Cryopreservation and Quality Assessment of Buffalo Bull Semen Collected from Farmer's Doorstep

Pawan Singh · D. Kumar · Pradeep Kumar ·
Inderjeet Singh · P. S. Yadav

Received: 2 November 2012 / Accepted: 26 March 2013 / Published online: 1 May 2013
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Abstract The present study was undertaken with the objective to evaluate the effect of transportation distance on freezability of buffalo semen collected at farmer's doorstep. In the study elite Murrah buffalo bulls of 3–6 years of age available with the farmers in the breeding home tract were used. Semen samples were divided into two groups based on distance of transportation viz. group I (80–100 km) and group II (130–150 km). Semen was collected at the owner's premises and kept in warm water at 30 ± 2 °C and extended 10–15 times based on its consistency and color. The extended semen was transported to laboratory in indigenously designed container. In the laboratory, after subjective assessment, the semen was cryopreserved successfully. The frozen–thawed semen was assessed for quality parameters using computer assisted semen analyzer and artificial insemination (AI) in field conditions. Post-thaw sperm total motility (51.06 ± 2.59 vs. 45.36 ± 2.43 %) and progressive motility (31.8 ± 1.75 vs. 26.5 ± 1.55 %) did not vary significantly between groups, whereas rapid motility and sperm kinetic parameters like average path velocity, straight linear velocity, curvilinear velocity, beat cross frequency, and straightness were significantly ($P < 0.05$) higher in group I than the group II. Parameters, such as amplitude of lateral head displacement, linearity, elongation, and head size of spermatozoa did not vary significantly between the two transportation distances. The conception rates, of the frozen semen used for AI at field level, were found to be 53.8 and 60.2 % in group I and group II, respectively. In conclusion, the proposed design of container for cooling and transportation of diluted semen, up to 150 km distance proved to be highly efficient for cryopreservation of semen of buffalo bulls from field conditions.

Keywords Buffalo semen · Sperm motility · Biodiversity conservation · AI · CASA

Introduction

Buffalo is the main milk producing animal in our country, as it contributes more than 55 % of the total milk produced in India. Therefore it plays a pivotal role in livestock sector of this country. Today, India is the only nation having substantial and varied biodiversity of buffalo germplasm, which includes about a dozen well defined breeds and a vast population of non-descript and low producer buffaloes. Among

the well established breeds of buffalo, Murrah is the breed of choice for improvement of non-descript and low producing buffaloes. Haryana is the home tract of this important breed, but due to its higher milk production this breed has been taken to other parts of the country for genetic improvement of their native breeds. Hence, a heavy migration of elite buffaloes from their home tract has taken place. As a result, the gene-pool of elite Murrah germplasm is depleting day by day [7, 8] and the numbers of elite animals are reducing very fast. To improve the low producing buffaloes and continue with the genetic improvement program in the country, there is urgent need to have quality frozen semen from genetically superior Murrah bulls from its home tract. Some of the most elite bulls are owned by the well-to-do farmers, NGOs, Gaushalas, etc. Therefore, involvement of such genetically

P. Singh · D. Kumar (✉) · P. Kumar · I. Singh · P. S. Yadav
Buffalo Physiology and Reproduction Division, Central Institute
for Research on Buffaloes, Sirsa Road, Hisar 125001, Haryana,
India
e-mail: vetdmkl@yahoo.co.in

superior elite Murrah germplasm available with the farmers needs to be more extensively utilized for animal improvement programs. Cryopreservation of semen from such bulls and its dissemination for improvement of the productivity of the buffalo is very much desired. There are no reports on collection of semen at farmer's doorstep and its transportation to the laboratory for cryopreservation. Therefore, this study was proposed with the following objectives: to evaluate the effect of transportation distance on freezability of semen collected at farmer's doorstep and evaluation of the transported and frozen-thawed semen quality parameters based on distance using computer assisted semen analyzer (CASA) and fertility through performing AIs at field level.

Materials and Methods

Semen Collection and Transportation

Six elite Murrah buffalo bulls of 3–6 years of age were selected from the breeding tract of Murrah during exhibition, buffalo fair, camps, or through farmers contact owning champion bulls for semen collection. These bulls were located at distance ranging from 80 to 150 km, from the Semen Freezing Laboratory of Central Institute for Research on Buffalo, Hisar (India) located at 29.10°N latitude and 75.46°E longitudes. Three ejaculates from each bull were collected with artificial vagina technique at the owner's premises. Immediately after collection, each ejaculate was kept in warm water at $30 \pm 2^\circ\text{C}$ and extended 10–15 times with Tris-egg yolk-glycerol diluent, based on subjective assessment of its consistency and color. The diluent contained Tris (3.02 % w/v), citric acid (1.67 % w/v), fructose (1 % w/v), egg yolk (20 % v/v), glycerol (6.4 % v/v), penicillin (500 IU/ml), and streptomycin (500 µg/ml). The extended semen was packed in sterile capped bottles and placed in an insulated box containing spongy foam having place to fix the filled bottle and then warm ($30 \pm 2^\circ\text{C}$) water was added to wet it and then the ice pack were kept surrounding the foam, which helped in reduction of temperature slowly during road transportation (Fig. 1). This time (~1–3 h), from collection of last ejaculate to transportation to the laboratory, was used as part of the total equilibration period of 3 h, including left over equilibration time at 5°C , if any, in an IMV cold cabinet in the laboratory. In the laboratory, subjective sperm motility was assessed at five different fields by same persons throughout the study. For studying the effect of distance of transportation during the equilibration period on quality and fertility of frozen semen, the samples were divided into 2 groups on the basis of distance of transportation viz. group I- 80–100 km and group II- 130–150 km.

Laboratory Processing and Freezing

On arrival of semen sample in the laboratory and completion of equilibration, pre-freezing sperm motility was assessed under a warm-stage phase contrast microscope as explained above. The extended and equilibrated semen was filled and sealed with straw filling and sealing machine into 0.25 ml plastic straws (IMV, L'Aigle, France) and frozen with a programmable bio-freezer (Mini Digi-cool, IMV Technologies, L'Aigle, France) for cooling down from 5°C to -140°C . Each semen sample was initially cooled at $-5^\circ\text{C}/\text{min}$ from 5°C to -10°C , from -10°C to -100°C at a freezing rate $-40^\circ\text{C}/\text{min}$, and then from -100°C to -140°C at $-20^\circ\text{C}/\text{min}$. From -140°C , semen straws were then plunged into liquid nitrogen (at -196°C) for storage.

Post-thaw Analysis

After 24 h of cryopreservation, three semen straws for each bull were thawed at 38°C for 40 s. Immediately after thawing, sperm motility characteristics and viability were analyzed by CASA (IVOS 12.1, Hamilton-Thorne Biosciences, Beverly, MA, USA). Details of CASA settings of software for assessing sperm motility parameters are summarized in Table 1. A 50-µl aliquot of the frozen-thawed semen was diluted with 100 µl pre-warmed Tris buffer to give a sperm concentration of about 26×10^6 spermatozoa/ml. Then a 3-µl aliquot was loaded in a chamber of a pre-warmed (38°C) Leja® four chamber slide (depth 20 µm; Leja, Nieuw-Vennep, The Netherlands) and analyzed. For each sample, five optical fields around the central reticulum of the chamber were read. The motion characteristics recorded were total motility (%), progressive motility (%), rapid motility (%), straight linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), lateral head displacement (ALH, µm/s), beat cross frequency (BCF) (Hz), straightness (STR) (%), linearity (LIN) (%), elongation (%), and head size (µm sq) of the spermatozoa. The percentage of sperm motility after freezing was also estimated by microscopic examination. Sperm abnormalities in frozen-thawed semen were microscopically assessed with Eosin-Nigrosin staining method. The mean of values from three samples per bull were recorded for comparative analysis.

Sperm Viability Assessment

The Viadent option of CASA was used to determine sperm viability, using bis-benzimide trihydrochloride (Hoechst 33258, 5 µg/ml) dye that stains only the sperm cells with damaged membrane. Semen sample was diluted properly at

Fig. 1 Indigenously designed semen carriage system for transportation of semen collected from farmer's doorsteps

Table 1 Set-up of CASA (Hamilton-Thorne IVOS 12.1) for motility assessment of frozen–thawed buffalo sperm

Parameters	Set value
Frame rate (Hz)	60
Frames acquired	30
Minimum contrast	35
Minimum cell size (pixels)	5
Cell size (pixels)	9
Cell intensity (pixels)	110
Path velocity (VAP) ($\mu\text{m/s}$)	50
Straightness (STR) (%)	70
VAP cut-off (μs)	30
VSL cut-off (μs)	15

a concentration of 20–60 Million sperms/ml, then add 500 μl of diluted semen sample to 500 μl of stain solution to create the final stain concentration of 5 $\mu\text{g/ml}$. After that, the stained sample was incubated at 37 °C for 2 min and then analyzed with blue light and Viadent filter block under CASA. Damaged membrane allows the stain to penetrate and stain DNA of the sperm cells. Stained sperm cells emit fluorescent light, which is recorded by camera and the viable sperm count is obtained.

Sperm Morphology

The sperm morphology was evaluated by Eosin–Nigrosin staining method. After washing sperm samples in PBS at 37 °C, one drop of the suspension containing $2\text{--}3 \times 10^5$ sperm/ml was placed in one corner of a pre-warmed glass-slide. Then a thin film smear was prepared using a spreader slide at 30° angle to disperse the semen suspension over the slide's length and fixed with air dry. The smear was stained with Eosin–Nigrosin solution (1.67 g Eosin, 10 g Nigrosin dissolved in 100 ml distilled water) for 3–5 min, excess stain was removed by rinsing the slide in distilled water, dried, and evaluated under a phase contrast microscope at

1,000 $\times$ magnification. Minimum 200 spermatozoa were analyzed for each sample.

Field Conception Rate

Frozen semen straws were distributed to five field inseminators in different districts of Haryana state for undertaking inseminations in farmers' buffaloes. A total of 384 inseminations were carried out (ranging from 22 to 171 per bull) and information on day 60–90 non-return rates (NRR) as indication of conception, was collected from farmers.

Statistical Analysis

A statistical analysis was performed using statistical analysis system (SAS) 9.2 for window (SAS Institute Inc. Cary, NC, USA) for obtaining mean $\pm$ standard error (SE). Differences in sperm motion and kinetic values due to distance of transportation during equilibration were tested with one way ANOVA test followed by Tukey's Studentized range test whenever significant differences were observed ($P < 0.05$).

Results

The means of pre-freeze motility, post-thaw motility, and abnormal spermatozoa of different bulls, divided into two groups as per the distance from the laboratory, assessed by light microscope, are presented in Table 2. The overall percentage of pre-freeze sperm motility was 72.56 ± 2.09 and 65.10 ± 1.91 , where as post-thaw it was 54.3 ± 1.46 and 50.33 ± 1.20 in group I and II, respectively. This indicates that $\sim 15\text{--}18\%$ sperms became immotile during freezing. The post-freezing percentage of abnormal spermatozoa was 12.3 ± 2.2 and 18.40 ± 2.2 in group I and II, respectively. The same semen samples post-thaw were also analyzed with the CASA. The results (Table 3) indicate

that post-thaw total sperm motility and progressive motility did not vary significantly between groups, whereas rapid motility and sperm kinetic parameters like VAP, VSL, VCL, BCF, and STR were significantly ($P < 0.05$) higher in group I than the group II (Table 3). Parameters, such as ALH, LIN, elongation, and head size of spermatozoa did not vary significantly with the transportation distance. These values are almost similar to the values obtained for semen samples collected from bulls at the farm processed immediately under lab conditions (unpublished data). Although very few information are available related to the freezing of semen from field bulls and the information on conception rates using field bulls frozen semen for AI. The percentage of live spermatozoa, evaluated by CASA, did not vary with distance. The conception rates of the frozen semen used for AI at field level were found to be 53.8 and 60.2 % in group I and group II, respectively (Table 4).

Discussion

The reduction in the animal populations causes a decrease in genetic variability. In this context, cryopreservation of sperm is an essential tool in the conservation of gametes of indigenous and/or endangered wild mammals and is suitable for genetic management. However, semen cryopreservation cannot be done at the site of collection located distantly from the semen freezing laboratory. Therefore, it is required to bring the collected semen to the laboratory. There is no such kind of strategy currently available that is specifically designed for cooling and transport of freshly collected buffalo semen. In the present study, we present an indigenous design of the container which can be easily assembled and successfully bring the collected semen to the semen freezing laboratory, without any reduction in quality. The wetted spongy foam surrounding the capped semen bottle successfully prevented cold shock by direct contact of ice pack and also helped in bringing the temperature slowly down from 30 to 5 °C during transportation and served for equilibration of semen. Care was taken to

ensure that all bottles/tubes were completely filled with semen to minimize agitation during road transportation. The technique proved to be inexpensive, easy to use and there was no requirement of power for cooling. Thus, this design will be useful for the people working in remote area. The container can be assembled easily in the field and can carry bulk semen samples to the laboratory successfully. The quality of the semen as assessed by CASA was of superior quality and at par with the value of semen collected and frozen at our semen freezing laboratory.

To see the efficacy of container for cooling and distance of transportation on semen quality, objective assessment of sperm motility after cryopreservation was made with the help of CASA. To the best of our knowledge, this is the first report on the collection and cryopreservation of the Murrah semen collected from farmers' doorsteps and frozen semen for use for AI at the field level and presents a novel approach for collection of rare elite germplasm from field. In the present study, no significant effect of post-collection equilibration (2–4 h) during transportation on total motility and progressive motility of frozen–thawed semen was obtained. Earlier reports also found that 2–4 h equilibration improved the total and progressive motility and fertility of frozen–thawed semen in bubaline [2, 3]. Based on the results of slow cooling and equilibration time at 5 °C and its relationship with fertility, several authors have recommended an equilibration of 4–18 h at 5 °C before freezing [4, 6]. Although equilibration time during transportation significantly affected sperm kinetics of VAP, VSL, VCL, BCF, and STR in the present study, which is not in agreement of previous study on effects of extender and equilibration time on post-thaw motility and membrane integrity of cryopreserved Gyr bull semen evaluated by CASA and flow cytometry [5]. Semen collected and frozen from a lesser distance of transportation had greater BCF values, suggesting that less damages of flagellar structures which stimulated ATP production and consequently beat frequency, as previously suggested by Celeghini et al. [1]. A greater value of STR indicates that the trajectory of sperm is more uniform and with low amplitude, was supported by

Table 2 Mean (mean $\pm$ SE) values of semen parameters evaluated by microscope

Group	Bull.	Pre-freezing sperm motility (%)	Post-thawed sperm motility (%)	Abnormal spermatozoa (%)
I	1	77.5 $\pm$ 2.5	54.3 $\pm$ 1.34	12.2 $\pm$ 2.1
	2	67.4 $\pm$ 1.93	52.5 $\pm$ 1.30	14.6 $\pm$ 2.6
	3	72.8 $\pm$ 1.84	56.1 $\pm$ 1.74	10.2 $\pm$ 1.9
	Overall	72.56 $\pm$ 2.09	54.3 $\pm$ 1.46	12.33 $\pm$ 2.20
II	4	66.87 $\pm$ 2.09	51.0 $\pm$ 2.10	22.5 $\pm$ 2.3
	5	66.25 $\pm$ 1.82	49.0 $\pm$ 0.42	16.5 $\pm$ 3.1
	6	62.3 $\pm$ 1.82	51.0 $\pm$ 1.08	16.2 $\pm$ 1.3
	Overall	65.10 $\pm$ 1.91	50.33 $\pm$ 1.20	18.40 $\pm$ 2.23

Table 3 Mean ($\pm$ SE) values of motility and kinetic parameters of frozen–thawed spermatozoa analyzed with CASA

Parameters	Group I	Group II
Total motility (%)	51.06 $\pm$ 2.59	45.36 $\pm$ 2.43
Progressive motility (%)	31.80 $\pm$ 1.75	26.50 $\pm$ 1.55
Rapid motility (%)	34.40 $\pm$ 2.30 ^a	27.04 $\pm$ 2.08 ^b
VAP (μ m/s)	82.29 $\pm$ 1.65 ^a	74.41 $\pm$ 1.42 ^b
VSL(μ m/s)	68.29 $\pm$ 1.48 ^a	58.95 $\pm$ 1.02 ^b
VCL(μ m/s)	141.78 $\pm$ 2.66 ^a	129.06 $\pm$ 5.38 ^b
ALH(μ m)	6.05 $\pm$ 0.10	5.79 $\pm$ 0.18
BCF (Hz)	33.95 $\pm$ 0.58 ^a	32.10 $\pm$ 0.69 ^b
STR (%)	82.06 $\pm$ 0.56 ^a	79.40 $\pm$ 1.20 ^b
LIN (%)	49.68 $\pm$ 0.55	49.23 $\pm$ 1.85
Elongation (%)	53.43 $\pm$ 1.06	53.13 $\pm$ 0.90
Size (μ m sq)	8.16 $\pm$ 0.79	6.92 $\pm$ 0.40
Viability (%)	63.70 $\pm$ 2.94	61.76 $\pm$ 9.99

Values with different superscript within row differ significantly ($P < 0.05$)

Table 4 Conception rates of buffalo cryopreserved semen collected from different distances of farmer's doorsteps

Group	Group wise Bull #	No. of buffaloes inseminated	No. of buffaloes not returning to estrus by D 90	NRR (%)	NRR (%) for the group
I	1	171	92	53.8	53.8 (162/301)
	2	99	53	53.5	
	3	31	17	54.8	
II	4	22	11	50.0	60.2 (50/83)
	5	26	16	61.5	
	6	35	23	65.7	
Overall		384	212	55.21	55.2 (212/384)

earlier study [5]. The success of this technique is evident by the fact that the overall field conception rate was observed to be 55.2 % which is almost equal to fertility rate (57.9 % on 90-d NRR) observed in cattle [2, 3], however, it was slightly lower than that observed by Singleton [9].

In conclusion, the proposed design of container for cooling and transportation of diluted semen, up to 150 km

distance, was suitable for cryopreservation of semen of buffalo bulls. Although we could successfully cryopreserve the semen, with acceptable fertility in the field, collected 150 km away from the laboratory, but the affect of transportation from a distance more than that needs to be studied on quality, freezability, and fertility of buffalo and other species semen. This study has also paved the path of exploring the possibilities of conservation of semen of domestic as well as wild animals which are at the verge/ risk of extinction in future. This technique may prove to be helpful in faster multiplication of their population through AI for maintenance of genetic diversity in the nature.

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