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STUDY ON POST THAWING QUALITY OF KUNDHI BUFFALO SEMEN

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ABSTRACT

This study was conducted to assess the post thawing quality of Kundhi buffalo semen diluted in tris based diluents. Before freezing, the ejaculates were checked for volume, colour, pH, sperm concentration, mass activity, progressive linear motility (PLM), normal morphological characteristics and % of membrane intact cells. The semen qualifying these tests was frozen. The frozen semen after thawing was incubated at 37oC and assessed for progressive linear motility (PLM) and % of membrane intact cells. It was observed that all the ejaculates were creamy white in colour. The mean (±SEM) mass activity, volume, pH, progressive linear motility (PLM), sperm concentration, normal morphological characteristics and % membrane intact cells were. +++, 2.25 ± 0.01 ml, 6.10 ± 0.007, 69 ± 0.34%, 1542 ± 9.20 x 106, 79 ± 1.37%, 55.56 ± 1.37% respectively for fresh semen. No significant difference (P>0.05) was observed between the bulls for the parameters recorded except normal morphological percentages and % of membrane intact cells where a significant difference (P<0.05) was observed. The mean (±SEM) post thawing progressive linear motility (PLM) and % of membrane intact cells was found to be 43±3.40% and 31.4 ± 3.07% respectively. A significant difference (P<0.05) was observed between the bulls for post thawing semen parameters. It was concluded that semen of Kundhi bulls tolerate freezing and thawing stress and maintained reasonably good score of parameters to be considered for A.I programme. The objective method Osmotic Resistance Test (ORT) was found to be useful parameter for assessment of in vitro quality of buffalo semen.

Key words: In vitro-fertility, Post thaw incubation, Buffalo, Bulls, Semen.

INTRODUCTION

The domestic water buffalo *(Bubbalis bubbalis)* is an important animal in the agriculture economy of many Asian countries especially India and Pakistan (Jainudeen *et al*. 1982, Qureshi and Ahmad, 2008). They are of two types, Riverine and Swamp. Seventy percent Riverine type water buffaloes are reared in South Asia, especially in India and Pakistan. There are about 158 million buffaloes in the world. About 97% of them (153 million) are water buffaloes essentially found in the Asian Region (Anonymous, 2000). Pakistan is blessed with two breeds of Riverine type water buffaloes namely Kundhi and Nilli Ravi possessing high qualities of adaptation under stressful environment, they have gained the reputation of being among the best multipurpose breeds in the tropical and subtropical world. Buffalo productivity under field conditions in the region has been low as already reported (Qureshi et al., 2002). They suggested that excess intake of crude protein, associated with higher serum urea levels and low energy intake, associated with poor body condition, are the key factors for low reproductive efficiency. It may be corrected by adopting a proper feeding strategy.

There are 28.4 million buffaloes in Pakistan contributing 71% of the national milk production is contributed by buffaloes. Meat is another important by-product of buffaloes. According to the recent estimates, more than 49% total of meat is produced by buffalo per annum in Pakistan. In addition to their use for milk and meat production, buffaloes are also an important source of draught power in many parts of the country. They are used for a variety of agricultural operations i.e. ploughing, especially in paddy fields, water lifting from wells, transportation of farm products to near by markets, etc. (Anonymous, 2006).

Artificial insemination (AI) is an important tool for genetic improvement of dairy animal. The productive efficiency of buffalo can be improved by intensive selection and use of genetically superior sires produced through artificial insemination. Laboratory assessment of sperm fitness before placing it in the female tract is one of the main concerns in semen technology. The assessment of semen quality includes motility, sperm morphology, metabolic activity, cell count, and structural integrity (cell membrane and acrosome).

The semen for artificial insemination use is always extended in a medium friendly to the sperm. Buffalo semen extender is usually composed on the basis of an energy source (sugars such as glucose, fructose and lactose) and a buffer medium of different inorganic substances (tris and sodium citrate). Milk and egg yolk, are basic ingredients of most extending media and are recognized as a protectant against cold shock through its lipoprotein and phosphatidylcholine (Salisbury *et al.* 1978). Glycerol is added for cryopreservation and is the

standard cryopreservative agent. Penicillin and streptomycin are added to prevent bacterial contamination. (Samad, 1985).

The present study was therefore designed to investigate and establish basic norms of quantitative and qualitative characteristics of the semen using objective methods of semen assessment. To establish basic norms of quality characteristics of frozen Kundhi buffalo semen. To apply and validate the objective semen assays for the evaluation of fresh and frozen thawed semen from buffalo bulls.

MATERIAL AND METHODS

Current study was conducted at the Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam. The data was collected over the period of three months, on volume, color, pH, concentration, mass activity, progressive linear motility (PLM), normal morphological characteristics and membrane integrity of cells.

Selection of animals: Four Kundhi Buffalo bulls of 3 to 4 years of age were used for the present study. The bulls were housed in individual bull pens having good ventilation. Seasonal green fodder was fed. Wheat straw, cotton seed cake and clean water were provided ad-libitum.

Collection of semen: Semen from each bull was collected twice a week for 3 months in an artificial vagina (AV). Preparation of AV was done by the procedure described by Salisbury *et al*. (1985) maintaining the inner temperature of AV at 42°C - 45°C. On each collection day, two ejaculates were collected in succession, and each ejaculate was preceded by a period of sexual preparation consisting of at least two false mounts separated by about two minutes restraint time.

Semen evaluation: Immediately after collection each sample was brought into laboratory and placed in a water bath at 37°C. It was examined for volume, color, pH, mass activity, progressive linear motility (PLM), concentration, morphological characteristics and membrane integrity of cells as described by Qureshi (2011). Semen colour was evaluated by visual examination direct from the collected tube and was categorized as fallow. Milky, creamy, creamy white, and translucent. Ejaculate volume per ejaculate was recorded directly from graduated collection tube. pH of the semen was obtained by using digital pH meter. The mass activity was evaluated in a drop of un-diluent semen without using cover slip under low magnification (20X) with phase contrast microscope. Wave patterns of the semen sample were recorded and graded as following: 0 = No mass activity; + = Slow wave motion; ++ = Rapid wave motion with formation of eddies at the end of wave; +++ = Eddies **.**

Progressive linear motility (PLM): Progressive linear motility was assessed by standard subjective ranking method. A wet mount of diluted semen was prepared by placing a drop of fresh semen under cover slip at magnification of 20x under phase contrast microscope. At least 100 spermatozoa, selected randomly, were observed for percent motile spermatozoa in straight forward progression movement. While sperms moving in circles, in backward direction were excluded. The results were expressed in motility%. The activity and motility % were recorded on warm stage maintaining the temperature about 37 ^{0}C. The sample having 60% and above PLM were used for freezing and further investigation.

Sperm concentration: Sperm concentration was determined using a hemocytometer by using fixing solution (3% Sodium chloride solution) described by Henry (1991) Fixing solution.

Sodium chloride 3%	30g
Farm aldehyde 37%	4ml
Distilled water	1000 ml

A test tube loaded with 9.99ml of fixing solution was incubated at 37^{o}c, 0.01ml of diluted semen was added and allowed fixation for 5 minutes, a small drop of suspension was dropped on both chambers of hemocytometer, covered with a cover slide and observed under light microscope at 40 x magnifications. Sperms were counted in 5 squares one middle and four corner squares. Following formula was used to determine the cell count. Sperms/ ml = N x 5 x df x10000, N = no of sperm counted, and 5= no of chambers counted on hemocytometer, df = dilution factor

Osmatic Resistant Test (ORT): Fresh semen sample was used for the Hypo Osmotic Swelling test (HOST) by the method developed by Revell and Mrode (1994) in cattle. The test solution for fresh semen was prepared as follows:

Fructose	13.51g
Tri-sodium-citrate	7.35g
Distilled water	1000ml

100 µl semen was added to 1 ml of a hypo-osmotic solution. After incubation for 60 min at 37^{o}C, sperm swelling was assessed by placing 15µl of well mixed sample on a warm slide (37^{o}C), covered with a cover slide and observed under light microscope at 40x magnification. The spermatozoa swell in the response of the test solution were the cells having intact membrane and were considered as normal fertile cells. Three hundred spermatozoa per slide were counted and expressed in percentage.

Morphological characteristics: These were estimated as per standard staining procedure described by Sidhu and Guraya (1985). The staining solution was prepared by

mixing, Eosin (0.67g) and Nigrosin (5.0 g) in 100 ml distilled water. A drop of fresh semen was mixed with eight drops of stain solution. After standard incubation time (3 minutes) at 37°C, a thin smear was made on pre warmed slides and allowed to dry at 30°C. The excess stain was washed off in running tap water. The slide was then immersed in Ethanol to remove water. The dried film was examined using the oil immersion lens of light microscope. 100 sperms were counted and scored as normal and abnormal morphological percentages.

Extension of semen: Each sample was diluted with Tris based extenders (Samad, 1985).

Composition of the Tris – egg yolk Extender:

Tris (hydroxymethyl -amino-methane)	3.81g
Citric acid	1.97g
D (-) fructose	1.25g.
Egg yolk	20ml.
Glycerol	7ml.
Penicillin	1000 mg /ml.
Streptomycin	1.00i.u. /ml
Distilled Water	100ml

Semen was diluted in cold cabinet by bringing both the semen and extender at same temperature to prevent from cold shock. Rate of dilution was based on initial sperm concentration observed by hemocytometer and adjusted to have 20 million spermatozoa per straw per insemination dose. Diluted semen was allowed to equilibrate in a cold cabinet to reach at 5°C for 5-6 hrs. The filling of straws was carried out with the help of manual suction machine in. 0.5-ml straws. Sealing of the open end of the straw was done by polyvinyl chloride powder.

Freezing and thawing: Freezing was carried out by holding the straws in liquid nitrogen vapors 5 cm above the surface of liquid nitrogen for 6 minutes as already reported (Ahmed and Chaudery, 1980). Then the straws were plunged into liquid nitrogen (-196°C). Small plastic goblets, filled with liquid nitrogen were used to collect the straws from freezing chamber. After filling, the goblets were shifted immediately to steel canisters, having sieve at the lower end. The frozen semen was stored in liquid nitrogen at least for 24 hours and then assessed for post thaw quality.

Thawing of the semen was carried out by immersing straws in a water bath at 37 °C for 15 seconds (Rasul *et al.* 2000).

Post Thaw Evaluation: The post-thaw examination of the semen samples was done for assessing motility percentage and membrane integrity of the cells. Membrane integrity was assessed by adding a drop of thawed semen in the solution developed by Revell and Mrode (1994) in cattle for freeze thaw semen.

Fructose	9.0g
Tri-sodium-citrate	4.9g
Distilled water	1000ml

Statistical Analysis: Analysis of variance (ANOVA) was applied for means comparison. The data are presented as mean ± standard error of mean. Least significant difference (LSD) test was applied where appropriate.

RESULTS

Progressive linear motility (PLM): The mean (± SEM) post thaw motility % of semen of each bull is presented in Table-4. Mean (± SEM) motility percentage of all four bulls was observed to be 43.25± 3.40 A significant difference was observed ($p< 0.05$) between the bulls for post thaw motility % (Appendix-VII). Post thawing motility % was significantly higher in bull 4 (50.8±1.49 %) followed by bull 1 (42±0.94 %), 2 (34.6±1.33 %) and 3 (45.6±1.21 %).

Membrane integrity (%): The mean (± SEM) the sperm cells having intact membrane of each bull are presented in Table-4. Mean (±SEM) % cells having intact membrane was observed to be 31.4±3.07. A significant difference ($p< 0.05$) was observed between the bulls for cells having intact membrane (Appendix-VIII). The % of sperm cells having intact membrane was significantly higher in bull 4 (38.6±1.43 %) than bull 1 (33.6 ±1.02 %), 2 (23.8±1.625%) and 3 (30.2, ±1.364%).

Table 1: Results of the Post thawing semen characteristics of Kundhi Buffalo semen.

Bull NO	Motility		M. integrity	
	Mean	SE	Mean	SE
1	42	0.95	33.6	1.36
2	34.6	1.33	23.8	1.62
3	45.6	1.21	30.2	1.02
4	50.8	1.49	38.6	1.44
Mean	43.25	3.40	31.4	3.07

LSD For motility %: 3.78; LSD for Membrane intact cells: 4.13

DISCUSSION

Buffaloes are sensitive to heat stress, thus a decline in semen quality is common in hot seasons. Breeding frequency in buffaloes is high in winter and lowest in summer. The current study was conducted during hot months of the year. the results are discussed under appropriate headings.

Post-thaw Assessment of Semen: Freezing and thawing of semen leads to the decrease in the percentage of intact sperms, and reduces 50% viable sperm (Zahid *et al. 2001*). Motility and plasma membrane are important aspects to assess the fertility of sperm after freezing and thawing. In the current study post thawing motility and integrity of plasma membrane was studied to assess the

post thawing fertilizing ability of sperm. The results are discussed as follow.

Progressive linear motility (PLM): The post thawing motilityscore mean (±SEM) was found as 43.25%±3.402 in the current study which was higher that of Rasul *et al.* (2001) in Nilli Ravi buffalo. The difference may be due to the breed variation. Sukhato *et al.* (2001) in swamp buffalo bulls found results similar to current study. This shows that motility score of post thaw semen of Kundhi buffalo semen falls in range of acceptance by A.I program in other buffalo breeds. The parameters however is of objective in nature may not guarantee fertilizing ability of sperm cells. Several sperms having broken cell cell membrane remain motile after freezing and thawing (Samo *et al.* 2004)

Osmotic resistance test (ORT): The integrity of the plasma membrane has been one of the most studied parameters, for its major role in acting as cell boundary and in cell-cell interactions. Intact cell membrane is considered to be key to the success of fertilization, when frozen semen is used. Mean (±SEM) intact membrane cells (31.4±3.07) observed in current study agrees with the results of Rasul *et al.* (2001) in Nilli Ravi breed. This indicates that semen from Kundhi buffalo freezes similar to those of other breeds of buffalo in hot season. Conformation of these findings is made through in vitro studies during different seasons of the year. Parameter is objective in nature and has positive correlation with fertility of bovine spermatozoa (Revel and Mrode, 1994).

Conclusion: On the basis of present studies, following conclusions could be drawn: i) Post thaw motility % and membrane intact cell was significantly different ($p< 0.05$) between the bulls, falling within the range of acceptance for A.I program; ii) Kundhi buffalo semen freeze similar to other breeds of buffalo in warm season; iii) Tris extender is still suitable for cryopreservation of Kundhi buffalo semen; iv) Objective methods of semen assessment developed for cattle semen were found suitable for buffalo semen.

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