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POST-THAW EVALUATION OF KUNDHI BUFFALO BULL SEMEN

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ABSTRACT

The study on *in-vitro* fertility assessment of frozen thawed semen from Kundhi bulls was undertaken during the months of July to September 2006 at the Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam. Before freezing, each ejaculate was assessed for volume, color, mass activity, and motility and sperm concentration. Twenty samples having motility 60% or above were frozen for post-thaw assessment. Frozen thawed semen was incubated at 25^0C for 5 hours and examined for progressive linear motility and live dead sperm count. It was observed that all the ejaculates were creamy white in color. The mean (± SEM) volume, mass activity, motility percentage sperm concentrations and pH of the semen from Kundhi buffalo bulls were found to be 2.79 ± 0.217 ml, 2.85 ± 0.111, 71.75 ± 2.621%, 11.35 ± 1.255 millions/ml and 5.82 ± 0.092 respectively for fresh semen. No significant (P>0.05) difference was found between the parameters except for pH value, which was significantly (P< 0.03) different between the bulls. The mean (± SEM) sperm motility percentage was found to be 20.46±1.62 and dead sperm count were 6.9± 0.2% for frozen semen of Kundhi buffalo bull. A significant (P< 0.05) difference was found between the bulls for post-thaw motility percentage. It was found that at one hour of incubation period motile sperms were 43.25 ± 2.95% having 11.78 ± 0.28% dead sperm count. After 5 hours incubation all sperms were found dead. It was concluded that sperms maintaining long term motility and having less dead sperm count are suitable for artificial insemination.

Keywords: *In vitro*-fertility, Post thaw incubation, Buffalo, Bulls, Semen.

INTRODUCTION

The water buffalo (*Bubalus bubalis*) is a unique domestic animal of developing countries, particularly in Asia and provides draught power, milk and meat (Chantalakhana and skunmun, 2000). India ranks first in production of buffalo milk followed by Pakistan, China, Egypt, and Nepal (Bandyopadhyay, *et. al*., 2000)

The estimated population of buffalos in Pakistan is 32 millions (Anonymous, 2009-10), comprising two breeds of Nili Ravi and Kundhi. Nili Ravi is found in the province of Punjab and Kundhi is the well know milch breed of buffalo in Sindh province of Pakistan.

There is always shortage of breeding bulls, especially in urban areas. The bull supply scheme, from Government farms to farmers (maldars), had been discontinued, resulting a shortage of breeding bulls specially, in remote areas. 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 farm community observes no specific breeding programme and resulted decline in number of purebred animals.

Artificial insemination (A.I.) is one of the biotechnological tools, which has resulted in a rapid 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 cows inseminated artificially in the province of Sindh, especially in buffaloes (Samo *et. al.*, 2004). A limited number of cows/buffaloes are breed artificially and the semen supply is irregular which has resulted in the lack of interest of the farm community. The farmers meet the requirements either by keeping or borrowing the bulls, which are not properly evaluated and the result is 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 if added more diluents, 200 to 300 cows can be inseminated. In natural services 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 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.

This study was therefore designed to asses *in-vitro* fertility of frozen semen from kundhi buffalo bulls incubated at room temperature (25^oC) after frozen storage in liquid nitrogen for 24 hours.

MATERIALS AND METHODS

Experimental Animals

Four Kundhi buffalo bulls of 22 to 30 months of age were kept at the Department of Animal Reproduction, Sindh Agriculture University, Tandojam, under intensive management and feeding conditions in the month of July to September 2006. Vaccination and deworming were undertaken as per farm routine.

Semen collection

Each bull was properly cleaned and all the hygienic measures were observed to obtain uncontaminated semen samples. Semen was collected with the help of artificial vagina AV following the technique of (Salisbury and Willet, 1985). The temperature of the AV at the time of preparation was maintained around 50^{o}C to 52^{o}C and ranged between 42^{o}C to 44^{o}C at the time of collection which was recorded with digital thermometer. Teasing of bull was usually practiced to increase the libido, concentration of the spermatozoa and the volume of the 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 after the first collection. Total 20 samples were collected and 100 semen aliquots (straws) were used in this study.

Evaluation and selection of ejaculates

The semen samples after collection were immediately transferred to a water bath at 37^{o}C and evaluated for general appearance like volume, pH, mass activity, progressive motility of spermatozoa (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 were done by using nigrosin eosin solution (Sindhu and Guraya, 1985). The ejaculates having a motility more than 60% and sperm concentration not less than 1200-1500 millions/ml was selected for further investigation. All the semen samples were subjected to the following tests.

Quality Characteristics of fresh Semen

The volume of the semen was measured by 5ml-graduated test tube immediately after collection. The color of the semen was assessed by naked eye and the pH of semen was measured by pH meter. Counting of sperms cell was made by Haemocytometer. Mass activity was evaluated using a drop of fresh semen under microscope without using cover slip under a low magnification 20x at 37^{0}C and the mass activity was graded as:

0 = No mass activity
+ = Progressive motion < 20%
++ = Progressive movement with slow wave 44 to 60 %.
+++ = Progressive movement with wave more intense 60 to 80%.
++++ = Progressive movement with rapid wave 80 to 100 %.

The motility was expressed in percentage of sperms moving in forward direction. Motility was evaluated using a drop of diluted semen under phase contrast microscope using cover slip at magnification of 200x at 37^{0}C. It was estimated as per standard staining procedure using nigrosin eosin solution (Sindhu and Guraya, 1985).

Dilution of Semen

Acceptable ejaculate was split into two equal portions, then one portion diluted at the rate of 1:20 with diluents used in the experiment (Table-1**).**

Preparation of diluents

The required amount of Tris, citric acid and fructose were weighed with the help of electrical weighing balance, and mixed with glycerol and distilled water. Egg yolk was separated by complete removal of albumin and yolk was procured in a cylinder by puncture the yolk membrane. Diluents were prepared by mixing required quantities of Tris, citric acid, fructose, glycerol, egg yolk and antibiotics. After preparation the diluents was stored in clean sterilized flasks, which were kept in a water bath at 37^{0}C until used for dilution of semen samples.

Filling and sealing of straws

One hundred French medium size white color straws made up of polyvinyl chloride were fixed in clips, filled with the help of automatic suction pump and sealed by using polyvinyl chloride powder were subjected to frozen.

Equilibration, freezing Storage of frozen semen and Thawing

The semen filled in straws was stored for equilibration period of 6 hours at 5^0C in a cold cabinet. The frozen straws were carried out in wide mouth freezing chamber containing liquid nitrogen. A wire net was used for holding the straws in vapors 6 centimeters above the surface of liquid nitrogen for 6 minutes and then straws were gradually dipped down in liquid nitrogen. Small plastic goblets filled with liquid nitrogen were used to collect the straws from freezing chamber. After plunging the straws into plastic goblets, they were transferred immediately to bigger steel goblets having a sieve at the lower end and held vertically in the storage container filled with liquid nitrogen. The straws remained dipped under the surface of liquid nitrogen, stored for at least 24 hours. The frozen semen was then thawed and carried out in a Luke warm water bath at 37^0C for 30 seconds. (Bodhipaksha and Limtrakul, 1967).

Post-thaw assessment

One hundred frozen straws from four bulls were incubated at room temperature 25^0C and assessed for post-thaw survival rate. The sperm maintaining long time motility and response to eosin staining was considered as fertile. Following parameters were recorded after every hour for five hours.

- Motility %
- Dead sperm ratio/percentage

Statistical Analysis

Data was subjected to Analysis of Variance (ANOVA) using soft ware packages of Minitab.

Table 1. Composition of extender used in the experiment (**Tris based diluents*** (Samad, 1984).

Ingredients	Quantity in gm/ml
Tris (Hydroxy-methylaminomethane)	**3.81gm/100ml**
Egg yolk	20ml
Glycerol	7ml
Citric acid	1.55gm
Fructose	2.O gm
Penicillin	1000 I.U/ml
Streptomycin	1.00mg/ml
Distill water	100ml

Table 2. The mean (±SEM) progressive linear motility% (PLM) of semen from each Kundhi buffalo bull.

Incubation time	Bull numbers				Incubation Mean ± SEM
	01	02	03	04	
01	42.00	34.60	45.60	50.80	43.25 ± 2.95
02	30.00	25.40	35.50	35.40	31.58 ± 2.05
03	15.30	15.20	28.20	20.00	19.68 ± 2.65
04	5.20	7.70	10.00	8.30	7.80 ± 0.86
05	00	00	00	00	0.00 ± 0.00
Bull Mean ± SEM	18.5 ± 2.20	16.62 ± 1.86	23.86 ± 1.20	22.9 ± 2.30	20.46 ± 1.62

RESULTS

The study was conducted to determine the effect of Post-thawing incubation on motility percentage and dead sperm count percentage of Kundhi Buffalo Bull Semen. Mean (±SEM) progressive linear motility (PLM) of frozen thawed semen after various timings of incubation at 25^oC showed a significant ($P<0.05$) and progressive decrease in

PLM of the sperm cells. Significantly higher PLM (43.25 ± 2.95%) was found to be at one hour incubation time followed by two hour (31.58 ± 2.05%), 03 hour (19.68 ± 2.65) and 04 hour (7.80 ± 0.86%) and the overall mean was 20.462 ± 1.67 % (Table-2).

Table 3. The mean (±SEM) live dead sperm count% of semen from each Kundhi buffalo bull.

Incubation time (Hours)	**Bull numbers**				**Incubation Mean± SEM**
	01	02	03	04	
01	11.33	13.66	12.16	11.33	11.78 ± 0.28
02	10.25	10.30	10.60	10.00	10.29 ± 0.21
03	8.70	8.40	8.30	7.80	8.30 ± 0.16
04	5.10	3.40	4.50	3.50	4.13 ± 0.35
05	00	00	00	00	0.0 ± 0.00
Bull Mean ± SEM	7.07 ± 0.14	7.152 ± 0.16	7.112 ± 0.13	6.526±0.12	6.9 ± 0.2

Table 4. Analysis of variance (ANOVA) for semen sperm motility% of Kundhi buffalo semen.

Source of Variation	*df*	*SS*	*MS*	*F*	*F crit.*
No of bulls (A)	3	177.318	59.106	4.756	
Incubation time (B)	4	4867.693	1216.923	97.911	SS
A x B	12	149.147	12.429		NS
Total	19	5194.158		0.001	

Table 5. Analysis of variance (ANOVA) for semen live and dead sperm count % of Kundhi buffalo semen.

Source of Variation	*df*	*SS*	*MS*	*F*	*F crit.*
No of bulls (A)	3	1.308	0.436	1.063	NS
Incubation time (B)	4	383.888	95.972	233.949	SS
A x B	12	4.923	0.410	0.001	NS
Total	19	390.119			

A non significant (P>0.05) and progressive decrease was observed under incubation period in live sperm count of semen. Non significant but slightly higher live sperm count (11.87 ± 0.28%) was found to be at one hour

incubation time followed by 02 hour (10.29 ± 0.21%), 03 hour (8.30 ± 0.16%) and 04 hour (4.13 ± 0.35%). The overall mean was 6.9 ± 0.2% (Table 03)**.**

DISCUSSION

Buffalo spermatozoa are more susceptible to hazards during freezing than cattle spermatozoa, thus a decline in semen quality is common in hot season affecting semen quality and fertility rate in buffalo (Raizada *et al*., 1990). 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±2.95%) recorded after one hour incubation falls in the range reported by others (Zahid *et. al.*, 2001) in various breeds of buffalo. However mean values (20.46 ± 1.62) after four hours incubation are poor than those of others. This alarms the early usage 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 mean values (6.9 ± 0.2) after four hour incubation were poor than those of others. Differential staining techniques have been used for determination of dead sperm ratio (Roachwerger and Cuaniscu, 1992). Higher results of post-thaw live sperms (43.51 ± 1.46%) were reported by other workers (Belorkar *et. al*., 1990) in crossbred and buffalo bulls. Thus greater the live ability of spermatozoa better would be the quality and motility pre and post-freezing.

CONCLUSIONS

1. There was decrease in PLM and live sperm count after freezing.
2. Tris based extender was suitable for Kundhi buffalo bull semen.

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