



***In vitro* fertility assessment of Kundhi buffalo bull semen**

Hamzo Khan Kunbhar, T.A. Raho and M.U. Samo

Faculty of Animal Husbandry Veterinary Sciences, Sindh Agriculture University TandoJam,

Abstract

The study was conducted on in-vitro fertility assessment of frozen thawed semen collected from Kundhi buffalo bull maintained at Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam. Before freezing of semen, each ejaculate was assessed for volume, sperm concentration, mass activity and mass motility percentage. Twenty semen samples having motility 60% or above were frozen for post-thaw assessment. Frozen thawed semen was incubated at 25°C for 5 hours and examined for progressive linear motility and live dead sperm count. The mean volume, mass activity, mass motility percentage sperm concentrations and pH of the semen were found to be 2.79±0.217 ml, 2.85±0.111, 71.75±2.621, 11.35±1.255 millions/ml and 5.8185±0.092 respectively of fresh semen. No significant difference was found between the parameters except pH, which was significantly different between the bulls. The mean sperm motility percentage and live dead sperm count % of Kundhi buffalo bull semen was found to be 20.46±1.62 and 6.9± 0.2% for frozen semen. A significant ($P < 0.05$) difference was found between the bulls for post-thaw motility percentage. It was found that at 01 hour incubation, 43.25±2.95% of sperms were motile having 11.78±0.28 % dead sperm count. It was gradually decline from 0 to 5 hours incubation, After 5 hours, all sperms were found dead. It is concluded that sperms maintaining long term motility and having less live dead sperms count were considered suitable for artificial insemination.

Keywords: Buffalo, Bulls, Semen, *In Vitro*, Fertility, Assessment, Live-dead Sperm

Introduction

Livestock is an important sector of Agriculture and plays a vital role in the economy of Pakistan, which accounts for 50% of the agricultural value added and about 11.4% of the GDP (GOP, 2005-06). Next to Agriculture, Animal husbandry is the most important economic activity in rural areas. These two sectors together provide employment and income to vast majority of rural population. The role of livestock in rural economy may be realized from the fact that 30-35 million rural populations is engaged in livestock raising, which help them to derive 30-40% of their income from it. Livestock produce a number of vital products and services. These can be classified into three broad groups: Energy, Food and Raw materials. Rapid economic development puts pressure on the livestock sector to increase its output, as to meet the demand of meat and milk.

The water buffalo (*Bubalus bubalis*) is the unique domestic animals of developing countries, particularly in Asia (Mustafa et al., 2001) and provides draught power, milk and meat (Chantalakhana and skunmun, 2000). In buffalo milk production, India ranks first

followed by Pakistan, China, Egypt, and Nepal (Bandyopadhyay et al., 2000). The estimated population of buffalos in Pakistan is 29.9 millions (GOP, 2009), comprising two breeds viz., Nili Ravi and Kundhi. Nili Ravi is found in the province of the Punjab and Kundhi is the well known milch breed in Sindh province of Pakistan. It has been observed that very few farmers are aware of the importance of pedigree of their animals, except those who participate in animal competition shows. A vast majority of the farming community observes no specific breeding programme and it is resulting in decline in number of purebred animals. There is always shortage of breeding bulls, especially in urban areas.

Artificial insemination (A.I.) is one of the biotechnological tools, which has resulted an improvement in the productivity of each individual animal. The A.I. programme is provided by Government, but there seems to be no visible increase in the number of animals inseminated artificially in the province of Sindh, especially in the buffaloes (Samo et al., 2004). A limited number of cows/buffaloes are bred artificially and the semen supply is remained irregular which has resulted in the lack of interest of the farm

community. The farmers meet their requirements either by keeping or barrowing the bulls, which are not properly evaluated and results in decline in the performance of the animals. The vast majority of breeding animals in the rural areas are almost completely deprived of breeding facility. The situation in buffalo is still worst and warrants for the establishment of a strong and reliable breeding plan to solve the problems.

In natural service, one bull is sufficient for 40-50 buffaloes, where as in case of A.I., from one ejaculation, 200 to 300 female animals can be inseminated. In natural service it is difficult to evaluate the bull semen for its fitness, while in A.I. programme semen can be checked properly and used either fresh or after freezing. The fresh semen has shorter shelf life, where as frozen semen can be stored and used for a longer period. A sufficient number of sperm cells die during freezing and the fertilizing ability of stored semen may be affected, if not stored properly. There is great need to evaluate the semen for fertility after freezing and thawing, so that it could be used extensively. The study was therefore designed to assess *in vitro* fertility of semen frozen at room temperature of 25°C for 24 hours of Kundhi buffalo bulls.

Materials and Methods

Four adult Kundhi buffalo bulls were used in this study. These bulls were kept at the Department of Animal Reproduction, Sindh Agriculture University, Tandojam, under intensive managemental conditions. Vaccination and deworming were undertaken as per farm routine scheduled.

Each bull was properly cleaned prior to semen collection. All the possible hygienic measures were observed to obtain uncontaminated semen samples. Semen was collected with the help of artificial vagina (AV) as per technique of Salisbury and Willet (1985). The temperature of the AV at the time of collection was maintained around 42°C. Teasing of bull was usually practiced to increase the *libido*, concentration of spermatozoa and the volume of the semen ejaculate. Each bull was given enough time for sexual stimulation and at least one false mount, to get complete ejaculate of good quality. The second ejaculate was collected 15-30 minutes intervals, after the first collection. 20 semen samples were collected and used for further assessment. 100 semen aliquots (straws) were used in this study.

After collection, the semen samples were immediately transferred to water bath at the temperature of 37°C and evaluated for like volume, pH, mass activity and progressive motility of spermatozoa as per technique described by Salisbury and Demark (1961) and for concentration of spermatozoa by adopting the procedure of Settergen (1967).

Post-thaw assessment for motility % and live dead percentage of sperms was done by using nigrosin eosin solution as per technique of Sindhu and Guraya (1985). The dead sperms stained red with blue background, whereas live sperms appeared transparent (Bearden, 1997).

Acceptable ejaculate was split into two equal portions, and then one portion was diluted at the rate of 1:20 with diluents used in the experiment (Samad, 1984). Doses were prepared and stored. The thawing of frozen semen straws was carried out in Luke warm water bath at the temperature of 37°C for 30 seconds only (Bodhipaksha and Limtrakul, 1967). One hundred frozen straws from four bulls were incubated at room temperature 25°C and assessed for post-thaw survival rate. The sperm maintaining long time motility and response staining was considered as fertile.

Statistical Analysis

Data was subjected to Analysis of Variance using soft ware package of Minitab.

Results

A non significant ($P>0.05$) difference was observed between the bulls for semen volume and sperm concentration, mass activity and progressive linear motility (Table 1). A significant ($P>0.03$) difference was observed between the bulls for semen pH. Significantly higher value of pH (6.03 ± 0.14) was found in bull number 01 followed by bull numbers 2, 3 and 4.

Mean progressive linear motility (PLM) of frozen thawed semen after various timings of incubation at 25°C was observed as $20.462\pm 1.67\%$ (Table-2). A significant ($P<0.05$) and progressive decrease was observed in PLM of the sperm cells. Significantly higher PLM ($43.25\pm 2.95\%$) was found at incubation time 01 hour, followed by timings 02 hours, 03 hours and 04 hours. The mean (\pm SEM) live sperm count of semen ejaculates after various incubation periods was recorded as $6.9 \pm 0.2\%$ in Kundhi buffalo bulls. Non significant but slightly higher live sperm count ($11.87\pm 0.28\%$) was found to be at incubation time 01 hour followed by timings 02 hour, 03 hour and 04 hours (Table 2). A non significant ($P>0.05$) and progressive decrease was observed under incubation period in live sperm count of semen (Table 3).

Discussion

Buffalo spermatozoa are more susceptible to hazards during freezing than cattle spermatozoa, thus a decline in semen quality is common in hot season, and affecting semen quality and fertility rate in buffalo (Raizada et al., 1990). The volume of semen recorded

Table 1: The mean semen volume, sperm concentration, pH, mass activity and progressive linear motility of Kundhi buffalo bulls

Bull No.	Volume (ml)	Sperm concentration $\times 10^6/\text{ml}$	pH	Mass activity	Progressive linear motility (%)
01	3.03 \pm 0.146	13.2 \pm 1.53	6.03 \pm 0.15 ^a	3 \pm 0.00	72 \pm 1.22
02	2.39 \pm 0.043	10.6 \pm 0.81	5.90 \pm 0.04 ^b	2.8 \pm 0.2	72 \pm 3.39
03	2.74 \pm 0.072	12 \pm 2.00	5.74 \pm 0.07 ^b	2.6 \pm 0.24	69 \pm 4
04	3.1 \pm 0.108	9.6 \pm 0.68	5.59 \pm 0.10 ^b	3 \pm 0.24	74 \pm 1.87
Overall mean	2.79 \pm 0.093	11.35 \pm 1.25	5.81 \pm 0.093	2.85 \pm 0.11	71.75 \pm 2.62

^{a,b} Values within a column having different superscript differ significantly (P<0.05)

Table 2: The mean progressive linear motility (PLM;%) of Kundhi buffalo bull semen

Incubation (hr)	Bull numbers				Incubation Mean
	01	02	03	04	
01	42.00 \pm 0.33	34.60 \pm 0.35	45.60 \pm 0.31	50.80 \pm 0.43	43.25 \pm 2.95 ^a
02	30.00 \pm 0.12	25.40 \pm 0.21	35.50 \pm 0.22	35.40 \pm 0.32	31.58 \pm 2.05 ^b
03	15.30 \pm 0.1	15.20 \pm 0.17	28.20 \pm 0.11	20.00 \pm 0.33	19.68 \pm 2.65 ^c
04	5.20 \pm 0.03	7.70 \pm 0.1	10.00 \pm 0.05	8.30 \pm 0.12	7.80 \pm 0.86 ^d
05	00 \pm 0.00	00 \pm 0.00	00 \pm 0.00	00 \pm 0.00	0.00 \pm 0.00
Bull mean	18.5 \pm 2.20	16.62 \pm 1.86	23.86 \pm 1.20	22.9 \pm 2.30	20.46 \pm 1.62

^{a-c} Values within a column having different superscript differ significantly (P<0.05)

Table 3: The mean live dead sperm count (%) of semen of Kundhi buffalo bulls

Incubation (hr)	Bull numbers				Incubation Mean
	01	02	03	04	
01	11.33 \pm 0.33	13.66 \pm 0.21	12.16 \pm 0.16	11.33 \pm 0.21	11.78 \pm 0.28
02	10.25 \pm 0.21	10.30 \pm 0.17	10.60 \pm 0.32	10.00 \pm 0.13	10.29 \pm 0.21
03	8.70 \pm 0.11	8.40 \pm 0.17	8.30 \pm 0.15	7.80 \pm 0.11	8.30 \pm 0.16
04	5.10 \pm 0.12	3.40 \pm 0.22	4.50 \pm 0.12	3.50 \pm 0.10	4.13 \pm 0.35
05	00 \pm 0.00				
Bull mean	7.07 \pm 0.14	7.152 \pm 0.16	7.112 \pm 0.13	6.526 \pm 0.12	6.9 \pm 0.2

as 2.8ml in the present study, was close to the findings (2-6 ml) of Brohi (1993) in Kundhi buffalo bull. Similar findings have also been reported by others (Bajwa et al., 1982; Ahmed et al., 1984; Sharma, 1988) in buffalo bulls. Vale (1994a) reported that young bull donates less volume (1-3 ml) than the older ones (6 ml). Although the bulls used in current study were young and still under training. The volume produced speaks the potential of this breed and could be used for A.I. programme. The semen volume however varies from breed to breed, managerial conditions and age of the bull. The mean (11.35 \times 10⁶/ml \pm 1.25) concentration found in the present study are in close agreements to the findings of Augiar et al., (1994), Jainuddin et al., (1982) and Bajwa et al., (1982), who reported 11.66 \pm 17.5 millions/ml in Swamp and Nili Ravi buffalo bulls respectively.

Buffalo bull semen has a pH, which ranges from 6.4-7.0 (Rattan, 1990; Kumar et al., 1993b). The pH of Kundhi buffalo semen was found as 5.24 \pm 6.53 during the present study is in close to previous results (6.73) reported by Brohi (1993) and Toquir (1994) in Kundhi

buffalo bull semen. However pH recorded during the present study varies with those of previous reports for the same breed. The discrepancy might have been due to the age of the bull or season of the year. However none of the pH was in the range of lethal level for the sperm cells.

Mass activity was observed +++ in all Kundhi buffalo bulls. The results was non significant among the bulls. Similar mass activity of sperms were recorded by Bajwa et al. (1982) in Nili Ravi buffalo bulls maintained at Qadirabad and Brohi (1993) in Kundhi buffalo bull semen, maintained at Tandojam, Pakistan.

The PLM is a parameter of paramount importance for the assessment of semen quality before and after freezing. It is the most useful criteria applied to ensure semen quality in A.I. programme. However it is subjective type of semen assessment and has poor correlation with fertility rate (Revell and Mrode, 1994). The mean PLM (71.75 \pm 2.62) values observed during this study, is in agreement with the findings of Jainuddin, et al. (1982) and Bajwa et al. (1982), who reported 70.7% and 63.85% sperm motility in Swamp

and Nili Ravi buffalo bull semen respectively, however somewhat higher results ($78.6\pm 5.6\%$) have also been reported by Augiar et al. (1994) and Galli et al. (1993) in other breeds of buffalo.

There was significant and progressive decrease in the number of cells after thawing and incubation in all the semen samples. This signifies the importance of immediate use of frozen thawed semen. The mean PLM ($43.25\pm 2.95\%$) recorded after one hour incubation falls in the range reported by Zahid (2001) and Sukhato, (2000 and 2001) in various breeds of buffalo. However values (20.46 ± 1.62) after four hours incubation are poor than others scientists, this situation alarms the early use of semen after thawing.

Semen with more than 30% initial dead spermatozoa may not be suitable for storage and freezing. The mean values after one hour incubation was found 11.78 ± 0.28 in the present study. However the values (6.9 ± 0.2) after four hour incubation were poor than those of others. Differential staining techniques have been used for determination of live and dead spermatozoa (Roachwerger and Cuaniscu, 1992). Higher results of post-thaw live sperm ($43.51\pm 1.46\%$) were reported by other workers (Sharma, 1988; Bhavsar et al., 1988 and Belorkar et al., 1990) in crossbred cattle and buffalo bulls. Thus greater the live ability of spermatozoa better would be the quality and motility for pre and post-freezing.

It is concluded that there was decrease in PLM and live sperm count after freezing. Tris based extender was suitable for freezing of semen of Kundhi buffalo bull. It can be suggested that the frozen semen should be used immediately after thawing. Latest technologies should be applied for evaluation of fresh and frozen semen. Further more research should be conducted to establish basic norms and quality characteristics of fresh and frozen semen of Kundhi buffalo to observe the effect of various seasons.

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