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INVESTIGATIONS ON SEMEN QUALITY AND FREEZABILITY OF CHOLISTANI BREEDING BULLS - A PRELIMINARY STUDY FROM CHOLISTAN DESERT OF PAKISTAN

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ABSTRACT

The present study was designed to generate a preliminary data regarding the semen quality and freezability of Cholistani breeding bulls (n = 06) maintained at the Semen Production Unit, Karaniwala, Punjab, Pakistan. Semen from experimental bulls was collected weekly from October to December, 2011, using homosexual mount, with an artificial vagina. A total of 132 ejaculates, with 22 ejaculates per bull, were collected. The mean ejaculatory volume (ml), mass activity (Scale 0-5), individual sperm motility (%) and sperm concentration (million/ml) were 4.45 ± 0.76, 1.94 ± 0.14, 64.38 ± 2.64 and 918.05 ± 65.79, respectively. The fresh semen attributes in terms of live and morphologically normal sperm, and those with intact acrosome (acrosome integrity) revealed mean values of 84.60 ± 1.27, 83.97 ± 0.61 and 83.85 ± 0.54%, respectively. Semen was diluted by slow and one step method in Tris egg yolk extender, filled in 0.5 ml straws, equilibrated at 4°C for 6 hrs, frozen and stored in liquid nitrogen for 24 hrs. Post thaw assessment in terms of sperm motility, live and morphologically normal sperm and acrosome integrity revealed quite encouraging results. This preliminary data envisages for further studies with a larger sample size expanded over all seasons and in correlation to age and testicular biometry in order to establish seasonality of reproduction (if any) in the Cholistani breed of cattle.

Key words: Semen quality, freezability, breeding bulls, Cholistani.

INTRODUCTION

Pakistan is blessed with a thriving indigenous livestock genetic diversity. Fifteen well recognized indigenous cattle breeds in Pakistan constitute about 43% of the total cattle population and belong to zebu (humped; *Bos indicus)* cattle (Khan *et al.*, 2008; Raziq *et al*., 2010). Their potential of adequate thermo-tolerance and tick-resistance has widely been documented (Hammond *et al.*, 1996; Farooq *et al.,* 2010; Khan *et al*., 2011). The reproductive indices of bulls of some indigenous cattle breeds like Sahiwal and Red Sindhi under local climatic conditions have been well documented (Ahmad *et al.*, 2003; Ahmad *et al.,* 2005). Similarly, the research work done on buffalo bulls in the last decade or so, has revealed its previously hidden potentials (Rasul *et al.*, 2007; Ijaz *et al.*, 2009).

Cholistani breed of cattle is a zebu *(Bos indicus)* or humped breed, which is a relatively newly emerged breed having originated from crosses of Sahiwal with the local non-descript cattle (Khan *et al.*, 2005). Some unconfirmed reports also quote it as an ancestor of Sahiwal cattle. It is a large sized flabby breed with small horns, long ears, well developed hump in males and large dewlap in both males and females (Farooq *et al.*, 2012). It is an excellent thermo-tolerant and tick-resistant breed, having potential to withstand severe heat stress without any compromise on its productive and reproductive performance. Approximately 5,34,944 heads of Cholistani cattle are being reared by the nomadic pastoralists of Cholistan desert of Pakistan and are the major source of socio-economic uplift in the area (Anonymous, 2006).

After an extensive research on Sahiwal and Red Sindhi breeds of cattle, it is the dire need of time to manipulate the potential of this previously neglected indigenous breed. The present study was, therefore, designed to generate preliminary data regarding the semen quality of Cholistani breeding bulls being reared in the Cholistan desert at the Semen Production Unit (SPU), Karaniwala, Bahawalpur, Pakistan and to assess the degree of damage occurred to the spermatozoa during their cryopreservation. The present work is the first of its kind in Cholistani breeding bulls and envisages for broader avenues of research on this neglected breed.

MATERIALS AND METHODS

Experimental site: The present study was conducted at the SPU, Karaniwala, Bahawalpur, located in the Cholistan Desert of Pakistan. Sprawling over an area of 26,000 Km^2, this desert is located at latitudes 27º42´and 29º45´North and longitudes 69º52´and 75º24´East and at an altitude of about 112m above the sea level. The

climate of this area is arid, hot subtropical and monsoonal with the average annual rainfall of 180 mm (Ali *et al.*, 2009). The inconsistency in rainfall results in periodic droughts in the area.

Experimental animals: Six adult Cholistani breeding bulls, aged 5-10 years, with clinically normal reproductive tract, donating semen of acceptable quality, and maintained at the SPU, Karaniwala, Bahawalpur, Pakistan were used in this study. These bulls included: KWC-19, KWC-21, KWC-24, KWC-25, KWC-27 and KWC-28. They were kept under naturally prevailing climatic conditions, fed good quality seasonal fodder at the rate of 10% of body weight per bull and two to three kg of concentrate per bull per day. Vaccination against Hemorrhagic Septicemia and Foot and Mouth disease was carried out as per schedule. Preventive measures against worm infestation were undertaken twice in a year or whenever felt necessary.

Semen collection and evaluation: Semen from each experimental bull was collected at weekly intervals for a period of 12 weeks from October to December 2011 through artificial vagina (42°C), using an intact male as a teaser. Two ejaculates were collected from each bull on each collection. However, occasionally some bulls refused to give the second ejaculate. Thus, a total of 132 ejaculates, with 22 ejaculates from each bull, collected over a period of 12 weeks were available for further processing.

Immediately after collection, each ejaculate was kept at 37°C and evaluated for physical characteristics including ejaculatory volume, mass activity, individual sperm motility, sperm concentration, and percentages of morphologically normal spermatozoa and those with intact acrosome (acrosome integrity). Ejaculatory volume was recorded directly from graduated semen collection tubes. For the estimation of mass motility, a small drop of undiluted semen was placed on a pre-warmed microscopic slide, placed on a heated stage and examined under phase contrast microscope (10X). A mass activity score of 0-5 was given to the samples according to their wave pattern, as described earlier (Nazir, 1988).

For estimation of percentage of individual motile spermatozoa, a small drop of fresh semen was diluted with a drop of 2.9% sodium citrate solution and examined under phase contrast microscope (40X) for progressive forward movement of spermatozoa. Sperm concentration was determined using the photometric method (Bovine photometer n° 1119, IMV, France) at 560 nm wavelength.

The percentage of live spermatozoa was determined by eosin-nigrosin differential staining technique. Spermatozoa that absorbed the stain partially or completely and appeared pink were considered to be dead, while those that did not absorb the stain at all were taken as alive. At least 200 spermatozoa were counted per sample to determine live spermatozoa percentage (Khan and Ijaz, 2008; Atiq *et al*., 2011). At the same time, percentages of morphologically normal spermatozoa and those with intact acrosome were also assessed (Jainudeen *et al.* 1982).

Semen extension and freezing: Each semen sample was diluted at 37°C through slow and one step method of dilution (Ahmad *et al.*, 1980). Tris-citric acid (TCA) was used as buffer for the experiment, which consisted of 1.56g citric acid (Fluka, Switzerland) and 3.0g Tris-(hydroxymethyl)-aminomethane (Sigma, USA) dissolved in 74 ml distilled water (pH 6.9, osmotic pressure 320 mOsmol/kg). Egg yolk (20%), fructose (0.2%), glycerol (6%), benzyl penicillin (1000 IU/ml) and streptomycin sulphate (1000 µg/ml) were also added to the experimental extender. After dilution, the semen was cooled to 4°C in 2 hrs and then filled in 0.5 ml straws with suction pump at 4°C in the cold cabinet unit (Minitub, Germany). After equilibration for 6 hrs at 4°C, semen was frozen in programmable cell freezer (Kryo 10 Series III, UK) from 4°C to -15°C at the rate of 3°C/min and from -5°C to -80°C at the rate of 10°C/min (Ahmad *et al.*, 2003). Straws were then plunged into liquid nitrogen for storage (-196°C).

Post thaw evaluation: After 24 hrs of storage in liquid nitrogen, the straws were thawed at 37°C for 30 sec in a water bath and examined for sperm motility under phase contrast microscope (Ijaz *et al.*, 2009), percentages of live and morphologically normal spermatozoa and those with intact acrosome, as described earlier (Rasul *et al.*, 2001).

Statistical analysis: Mean values (± SD) were computed for various parameters of semen quality for each bull. Paired Sample T-test was used to assess the magnitude of difference in various spermatozoa characteristics of fresh and post thaw semen. Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS for Windows Version 12, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

The mean values (± SD) for various characteristics of fresh semen of Cholistani breeding bulls, viz. ejaculatory volume, mass activity, individual sperm motility, sperm concentration, percentages of live and morphologically normal spermatozoa and those with intact acrosome are presented in Table 1.

Ejaculatory volume: The overall mean ejaculatory volume of six Cholistani breeding bulls was 4.45 ± 0.76 ml, ranging from 1.25 to 8.75 ml. These results are in line with the findings of Ahmad *et al.* (2003), who reported a mean ejaculatory volume of 4.38 ± 0.09 ml in adult (5-years old) Sahiwal bulls reared at SPU, Qadirabad,

Sahiwal, Pakistan. However, Sarder (2005) recorded a higher mean volume of 5.15 ± 1.70 ml for Sahiwal bulls used for AI in India. A lower mean volume of 3.4 ± 1.3 ml was recorded for indigenous non-descript zebu bulls in India (Siddiqui *et al.,* 2008). Similarly, relatively lower mean values of 4.04 ± 0.03 and 2.92 ± 0.03 ml were recorded for locally born Holstein-Friesian and Jersey bulls in Pakistan, respectively (Fiaz *et al.,* 2010). Variations in age and breed of bulls, seasonal effects and nutritional status can be attributed to these differences (Sarder, 2005).

In Cholistani bulls, there was a wide variation in ejaculatory volume, with bulls KWC-19 and KWC-24 having the highest and lowest mean ejaculatory volumes of 7.20 ± 0.38 and 1.75 ± 0.19 ml, respectively. The difference in age can be attributed to this variation. Mosatri *et al.* (2004) also reported significant bull to bull variation among Sahiwal bulls imported to Bangladesh.

Mass activity: The overall mean mass activity of semen collected from six Cholistani bulls was 1.94 ± 0.14, the range was 0.50-3.75. The highest mean mass activity of 2.31 ± 0.18 was recorded for the bull KWC-25, while the lowest (1.50 ± 0.11) was for the bull KWC-21. The results of mean mass activity are lower than 2.29 ± 0.03 recorded for Sahiwal bulls in Pakistan (Ahmad *et al.,* 2003) and 2.83 ± 1.21 for Sahiwal bulls in Bangladesh (Sarder, 2003). Mean mass activity of 1.75 ± 0.01 was recorded for locally born Holstein-Friesian and Jersey bulls in Pakistan (Fiaz *et al.,* 2010). Variations among results of different studies can be attributed to differences in age of breeding bulls at the time of study along with seasonal effects (Ahmad *et al.,* 2003). Since mass activity is assessed through naked eye under the microscope, the experience of the worker can also affect the results.

Individual sperm motility: The overall mean individual sperm motility in the present study was 64.38 ± 2.64%, being highest for the bull KWC-28 *i.e.* 70.00 ± 1.06%. These findings are in accordance with the work of Ahmad *et al.* (2003) and Sarder (2003), who reported mean individual sperm motility as 65.14 ± 0.34 and 64.0 ± 0.35% for Sahiwal bulls kept in Pakistan and India, respectively. Siddiqui *et al.* (2008) reported a lower individual sperm motility of 50.8 ± 17.2% for indigenous non-descript zebu bulls in India. The difference in individual motility in various reports can be due to variations in handling of semen, number of bulls studied and environmental influences (Bailey *et al.,* 2003). Moreover, the results can also vary from person to person, as sperm motility is usually assessed under microscope through naked eye.

Sperm concentration: The overall mean sperm concentration in Cholistani breeding bulls was 918.05 ± 65.79 million/ml. There was a wide variation in the mean sperm concentration among bulls, being highest for the bull KWC-28 (1062.22 ± 101.26 million/ml) and lowest for the bull KWC-21 (688.35 ± 97.04 million/ml). The difference in age of the bulls can be attributed to this variation. The mean sperm concentration in Cholistani bulls is lower than 2541.9 ± 1699.2 million/ml for indigenous non-descript zebu bulls in India recorded by Siddiqui *et al.* (2008). Similarly, Sarder (2003) reported a sperm concentration of 1471 ± 37 million/ml for Sahiwal bulls in India. However, a sperm concentration of 990 ± 0.01 million/ml was reported by Ahmad *et al.* (2003) for Sahiwal bulls in Pakistan. These variations in sperm concentration among different studies may be the result of variable techniques used, season of the study, age and feeding regimen for the bulls (Ijaz *et al.,* 2009).

Live sperm: The overall mean percentage of live sperm in the present study was 84.60 ± 1.27. Comparing the results with those for Sahiwal bulls in Pakistan, varying reports have been published. Lodhi *et al.* (2008) reported mean live spermatozoa of 84.85%, whereas Haq *et al.* (2003) reported a lower live sperm percentage of 72.27 ± 2.38 in Sahiwal bulls. Ahmad *et al.* (2011), however, reported that the percentage of live sperm (67.7 ± 0.7) was almost similar in adult Sahiwal breeding bulls, except in 55-60 months old group, where it was lower ($P \leq 0.05$).

Morphologically normal sperm: The mean percentage of morphologically normal sperm in Cholistani breeding bulls included in the present study was 83.97 ± 0.61. Lodhi *et al.* (2008) also observed a mean normal sperm percentage of 86.25 for Sahiwal bulls. However, Ahmad *et al.* (2011) reported a mean percentage of abnormal sperm as 1.81 ± 0.3, indicating 98.19% normal sperm in Sahiwal bulls of various age groups.

In the ejaculates of Cholistani bulls, 83.97% sperm were morphologically normal, indicating that 16.03% sperm would be morphologically abnormal, which is within the acceptable limit, as it is generally believed that up to 20% abnormal sperm in the ejaculate would not affect fertility. However, type of sperm abnormality is more important in this regard than the total number of abnormal sperm.

Sperm acrosome integrity: The presence of intact acrosome is vital for the acrosome reaction in order to facilitate fertilization. Sperm aging or injury is a major cause of damaged acrosome (Akhter *et al.,* 2008). The present study revealed a mean number of sperm with intact acrosome as 83.85 ± 0.54%. Lower mean acrosome integrity of 74.85 ± 0.5% has been recorded in Sahiwal bulls of various age groups by Ahmad *et al.* (2011). These variations among different studies can be attributed to differences in the study protocol as well as seasons of the study.

Post thaw semen quality: Cryopreservation of bovine semen involves many critical steps such as extension,

cooling, freezing, storage and thawing, which can affect the sperm both physiologically and structurally (Bailey *et al.*, 2003). The chemical, osmotic, thermal and mechanical stresses during these steps result in cryoinjuries of varying degrees to the spermatozoa. Therefore, one objective of the present study was to assess the degree of damage occurred to the spermatozoa during their cryopreservation. Comparative mean values of sperm motility and percentages of live and morphologically normal spermatozoa and those with intact acrosome for fresh and post thaw semen are presented in Table 2.

The overall mean values for sperm motility (%), and percentages of live and morphologically normal spermatozoa and those with intact acrosome of post thaw semen were 54.02 ± 2.71, 71.89 ± 2.05, 75.64 ± 1.19 and 79.90 ± 1.69 which were 16.09, 15.02, 9.92 and 4.71% lower compared to those for fresh semen, respectively. Statistical analysis revealed that decrease in sperm motility, percentages of live and morphologically normal sperm was significant ($P<0.05$), while the freezing had a non-significant effect on acrosomal integrity of Cholistani bull spermatozoa. Ahmad *et al.* (2011), while assessing the damage to the buffalo bull semen during various stages of cryopreservation, reported a lower acrosomal integrity of 61.8 ± 2.40% in post thaw semen as compared to 73.2 ± 2.4% before freezing. Similarly, a lower mean post thaw live sperm (61.75 ± 1.5%) compared to pre freezing value was reported by Ansari *et al.* (2011). The various stages of cryopreservation, hence, infer varying degree of cryoinjury to the spermatozoa which can be breed specific, laboratory work module dependant and age related.

In conclusion, the present study reveals that Cholistani bulls are quite at par with Sahiwal bulls with reference to their semen quality. Furthermore, the comparison between the fresh and post thaw semen characteristics reveals quite encouraging results. This preliminary data envisages for further studies with a larger sample size expanded over all seasons and in correlation to age and testicular biometry in order to establish seasonality of reproduction (if any) in the Cholistani breed of cattle.

Table 1: Mean values (± SD) for characteristics of fresh semen collected from six Cholistani bulls

Bull No.	Ejaculatory volume (ml)	Mass activity (0-5)	Sperm motility (%)	Sperm conc. (10^6/ml)	Live sperm (%)	Normal sperm (%)	Acrosome integrity (%)
KWC-19	7.20 ± 0.38	2.08 ± 0.14	68.54 ± 2.07	739.45 ± 84.08	79.54 ± 5.10	81.09 ± 4.41	81.81 ± 4.42
KWC-21	5.85 ± 0.47	1.50 ± 0.11	56.04 ± 3.10	688.35 ± 97.04	88.10 ± 2.29	83.70 ± 2.92	83.30 ± 2.74
KWC-24	1.75 ± 0.19	1.52 ± 0.24	56.11 ± 5.20	1022.06±101.87	84.77 ± 2.13	84.33 ± 1.67	85.00 ± 2.21
KWC-25	4.02 ± 0.23	2.31 ± 0.18	67.91 ± 2.83	1018.12 ± 72.44	86.66 ± 2.32	84.91 ± 2.03	85.5 ± 1.58
KWC-27	3.91 ± 0.28	2.25 ± 0.13	67.70 ± 2.22	978.12 ± 65.39	86.08 ± 2.46	84.66 ± 2.16	83.41 ± 2.46
KWC-28	3.97 ± 0.20	2.02 ± 0.20	70.00 ± 1.06	1062.22±101.26	82.45 ± 3.69	85.18 ± 1.79	84.09 ± 2.76
Overall means	**4.45 ± 0.76**	**1.94 ± 0.14**	**64.38 ± 2.64**	**918.05 ± 65.79**	**84.60 ± 1.27**	**83.97 ± 0.61**	**83.85 ± 0.54**

Table 2: Showing the effect of freezing on post-thaw characteristics of semen collected from six Cholistani bulls (Mean ± SD)

Sperm characteristics	Fresh semen	Post-thaw semen	Decrease (%)
Sperm motility (%)	64.38 ± 2.64	54.02 ± 2.71	16.09*
Live sperm (%)	84.60 ± 1.27	71.89 ± 2.05	15.02*
Normal sperm (%)	83.97 ± 0.61	75.64 ± 1.19	9.92*
Acrosome integrity (%)	83.85 ± 0.54	79.90 ± 1.69	4.71NS

*Significant ($P<0.05$). NS Non-significant.

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