MORPHOLOGICAL STUDIES OF CRYOPRESERVED TODA BUFFALO SPERMATOZOA BY CASA

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ABSTRACT

Toda buffaloes are the endangered species of the Nilgiri Hills of South India. Successful attempts have been made in cryopreservation of semen of Toda bulls. Thawed semen samples from Toda buffalo bulls were used to analyze the spermatozoal morphometry by Computer assisted semen analyzer (CASA). Head length (major axis), width (minor axis), area, perimeter, elongation and tail length were the morphometrical characteristics measured. The mean (±SE) length, width, area, perimeter, elongation percentage and tail length were 7.38±0.02 µm, 4.68±0.02 µm, 28.53±0.17 µm^2, 20.32±0.19 µm, 62.95±0.22% and 52.93±0.41 µm respectively. Overall spermatozoal abnormality was 30.75% and coiled tail was the most common (50.49%) abnormality. The spermatozoal morphometry reported in this study may form a preliminary data in Toda buffalo semen analysis and helps for comparison with other breeds and can complement sperm motility assessment.

Keywords: buffaloes, *Bubalus bubalis*, Toda buffalo, cryopreserved semen, morphometry, CASA

INTRODUCTION

Toda buffaloes are semi wild and endangered group of buffaloes traditionally maintained by the Toda tribes of Nilgiris district of South India. Toda buffaloes are closely associated with the Toda tribal population economically and socially. Usually the herd consists of a few females with rarely one or two males. Toda bulls are known to stay in dense forests and will come out during the breeding season. In most of the herds no calvings have been reported for the past 5 to 6 years due to paucity of bulls. The breedable female population has also decreased over time. A project was carried out with the aim of collection and cryopreservation of Toda semen. Semen was successfully collected using buffaloes in estrum by intervening during natural service by AV method and cryopreserved.

Motility of the sperm (qualitative trait) and the concentration (quantitative trait) of the spermatozoa are the most commonly evaluated sperm parameters, which provide limited information regarding the potential fertility of sires (Rodriquez-Martinez and Larsson, 1998 and Brahmkshtri *et al*., 1999). Assessment of spermatozoal morphology reflects the testicular,

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epididymal and accessory glands physiology and pathology and also the functioning and handling of semen during cryopreservation (Rodriquez-Martinez, 2003). In addition the sperm head morphology has been suggested as an indicator of fertility (Ombelet *et al*., 1995; Casey *et al*., 1997 and Hirai *et al*., 2001). However, it is not used in regular assessment of semen.

Computer assisted semen analysis has been used for analysis of spermatozoa morphology in several species (Gravance *et al*., 1996; Sundararaman and Edwin, 2004; Sundararaman, *et al*., 2006; Aggarwal *et al*., 2007; Sundararaman, *et al*., 2007; Roy *et al*., 2008) including buffalo, which gives a clear picture about the shape and size of the spermatozoa. Recently Aggarwal *et al*. (2007) have studied the sperm biometry of eight breeds (Murrah, Surti, Tarai, Mehsana, Jafferbadi, Bhadawari, Pandharpuri and Nili Ravi) of Indian buffaloes. The aim of the present study was to characterize the sperm morphology using CASA and to assess the morphological abnormality in semi wild Toda buffaloes.

MATERIALS AND METHODS

Three Toda bulls were raised under organized farm conditions from a young age of 10 to 12 months to about 4 to 5 years at Sheep Breeding Research Station, Sandynallah, The Nilgiris district, Tamil Nadu, India. Semen collected using artificial vagina (AV) was diluted in Tris based diluent and cryopreserved in 0.25 ml straws using French straw technique.

Morphological characteristics of spermatozoa assessed by CASA

The frozen semen samples were transferred to the Semen Bank of the Department of Animal Genetics and Breeding, Madras Veterinary College, Chennai-7 for analysis with computer assisted semen analyzer. The samples were thawed at 37^{o}C for 30 seconds and further extended to minimize the sperm concentration for analysis by CASA.

Staining of semen slides

Semen slide was prepared by placing 4 µl of frozen thawed and diluted semen sample on a grease free glass slide. The slide was air dried and stained, using STAT III andrology stain (Mid-Atlantic Diagnostics Inc., NJ). Fixation of dried slide was done by immersing in methanol for 30 seconds. After air-drying, the slide was dipped in Thiazine dye mixture for 60 seconds. The excess stain on edges was blotted during staining operation and finally the slide was washed with distilled water and air dried. Several such slides were prepared from each sample.

CASA analysis

Hamilton Thorne Integrated Visual Optical System (HT-IVOS) version 10.9 was the computer assisted semen analyzer (CASA) used for morphology evaluation. The semen slide was loaded in the CASA system. Using 60 x objective, the sperm morphometrics were measured. From each slide, only properly digitized sperm heads were considered for morphometric measurement. A total of 992 properly digitized spermatozoa were analyzed. By using the metric software option of CASA, the morphological classification of sperm was made as normal / abnormal based on the morphometric traits of head and tail. Length (Major axis) and width (Minor axis) of the spermatozoal head, head area, perimeter, elongation and tail length were the morphometric traits studied in the present investigation. The spermatozoal tail

abnormality and other abnormalities of head were noted and analyzed.

Statistical analysis

The mean and standard error for all variables were calculated and presented. Differences between the bulls and ejaculates were tested by least squares procedure (Harvey, 1990). All possible interactions with a set of fixed effects were fitted initially and insignificant interaction effects were omitted. The linear statistical model was used for analysis of various traits. The differences between the least squares means for subclasses under a particular effect were tested by Duncan's multiple range test modified by Kramer (1957).

RESULTS AND DISCUSSION

For the first time, semen collection was achieved from the wild and endangered Toda buffalo bulls for cryopreservation and to use it as a method of conservation of the breed, which facilitated this first ever report on morphology of Toda spermatozoa. The average length (major axis) and width (minor axis) of the spermatozoa were 7.38±0.02 µm and 4.68±0.02 µm (Table 1) and were similar to the reports by Kodagali *et al*. (1973) in Surti buffaloes (7.62±0.22 and 4.59±0.51 µm), Harapanhalli and Mukherjee (1973) in Murrah buffaloes (7.32 and 4.90 µm) and Roy et al. (2008) in Murrah (7.59±0.01 and 4.91±0.01 µm) buffaloes. However, the spermatozoal head length was higher in all the eight Indian breeds of buffaloes (Aggarwal *et al*., 2007). The width of the sperm head was narrower in Toda bulls than in other breeds as reported earlier (Aggarwal *et al*., 2007) except Murrah buffaloes (4.75 µm).

The area of the spermatozoal head of Toda buffaloes was similar to those reported by Kodagali *et al*. (27.21±2.78 μm^2 : 1973) and Harapanhalli and Mukherjee (28.96 μm^2 : 1973). Aggarwal *et al*. (2007) observed a larger area in eight Indian breeds of buffaloes and Roy *et al*. (24.41±0.05 μm^2: 2008) observed smaller head area than the present study.

Significant difference ($P<0.01$) among the bulls were observed in all the parameters studied. Bull TM3 has longer major axis, larger area and higher perimeter, whereas, bull TM2 has broader minor axis and higher elongation percentage. For bull TM8, the mean values for all the parameters were the lowest except for elongation.

Spermatozoa with normal morphology were significantly longer (major axis) (7.42±0.02 vs. 7.35±0.03). However, elongation percentage was higher in spermatozoa with abnormal morphology (62.30±0.23 vs. 63.60±0.28).

The perimeter observed in the present study was equivalent to those observed by Roy *et al*. (19.65±0.02 µm; 2008) for Murrah buffaloes. The values were lower than the values observed by Aggarwal *et al*. (2007) for the eight Indian breeds of buffaloes.

The tail length (52.93±0.41 µm) observed in this study was similar to the tail length in Pandarpuri buffaloes (Aggarwal *et al*., 2007). The length of the tail was less than those studied by Kodagali *et al*. (1973) in Surti buffaloes, Aggarwal *et al*. (2007) for other seven Indian breeds of buffaloes Roy *et al*. (2008) for Murrah buffaloes.

The elongation percentage of the head of Toda buffalo spermatozoa was 62.95±0.22 percent. The length was comparatively lower than all the breeds of buffaloes studied and width was similar to Murrah buffaloes giving the sperm an oblong shape.

Table 1. Morphometry of the Toda buffalo spermatozoa.

Effect	N	Morphometrical parameters of the head of sperm					Tail length (µm)
		Major axis (µm)	Minor axis (µm)	Elongation (%)	Area (μm^2)	Perimeter (µm)	
2	521	7.29±0.02[a]	4.76±0.15[b]	64.67±0.22[b]	28.70±0.14[b]	20.33±0.08[b]	54.43±0.37 (60)
3	64	7.65±0.05[b]	4.64±0.04[a]	60.17±0.52[a]	29.33±0.40[b]	20.65±0.25[b]	52.04±0.81 (11)
8	407	7.20±0.02[a]	4.64±0.04[a]	64.01±0.25[b]	27.57±0.19[a]	19.98±0.11[a]	52.31±0.43 (88)
I	431	7.38±0.03	4.69±0.02	62.93±0.27	28.55±0.20	20.29±0.12	53.20±0.39 (77)
II	446	7.39±0.02	4.67±0.02	62.95±0.22	28.66±0.17	20.34±0.10	53.68±0.34 (75)
III	115	7.38±0.04	4.68±0.04	62.97±0.42	28.39±0.32	20.32±0.19	51.90±0.45 (7)
Normal	687	7.42±0.02[b]	4.66±0.02	62.30±0.23[a]	28.48±0.18	20.26±0.10	52.68±0.41 (125)
Abnormal	305	7.35±0.03[a]	4.70±0.02	63.60±0.28[b]	28.59±0.21	20.38±0.13	53.18±0.52 (34)
Overall	992	7.38±0.02	4.68±0.02	62.95±0.22	28.53±0.17	20.32±0.19	52.93±0.41 (159)

N : Number of spermatozoa

Means bearing different superscripts within each set of columns (effect) differ significantly (P≤0.01X

Figures in parenthesis indicate number of observations.

Table 2. Spermatozoal abnormalities in cryopreserved Toda buffalo semen.

Effects	Total Abnormalities (%)	Abnormalities of the tail (%)			Other defects (%)
		Bent tail	Coiled tail	Absent tail	
Bulls					
2	39.92[b] (208/521)	4.41 (23/521)	20.73 (108/521)	4.03 (21/521)	10.75 (56/521)
3	21.88[a] (14/64)	0.00 (0/64)	15.63 (10/64)	3.13 (2/64)	3.13 (2/64)
8	20.39[a] (83/407)	4.18 (17/407)	8.85 (36/407)	1.72 (7/407)	5.65 (23/407)
Ejaculate number					
I	25.75[a] (111/431)	3.71 (16/431)	8.35 (36/431)	3.94 (17/431)	9.745 (42/431)
II	28.92[a] (129/446)	3.36 (15/446)	16.82 (75/446)	2.24 (10/446)	6.50 (29/446)
III	56.52[b] (65/115)	7.83 (9/115)	37.39 (43/115)	2.61 (3/115)	8.70 (10/115)
Overall	30.75 (305/992)	4.03[a] (40/992)	15.52[c] (154/992)	3.02[a] (30/992)	8.17[b] (81/992)

Figures in parenthesis indicate number of observations.

Means bearing different superscripts within each set of columns (effect) differ significantly (P≤0.01).

Overall spermatozoal abnormality percentage in the Toda buffalo bulls was 30.75 percent (Table 2). Bull TM2 had significantly higher percentage of abnormality (39.92 percent) than the other two bulls (21.88 and 20.39 percent in TM-3 and TM-8 respectively). Similarly semen obtained by third ejaculate has more abnormal spermatozoa (56.62%) than the first (25.75%) and second (28.92) ejaculates.

Coiled tail was the most (15.52%) common abnormality observed. Bent tail and tail absent were the least to occur. The coiled tail defect was predominant in all bulls and ejaculates with bull TM2 having a higher coiled tail defect than other (20.73%) bulls. Several studies (Nordin *et al*.,1990 and Koonjaenak *et al*., 2007) has shown that spermatozoal abnormality in buffaloes used for AI should not exceed 15 percent and a healthy buffalo should not have >10% of tail defects. In the present study the spermatozoal abnormalities were higher.

Semen was collected from the wild Toda buffalo bulls during the embryo collection programme when all the bulls were allowed for natural breeding several times in that week. In addition semen was collected more than twice in a day when she buffaloes in estrum were available as dummy. Attempts have been made to collect and cryopreserve as much semen samples as possible for future use. More frequent collection of semen samples and allowing the bulls for natural service in between collections may be the reason for more tail defects in these samples. Bull TM-2 gave watery semen, the percentage of motility and concentration were lower in this bull and has also contributed for more abnormalities. More than 50% of the abnormality was contributed by coiled tail.

The spermatozoal morphometry reported in this study may form a preliminary data in Toda buffalo semen analysis and helps for comparison with other breeds. Similarly it can complement sperm motility assessment. Correlation between fertility and sperm morphology has been observed in human (Ombelet *et al*., 1995), stallion (Casey *et al*., 1997), boars (Hirai *et al*., 2001) and bulls (Januskauskas *et al*., 1995). In addition, the head area and shape affect the sperm freezability and cryoresistance (Estero *et al*., 2006). Thus the sperm morphometry may form the basis for formulating the fertility index in these bulls.

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REFERENCES

Aggarwal, R.A., S.P. Ahlawat, Y. Kumar, P.S. Panwar, K. Singh and M. Bhargava. 2007. Biometry of frozen-thawed sperm from eight breeds of Indian buffaloes *(Bubalus bubalis*). *Theriogenology*, **68**(4): 682-686.

Brahmkshtri, B.P., M.J. Edwin, M.C. John, A.M. Nainar and A.R. Krishnan. 1999. Relative efficacy of conventional sperm parameters

and sperm penetration bioassay to assess bull fertility in vitro. *Anim. Repro. Sci*., **54**: 159-168.

Casey, P.J., C.G. Gravance, R.O. Davis, D.D. Chabot and I.K.M. Liu. 1997. Morphometric differences in sperm head dimensions of fertile and sub-fertile stallions. *Theriogenology*, **47**: 575-582.

Esteso, M.C., A.J. Soler, M.R. Fernandez-Santos, A.A. Quintero-Moreno and J.J. Gardle. 2006. Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian Red Deer (*Cervus elaphus hispanicus*) epididymal sperm samples. *J. Androl*., **27**: 662-670.

Gravance, C.G., I.K.M. Liu, R.O. Davis, J.P. Hughes and P.J. Casey. 1996. Quantification of normal head morphometry of stallion spermatozoa. *J. Reprod. Fertil.,* **108**: 41-46.

Harvey, W.R., 1990. *Mixed Model Least-squares and Maximum Likelihood Computer Programme. PC-2 version*. Ohio State University, Columbus.

Harapanhalli, M.D. and D.P. Mukherjee. 1973. Effect of different diluents on the cytomorphology of the buffalo spermatozoa. *Indian J. Anim. Prod*., **4**: 128-135.

Hirai, M., A. Boersma, A. Hoeflich, E. Wolf, J. Foll and R. Aumuller. 2001. Objectively measured sperm motility and sperm head morphometry in boars (*Sus scrofa*): relation to fertility and seminal plasma growth factors. *J. Androl.,* **22**: 104-110.

Januskauskas, A., J. Gil, H. Rodriguez-Martinez, L. Soderquist and N. Lundeheim. 1995. Effects of a brief elevation of scrotal temperature on the post thaw viability of bull semen. *Reprod. Domest. Anim.*, **30**: 271-277.

Koonjaenak, S., V. Chanatinart, H. Ekwall and H. Rodriguez-Martinez. 2007. Morphological features of spermatozoa of swamp buffalo AI bulls in Thailand. *J. Vet. Med. A.*, **54**: 169-178.

Kramer, C.Y. 1957. Extension of multiple range tests to group correlated adjusted means. *Biometrics*, **13**: 13-18.

Kodagali, S.B., B.K. Bhavsar and A.D. Deshpande. 1973. Biometrics of Surti buffalo spermatozoa. *Indian Vet. J.*, **50**: 50-54.

Nordin, W., M. Hilmi and T.A. Bongso. 1990. Semen characteristics related to age in growing swamp buffalo (*Bubalus bubalis*). *Buffalo Journal*, **2**: 161-166.

Ombelet, W., R. Menkveld, T.F. Kruger and O. Steeno. 1995 Sperm morphology assessment: historical review in relation to fertility. *Hum. Reprod. Update*, **1**(6): 543-557.

Ostermeier, G.G., G.A. Sargeant, B.S. Yandell, D.P. Evenson and J.J. Parrish. 2001. Relationship of bull fertility to sperm nuclear shape. *J. Androl.*, **22**: 595-603.

Rodriquez-Martinez, H. 2003. Laboratory semen assessment and prediction of fertility: still utopia?. *Reprod. Domest. Anim.*, **38**: 312-318.

Rodriquez-Martinez, H. and B. Larsson. 1998. Assessment of sperm fertilizing ability in farm animals. *Acta. Agric. Scand. Suppl.*, **29**: 12-18.

Roy, B., P.K. Nagpaul, P.K. Pankaj, T.K. Mohanty, V.S. Raina and A. Mishra. 2008. Studies on biometry of sperm of Murrah buffalo bulls (*Bubalus bubalis*). *Buffalo Bull*., **27**: 161-164.

Sundararaman, M.N. and M.J. Edwin. 2004. Morphometric evaluation of caprine spermatozoa by HT-IVOS system at

pre-freeze and post-thaw stages of cryopreservation. *Indian J. Anim. Reprod.*, **25**: 91-93.

Sundararaman, M.N., K.S. Subramanian, A. Meenakshisundaram and M.J. Edwin. 2006. Computer automated analysis of morphometric characteristics of epididymal spermatozoa of spotted deer (*Axis axis*). *Indian J. Anim. Reprod.*, **27**: 77-79.

Sundararaman, M.N., J. Kalatharan and K. Thilak Pon Jawahar. 2007. Analyses of morphological and morphometrical deviations of bull spermatozoa by computer assisted semen analysis technique. *Asian J. Anim. Vet. Adv.*, **2**: 196-204.