12) used to assess sperm kinetics were 30 frames at a rate of 60 frames/sec, minimum contrast of 80, and minimum cell size (pixels) of 5. For progressive cells, the velocity average pathway (VAP) was 70µm/s and straightness was 75%. Spermatozoa with a VAP of more than 30µm/s were considered motile. Five randomly selected microscopic fields were scanned five times each. The mean of these 25 scans was used for statistical analysis. The following parameters were analyzed: total motile spermatozoa (%); spermatozoa with progressive motility (%); velocity average pathway (VAP, µm/s, the average velocity of the spermatozoon through a smoothed cell path); velocity straight line (VSL, µm/s, the minimum distance a spermatozoon travelled during the time between two scans); velocity curvilinear (VCL, µm/s, the mean of the sperm's velocity measured over the actual tracks followed); amplitude of lateral head displacement (ALH, µm, the mean width of the sperm's head oscillation); beat cross frequency (BCF, Hz, a measure of the frequency of lateral movement, causing a sperm head to cross the average path in either direction); straightness of the sperm's movement (STR, %, VSL/VAP); linearity of the sperm movement (VSL/ VCL = LIN, %). Furthermore, spermatozoa were classified into four groups based on their velocity of movement, viz: rapid (VAP > 50 µm/s, %), medium (30 µm/s < VAP < 50 µm/s, %), and slow (VAP < 30 µm/s, or VSL < 15 µm/s, %) moving spermatozoa and static spermatozoa.

Hypo-osmotic swelling test

Sperm plasma membrane integrity was evaluated using the hypo-osmotic swelling test (HOST) according to the methods of Correa and Zavos[6]. A hypo-osmotic swelling solution (100 mOsm/kg) was prepared by dissolving 0.49 g sodium citrate and 0.9 g fructose in 100 ml distilled water. For the HOST test, 100 µl of semen was added to 1ml of pre-warmed HOST medium and incubated at 37°C for 1 h. After incubation, a 5 µL drop from each sample was transferred to a warm, clean microscope slide and covered with a coverslip. This preparation was examined microscopically using a warm stage at 400x magnification and phase contrast optics. Two hundred spermatozoa were counted per sample and the number of spermatozoa showing characteristic swelling of the tail, indicative of an intact plasma membrane, was recorded.

Sperm acrosomal integrity and sperm abnormalities

The sperm acrosomal integrity and abnormalities were assessed according to the methods of Watson.[7] A 100µL sample of semen was fixed in 500µL of 1% formal citrate (2.9 g tri-sodium citrate dihydrate, 1 ml of 37% solution of formaldehyde, dissolved in 100 ml of distilled water); one hundred spermatozoa were examined under phase contrast microscopy at 1000x magnification. Normal acrosomes were characterized by a normal apical ridge. Sperm abnormalities were recorded as abnormalities of the sperm head, mid piece and tail.

Statistical analysis

The semen characteristics were analyzed using descriptive statistics and all results were expressed as a mean ± SEM.

## Results

Table1 summarizes the mean (±SE) of the BSE in the native Omani bulls (n=12), namely, BCS, body weight, SC and semen evaluation parameters (volume, mass activity, initial motility, sperm

concentration, normal sperm, primary and secondary abnormalities). Fifty percent of the bulls were found to be satisfactory potential breeders, while the remaining six bulls were declared unsatisfactory due to several factors such as, low SC (26 to 27.5 cm), azoospermia, poor sperm motility (30 to 60%) or high percent (15 to 22%) of morphological abnormalities.

The mean volume of semen and the sperm concentration observed during the study were 9.08 ± 0.9 ml and 284.6 ± 24.8 x $10^6$/ml, respectively. The mean values of sperm motion characteristics observed during the present study for diluted semen and frozen- thawed sperm are presented in Table 2. The percentage of HOST-positive spermatozoa was 27.1 ± 3.5, intact acrosomes was 90.7 ± 1.41 and sperm abnormalities was 8.3 ± 1.0 % in post-thawed semen.

## Discussion

### Breeding soundness examination and semen collection

Satisfactory potential breeding bulls play an important role in successful breeding outcomes and a positive impact on overall reproductive efficiency. There are obvious economic losses when bulls with potentially low fertility are used for artificial insemination. The BCS of bulls in the present study (3 ± 0.07 v/s 3.2±0.5) was similar to that reported by Chacon et al,[8] however, the average SC in the present study was much lower than their study (28.3 ± 0.47 v/s 36.2±4) in *Bos indicus* bulls. Native Omani bulls are a beef breed with a small frame and body weights ranging between 276 and 431kg. Bulls with lower body weights (276 to 358 kg) had a smaller scrotal circumference (26 to 27.5 cm); however, bulls found to be satisfactory as potential breeders (n=6), had greater body weights (364 to 431kg) and SC (29.5 to 30.5 cm). The present study demonstrated the successful collection and cryopreservation of semen in native Omani bulls. An EE is usually used to collect semen from animals that are unwilling or not trained to mount and ejaculate semen into an artificial vagina In this study, 90% of the ejaculates collected by an EE were qualified for freezing. The semen volume collected in the present study was comparable but sperm concentrations were lower than that reported for Curraleiro bulls.[9] However, the sperm concentrations obtained in the present study were higher than in beef bulls reported by Palmer et al.[10] These findings indicate that an EE can be used as a reliable method to collect semen in the aggressive native Omani bulls.

### Semen freezing and thawing

Artificial insemination has made a profound contribution to genetic improvement, particularly in cattle, and this impact would not have been possible without successful freezing of bull semen. The process of cryopreservation causes damage to sperm membranes, cytoskeleton, and motile apparatus and nucleus, and can alter cell metabolism.[7,11] The post-thaw quality of spermatozoa is of prime interest for the AI industry, since it can provide insights into the fertilizing capacity of the cryopreserved spermatozoa. Sperm motility is one of the commonly used criteria to assess sperm quality, as it is essential for sperm transport and fertilization in the female reproductive tract. However, subjective or visual assessment of post-thaw sperm motility has been reported to be poorly correlated with fertility. Variations of 30 to 60% have been reported in the estimation of motion parameters of the same ejaculate.[12] In the past several years, new criteria for evaluating spermatozoa have emerged, made possible by the development of imaging equipment for performing CASA. At present, CASA has been widely used in most species to obtain accurate and objective kinetic sperm measurements. The sperm motion parameters assessed by CASA in the present study were comparable to the previous report in bulls.[13] The motility of freshly diluted semen in the present study with tris extender (74.1% vs 73.93%) was similar to that reported by Vera Munoz et al[14] using a commercially available extender, Triladyl. However, the post-thaw motility (50.6% vs 46.4%) was higher in the present study. The total motility, VSL, BCF, LIN and STR in the current study with tris extender were higher (50.4, 58, 32, 60.8, 85.6 v/s 41, 55.8, 22, 48.6, 79.6, respectively) than those reported by Leite et al[15] using tris extender with four hour equilibration in cryopreserved semen from Gyr bulls. However, the progressive motility and ALH were lower in our study (27.4, 4.8 v/s 30.8, 6.0). The total and progressive motility of post-thawed semen

in the present study were higher than those reported for HF bulls.[14] The sperm velocity parameters (VAP, VSL and VCL) were lower in the current study than those reported by Chaverio et al.[16] These variations could be attributed to different settings of the CASA system.

Semen evaluation

The sperm plasma membrane is the primary site where damage occurs during freezing and thawing of semen.[11,17] An intact and functionally active plasma membrane is essential for the spermatozoon to sustain metabolism, undergo capacitation and acrosome reaction and to attach to and penetrate the oocyte zona pellucid.[18,19] The HOST has been successfully employed for evaluating the plasma membrane integrity of spermatozoa in bulls.[6,14,20-22] There was a positive correlation between HOST reacted sperms and progressive motility ($r = 0.705$).[21] The mean percentage of HOST positive spermatozoa in the current study was similar to that reported in previous studies;[14,22] however, it was lower than those reported by some authors.[6,20,21] The percentage of intact acrosomes in the current study was similar to those reported in the studies of Tuncer et al.[23] The percentage of sperm abnormalities was less than the values reported for Jersey, Tharparkar, and Holstein-Friesian bulls.[24-27] Overall, the quality of the post-thawed semen in the present study was similar to that obtained from crossbred and exotic bulls. In conclusion, BSE was essential in bull selection, with an average or above average body weight and SC recommended for potential breeders. Semen was collected successfully using an EE and frozen in native Omani bulls. The frozen-thawed semen was suitable for AI procedures.

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**Table 1:** Breeding soundness examination (BSE) parameters of native Omani bulls (n=12).

| **Parameters of BSE** | BCS | BW | SC | Semen Volume | MA | Initial motility | Sperm conc. | Normal sperm | Abnormal sperm | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Primary | Secondary |
| | | Kg | cm | cc | + | % | M/ml | % | % | % |
| **Mean±SE** | 3 ± 0.07 | 360.5 ±11.4 | 28.3 ± 0.4 | 4.6 ± 0.7 | 1.7 ± 0.3 | 55.4 ± 4 | 869.8 ± 119.5 | 89 ± 4.1 | 3 ± 1.9 | 8 ± 2.6 |
| **Range (min – max)** | 2.5-3.0 | 276 – 431 | 26-30.5 | 1.5-9.5 | 0-3 | 30 to 80 | 364 - 1856 | 57-99 | 0-21 | 1-22 |

BSC- Body condition score (1= thin, 5= too fat or obese), BW – Body weight, SC- Scrotal circumference, MA - Sperm mass activity (semen wave motion observed under microscope, 0= no MA to 4= max)

**Table 2:** Sperm motion characteristics of freshly diluted and frozen-thawed native Omani bull semen measured by CASA. (Mean ± SEM).

| Parameters | Freshly diluted | Frozen thawed |
|---|---|---|
| Velocity average pathway (VAP, µm/s) | 71.3±4.7 | 67.9±6 |
| Velocity straight line (VSL, µm/s) | 57.7±3.3 | 58± 4.6 |
| Velocity curvilinear (VCL, µm/s) | 111.2±8 | 101 ± 10.6 |
| Amplitude of lateral head displacement (ALH, µm) | 5.8±0.3 | 4.8 ± 0.4 |
| Beat cross frequency (BCF, Hz) | 27.9±1.1 | 32 ± 0.9 |
| Straightness (STR, %) | 81.7±1.3 | 85.6 ± 1.6 |
| Linearity (LIN, %) | 54.9±1.6 | 60.6 ± 2.2 |
| Total motility (%) | 74.1±5.4 | 64.6 ± 5.2 |
| Progressive motility (%) | 38.3±4.5 | 34.2 ± 4.1 |
| Rapid moving spermatozoa (%) | 50.8±6.1 | 33.1 ± 6.3 |
| Medium moving spermatozoa (%) | 23.1±3.9 | 17.2 ± 3.8 |
| Slow moving spermatozoa (%) | 16.6±2.6 | 15.7 ± 2.3 |
| Static spermatozoa (%) | 9.4±3 | 33.5 ± 9.2 |