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Characterization of Semen of Nili-Ravi Buffalo Bulls Using Standard and Modified Techniques

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ABSTRACT. A study was undertaken to characterize semen of buffalo bulls (Bubalus bubalis) using standard and modified laboratory techniques. Semen samples (n=132) were collected at weekly intervals for twelve months from three Nili-Ravi bulls aged 5 to 6 years using an artificial vagina. Following the initial evaluation of semen (n=132) for volume, colour, density, mass activity, motility and concentration, a detailed evaluation was done using the last sample collected at each month (n=36) to study the sperm morphology and the presence of cells other than sperms. Sperms were examined for morphology either unstained or stained with modified William's stain, and for live and dead status with Nigrosin-Eosin (N/E). Cells other than sperms were identified using Haematoxylin-Eosin (H/E) stain. The average volume of semen collected during each month ranged from 1.10±0.14 to 7.00±2.16 ml/ejaculate (mean 2.94±1.96) and concentration from 607.33±186.01 to 2050.00±353.44 million/ml (mean 1389.71 ± 567.50). The colour of semen varied from cloudy to creamy and density from 2D to 4D. Mass activity and motility of sperms ranged from 2+ to 4+ and 70 to 90%, respectively. The percentage of dead sperms in ejaculates ranged from 2 to 24 (mean 8.19 ± 5.03). The percentage of head, mid piece and tail abnormalities were found to be 3.21 ± 1.83, 3.16 ± 1.61 and 7.00 ± 4.04, respectively. Desquamated epithelial and spermatogonial line cells (1 to 2 cells/microscopic field) also were found in the ejaculates. The monthly motility percentages of spermatozoa during January (88%), May (89%) and August (90%), showed significant monthly variations ($p< 0.05$), whereas no such variations were observed in the percentages of head, mid piece and tail abnormalities.

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INTRODUCTION

Artificial breeding has been adopted as a primary tool for improving productivity of indigenous cattle and buffaloes in Sri Lanka. The success of an artificial breeding programme depends on many factors and among them the quality of semen used plays a very important role. The quality of semen is assessed conventionally by examining the colour, density, mass activity, motility and morphology. Morphology of sperms are assessed using various staining techniques. In this context, various laboratories have developed and used a wide variety of techniques which have their own merits and demerits (Galloway and Norman, 1980; Rajamahendran and Dharmasena, 1984). Thus, using standard and modified tèchniques, a study was undertaken to evaluate and monitor the monthly variation in the semen quality of Nili-Ravi buffalo bulls reared in the mid-country region of Sri Lanka, where the rainfall, temperature and relative humidity ranged from 24.2 mm (August) to 325.1 mm (December), 18.7°C (minimum in February) to 31.0°C (Maximum in May), and 77 to 91%, respectively.

MATERIALS AND METHODS

Animals

Nili-Ravi buffalo bulls (n=3) maintained as semen donors at the Central Artificial Insemination Centre, Kundasale in the mid country of Sri Lanka were used. The age of the bulls ranged from 5 to 6 years, and their weight ranged from 680-800 kg. They were stall fed with grass, fodder and concentrates. Splashing of water was done over the body once a day for a few minutes as a substitute for wallowing.

Semen Collection

Semen samples were collected between 0800-0900 h on a given day at weekly intervals for a twelve month period using an artificial vagina (AV). The AV was assembled and maintained at 42°C until used for collection. A lubricant (Isto gel) was applied to the inner sleeve of the mounting side of the AV. Prior to semen collection 3 false mounts were allowed on a restrained male dummy. At the fourth mount, semen was collected into a graduated collection tube attached to the AV which was covered by an insulator to prevent sperms from cold shock. Semen samples were maintained at 37°C in a water bath during the initial evaluation (n=132). The

ejaculate of each bull obtained during the fourth week of each month was used for detailed evaluation (n=36).

Initial evaluation of semen

The volume of semen collected was directly read from the calibrated collection tube and colour and density were visually judged. The colour was graded from transparent to thick creamy and density on a scale from D to 4D, which was correlated with sperm concentration ranging from less than 250×10^6 to more than 3000×10^6 per ml of semen.

For the assessment of mass activity, a drop of semen was placed on a warm slide (37°C) soon after collection, and examined at x100 magnification using a microscope attached with a warm stage set at 37°C. The mass activity was graded from individual sperm movement to very rapid circular waves, on an ascending scale from 1+ to 4+. For the evaluation of percentage motility, 1-2 drops of fresh semen were diluted in 2ml of skimmed milk diluent. A drop of diluted semen was placed on a warm slide at 37°C, covered with a cover slip and examined under a phase contrast microscope. The motility was expressed as a percentage. For counting sperms, 25μl of semen was added to 4975μl of sodium bicarbonate buffer and a dilution of 1:200 was obtained. The diluted sample was charged in a Neubauer' type counting chamber and counted under a phase contrast microscope at x400 magnification. The concentration was expressed in millions per ml semen.

Detailed evaluation of semen

Live and dead sperms

Nigrosin-Eosin (N/E) stain was prepared by adding one part of 5% Eosin - B (Gurr, BDH) aqueous solution to four parts of 10% Nigrosin (Gurr, BDH) aqueous solution. A drop of fresh semen was placed on a warm glass slide (37°C) and 4 to 5 drops of Nigrosin-Eosin stain were added and incubated at the same temperature for 2 minutes. Then a thin smear was made and allowed to dry at room temperature. Slides were examined under a light microscope (x1000) for dead (pinkish sperms stained with eosin) and live sperms (unstained colourless sperms) in a bluish purple background. Two hundred sperms were counted and live and dead sperms were expressed as percentages.

Head abnormalities

Modified William's stain was prepared by adding 10% carbol fuchsin solution in 10% ethyl alcohol (Analar, BDH), and 10% eosin-B (Gurr, BDH) solution in 10% ethyl alcohol at 1:1 ratio. Using a drop of fresh semen sample, a thin smear was made on a glass slide. The dried smear was immersed into 100% ethyl alcohol for 2 minutes, and then allowed to dry in air. The slide was placed in William's stain for 8-10 minutes, and then washed in distilled water and allowed to dry at room temperature. The smear was examined under a light microscope at x1000 magnification, and 200 sperms were counted for the estimation of head abnormalities, which was expressed as a percentage.

Acrosomal, mid piece and tail abnormalities

One to two drops of semen were added to one ml of formal saline (6.19g $Na_2HPO_4.2H_2O$; 2.54g KH_2PO_4; 5.41g NaCl; 125ml of 40% Formalin and 1000ml of distilled water) and a drop of this was placed on a glass slide and covered with a cover slip. The slide was examined under a phase contrast microscope at x1000 magnification. Two hundred sperms were counted and the different abnormalities were expressed as percentages.

Cells other than sperms

A drop of fresh semen was placed on a glass slide and a thick smear was made and allowed to dry. The smear was then immersed sequentially in 100%, 95%, 70% and 35% ethyl alcohol for 2 minutes at each concentration and was stained with Ehrlich's haematoxylin (Gurr,BDH) for 20 min. The stained slide was rinsed in distilled water and kept in distilled water for 30 min; and then counter stained with 1% Eosin Y (Gurr, BDH) in 90% ethyl alcohol for 2 min. Then, it was rinsed in distilled water and dehydrated, by dipping sequentially in 35%, 70%, 95% and 100% ethyl alcohol for 2 min in each concentration. The slide was dried and examined under a light microscope at x1000 magnification. The cells other than sperms were expressed as cells per microscopic field.

RESULTS AND DISCUSSION

The seminal characteristics of Nili-Ravi buffalo bulls are summarized in Table 1. The mean volume of ejaculate is in agreement with that reported by Kumar *et al.*, (1993) for buffalo bulls. However, this value is lower than those reported by Dixit *et al.*, (1984) for Murrah bulls. In comparison, the volume of the ejaculate of buffalo was lower than that of cattle which ranged from 1 to 15 ml (Galloway and Norman, 1980). In this study, the overall monthly mean volumes of semen of bulls showed three peaks namely, during December(3.62±3.20), June (3.43±2.06) and July (3.44 ±2.11). The monthly variation was not significant (P>0.05) and this finding is in agreement with the observations of Dixit *et al.*, (1984). However, Gill *et al.*, (1974) noted monthly variations in volume of semen.

Table 1. Mean (±SD) values and the ranges of the semen parameters of Nili-Ravi buffalo bulls.

Parameters	Mean	Range
Seminal volume (ml/ejaculate)	2.97 ± 1.96	0.5 - 9.0
Colour	-	Cloudy - Creamy
Density	-	2D - 4D
Mass activity	-	2+ - 4+
Sperm Concentration (million/ml semen)	1389.7 ± 567.5	440.0 - 2915.0
Sperm motility (%)	86.3 ± 5.33	70.0 - 90.0
Dead sperm (%)	8.2 ± 5.03	2.0 - 24.0

The concentration of Nili-Ravi semen in this study was 1389.71±567.50 million per ml semen which was lower than the values reported by Rajamahendran and Dharmasena (1984) for local, Surti and Murrah buffaloes, but greater than the values reported by Kumar *et al.*, (1993), Dixit *et al.*, (1984) and Zafar *et al.*, (1988) for Nili-Ravi and Murrah buffaloes. The monthly mean concentrations of sperms showed two peaks during April (1581.10±443.64) and July (1529.33±642.40), but the variations were not significant (P>0.05). The findings of Dixit *et al.*, (1984) indicating non existance of seasonal variation in the sperm concentration in Murrah bulls support the results of the present study. On the contrary, Zafar *et al.*, (1988),

and Gill *et al.*, (1974) reported monthly or seasonal variation in sperm concentration in the ejaculate of this species.

The motility of sperms ranged from 70 to 90% with a mean of 86.3%. Dixit *et al.*, (1984) reported a mean sperm motility of 74.6% with a range of 70 to 80% in Murrah bulls. In the same species, Kumar *et al.*, (1993) also reported 77.8% initial motility. In the present study, motility percentage was higher than that reported by the others. This could be due to the addition of extenders which resulted in a dilution effect on motility inhibiting factors present in the seminal plasma (Bass *et al.*, 1983). The monthly means of sperm motility of bulls were lower during December (81%) and March (78%) than during the other months (85 to 90%), and showed three peaks during January (88%), May (89%) and August (90%). The monthly variations observed in motility were significant ($P<0.05$). In buffaloes Gill *et al.*, (1974) reported a significant monthly variation in motility percentage. This supports the findings of the present study. However, in some other studies with buffaloes no significant seasonal variation in sperm motility was observed (Dixit *et al.*, 1984; Zafar *et al.*, 1988).

The colour and the density of the semen varied from cloudy to creamy and 2D to 4D, respectively. The mass activity ranged from 2+ to 4+. As these values are subjective, no attempt was made to compare with other reports. Mass activity of the sperms ranged from 2+ to 4+ and no monthly variations were to be seen. This was in agreement with the findings of Dixit *et al.*, (1984) and Zafar *et al.*, (1988). However Gill *et al.*, (1974) reported monthly variations in mass activity.

During the experimental period, the dead sperms ranged from 2 to 24% with a mean of 8.2±5.03%. Live sperms were impermeable to vital stains (eosin) and remained colourless, whereas the dead sperms were stained pink with eosin. Kumar *et al.*, (1993), and Rajamahendran and Dharmasena (1984) observed 14% and 17% dead sperms in the semen of Murrah and Surti buffalo bulls, respectively. Dixit *et al.*, (1984) observed 10% dead sperms in the same species which is in close agreement to the mean value obtained in the present study. Most of the studies reported a seasonal variation in the percentage of dead sperms (Rajamahendran and Dharmasena, 1984; Gill *et al.*, 1974), although no such variation was observed in the present study. The microscopic characteristics of buffalo spermatozoa are similar to that of cattle, except in the shape of the sperm head (Mukherjee, 1966), which is shorter and broader than that of cattle. The head morphology of cattle sperms were studied with modified William's stain and mid piece and tail with formal saline (Galloway and Norman, 1980). With William's

stained, the stained sperms gave a dark pinkish red colour and with formal saline they were colourless. The morphological abnormalities are summarized in Table 2.

Table 2. The sperm abnormalities found in the semen of Nili-Ravi bulls.

Abnormality	% Mean ± S.D
Head:	
Pear shaped	0.11 ± 0.16
Narrow at base	0.42 ± 0.49
Abnormal counter	0.22 ± 0.22
Undeveloped	0.67 ± 0.45
Narrow	0.25 ± 0.49
Loose	1.44 ± 0.54
Abaxial	0.11 ± 0.22
Overall	3.21 ± 1.83
Mid piece:	
Proximal cytoplasmic droplets	2.61 ± 0.60
Distal cytoplasmic droplets	0.25 ± 0.32
Abnormal mid piece	0.31 ± 0.30
Overall	3.16 ± 1.61
Tail:	
Single bent	3.58 ± 1.06
Double bent	0.75 ± 1.02
Coiled under head	2.22 ± 0.83
Others	0.44 ± 0.61
Overall	7.00 ± 4.07

In this study, the percentages (mean ± s.d) of head, mid-piece and tail abnormalities were 3.21±1.83, 3.16 ±1.61 and 7.00 ±4.07, respectively. The comparable values observed in Nili-Ravi bull semen by Ahamad *et al.*, (1987) were 6.5, 4.5 and 3.4 and that of Saeed *et al.*, (1989) were 7.82, 0.34

and 5.58, respectively. The values reported for Murrah bulls (Saxena and Vripathi, 1983) for head, mid piece and tail abnormalities were 3.06, 9.80 and 2.66, respectively. Although these reports reveal considerable variations in the percentage abnormalities in the head, mid piece and tail, the overall total abnormalities are closer. Rajamahendran *et al.*, (1984); Ahamad *et al.*, (1987); Gill *et al.*, (1974); Saxena and Vripathi, (1983) and Bhosrekar, (1981) reported monthly or seasonal variations in sperm abnormalities in the ejaculates of buffalo bulls. In the present study the head abnormalities ranged from 1.7% (August) to 6.3% (September) and appeared in three peaks during June (5.7%), September (6.3%) and November (4.3%). The percentage of mid piece abnormalities occurred in three peaks during March (4%), August (4%) and November (4%), and the tail abnormalities in four peaks during February (8%), June (9%), August (9%) and November (8%). The monthly variations observed in head, mid piece and tail abnormalities were not significant ($P>0.05$). However, much attention has paid so far to determine the upper and lower limits of various abnormalities to be used as criteria for semen evaluation.

Single and polynucleated spermatogonial lines and epithelial cells were also observed with H/E stain. These cells were found to be very few in number (1-2 cells per microscopic field), which is presumably a reflection of the healthy state of the reproductive system.

CONCLUSIONS

Though in this study the values obtained and the monthly variations noted for different seminal parameters were in agreement with the reports available in buffaloes, any relationship with the parameters and the climatological changes could not be established. Therefore, to acquire more information, further studies with (more animals) should be carried out for a longer duration.

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REFERENCES

Ahamad, M., latif, M. and Ahamad, M. (1987). Morphological abnormalities of spermatozoa of Nili-Ravi buffalo. Buffalo J. 2: 153-160.

Bass, J.A., Molan, P.C. and Shannon, P. (1983). Factors in seminal plasma of bulls that affect the viability and motility of spermatozoa. J. Reprod. Fert. 68: 275-280.

Bhosrekar, M. (1981). Studies on buffalo semen. Indian Vet. J. 58: 784-789.

Dixit, N.K., Agarwal, V.K. and Dwaraknath, P.K. (1984). Seasonal variation in serum levels of thyroid hormones and their relation with seminal quality and libido in buffalo bulls. Therioginol. 22: 497-507.

Galloway, D.B. and Norman, J.R. (1980). Laboratory examination of semen as a diagnostic aid. 9th Int. cong. on Artificial Insemination. Madrid, Spain, IV: 714-717.

Gill, R.S., Gangwar, P.C. and Takkar, O.P. (1974). Seminal attributes in buffalo bulls as affected by different seasons. Indian J. Anim. Sci., 44: 415-418.

Kumar, S., Sahni, K.L. and Bistha, G.S. (1993). Cytomorphological characteristics of motile and static semen of buffalo bulls. Buffalo J. 2: 117-127.

Mukherjee, D.P. (1966). Study of the quantitative characteristics of live and dead spermatozoa of bulls, buffalo bulls, rams and goats. World Rev. Anim. Reprod. 1: 74-82.

Rajamahendran, R. and Dharmasena, L. (1984). Preservation of buffalo semen in citric acid whey and tris buffer extender at -196°C. J. National Sci. Coun. Sri Lanka. 12: 45-51.

Saeed, A., Chaudhry, I.H., Khan, I.H. and Khan N.U. (1989). Studies on morphology of buffalo bull semen of different age groups. Buffalo J. 1: 99-102.

Saxena, V.B. and Vripathi, S.S. (1983). Sperm abnormalities in Murrah bulls as affected by different season. Indian J. Anim. Res. 17: 25-30.

Zafar, A.H., Ahamd, N. and Shah, S.K. (1988). Effects of seasonal variation on semen production of Nili-Ravi bulls. Buffalo J. 1: 61-68.