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Preservation of Buffalo Semen in Citric Acid Whey and Tris Buffer Extenders at -196° C

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1. Introduction

Our present population of local buffaloes is estimated to be around 0.4 million. In order to meet our draught-power, milk and meat requirements the local buffalo population must be multiplied and upgraded to Indian River Buffalo standards. With this objective in mind, in 1967, large scale importation of Murrah and Surti buffaloes were made from India and are maintained as a closed herd in state farms. Bull calves born in these farms are being issued to the farmers to upgrade their local animals. The genetic improvement achieved by this practice is very minimal. One of the quickest ways of achieving rapid genetic improvement is by artificial breeding. Artificial breeding in buffaloes is not practiced in Sri Lanka due to lack of trained and proven bulls for semen collection and further no work has been done on the evaluation of different diluents for the preservation of buffalo semen in Sri Lanka.

Recent studies on buffalo semen preservation at 4° C[6] and at room temperature,[7] in Sri Lanka, indicate that buffalo semen could be preserved only upto 5 days with 50% motility. This problem of storage length could be overcome by storing semen in a good extender at —196°C in liquid nitrogen. At this temperature semen could be kept for a number of years without affecting the motility and the conception rate in inseminated cows. A further advantage of deep frozen semen is that it is easy to handle and could be transported to any part of the island in liquid nitrogen. Citric acid whey, egg yolk citrate and TRIS buffer have been used as the diluents by the Indian animal scientists[1,2,9] for the preservation of buffalo semen at -196°C. In this study two diluents, citric acid whey and TRIS buffer were compared for their ability to preserve buffalo semen at -196°C, under Sri Lanka conditions.

2. Experimental

2.1 Animals and semen collection

Four Murrahs, three Surtis and one indigenous buffalo belonging to the Department of Animal Husbandry of the University of Peradeniya, which were maintained under normal standard conditions of feeding and management, were used in this

study. Semen was collected once a week from each bull using an artificial vagina and a dummy. A separate artificial vagina was used for each buffalo and this was designed to suit the bull. Warm water at a temperature of 40°C was poured outside the inner lining of the rubber tube and by inflating sufficient air optimum pressure was created to simulate a vagina of a cow in heat. In order to provide a smooth surface for the buffalo bull a lubricant namely a jelly or vasoline was applied at the mounting end of the artificial vagina. Animals after teasing were allowed to mount on a dummy and semen was collected by the operator into a graduated collection tube.

2.2 Evaluation of samples

Immediately after collection the colour, volume, wave pattern or mass movement, the general motility and the live and dead counts of sperms and abnormal sperms. were assessed. Evaluation for wave pattern or mass movement was done as follows. Two drops of each sample was kept on a slide, warmed to body temperature and viewed under the lowest power of the microscope for forward and progressive movement which simulates a wave motion in the sea. In order to evaluate the general motility a thin smear with a coverslip was viewed under the same powered lens of microscope. To ascertain the live and dead counts of sperms, Nigrosin and Eosin stained slides were used under the oil immersion lens of the microscope where the dead sperms were stained violet and the live sperms remained unstained. The abnormal sperms were determined in these stained preparations.

2.3 Preparation of citric acid whey diluent and semen extension

Citric acid whey packets supplied by the National Dairy Research Institute Karnal were used directly in the following way: Ten grams of citric acid whey with penicillin (1000 I.U. per ml of the diluent) and streptomycin (1 mg per ml of the diluent) were dissolved in 100 ml of double distilled water. The suspension was allowed to stand for 5 - 10 minutes and filtered through cotton wool. The pH of this solution was adjusted to 6.8 with freshly prepared 10% sodium hydroxide solution. The solution thus prepared was divided into two equal fractions A and B. Glycerol was added to these fractions so that 3% of fraction A and 11% of fraction B constituted undiluted glycerol. Fresh semen was added to fraction A, keeping in mind after the final mixing of A and B, the ratio of semen of the dilution be maintained at 1:10. Fractions A and B were cooled to 5°C in a refrigerator and part B was added to part A in fractions at a time (about 1/5th of B added to A) at an interval of 10 - 15 minutes and the dilution was completed in 50 - 75 minutes.

2.4 Preparation of TRIS diluent and semen extension

In this preparation following chemicals were used: TRIS - 1.52 gms, citric acid -0.85 gms, fructose - 0.625 gms. These were dissolved in 42.5 ml of double distilled

water. To 37.5 ml of the above solution was added 10 ml of egg yolk, 3 ml of glycerol, penicillin (at the rate of 1000 I.U. per ml of the diluent) and streptomycin (1 mg per ml of the diluent), mixed well, and pH was adjusted to 6.8. This was used as a single step diluent where A and B fractions were not involved. Here again, semen dilution rate was 1:10. Motility percent and live/dead sperm percent was assessed after extending the semen with citric acid whey and TRIS buffer.

2.5 Equilibriation of extended semen

This is the time taken by the extended semen at 5°C to acquire the resistance power to cold shock. Normally it varies from 4 - 6 hours. Hence the extended semen (with citric acid whey and TRIS buffer diluents) was kept in the refrigerator at 5°C for 4 - 6 hours for equilibriation, after which time motility as well as live and dead count of spermatozoa were assessed.

2.6 Semen freezing

The diluted semen samples after a period (4 - 6 hours) of equilibriation were packed in ice, kept in a regiform box and transported by a vehicle to Central Artificial Insemination Centre, Kundasale, 16 km away from the place of collection. Diluted semen was tested for general motility, live and dead sperm counts, soon after transport. At the Artificial Insemination Centre the samples were kept inside a cold cabinet maintained in a temperature of 5°C. Prior arrangements were made in such a way that all the equipment were sterilised and kept inside the cold cabinet so that all equipment required would attain a uniform temperature of 5°C, to prevent any temperature shock to sperms. French ministraws of 0.25 cc.capacity were used for freezing semen. The straws were filled with diluted semen and the open end of the straws were dipped in polyvinyl powder to make a satisfactory seal. The straws were then placed in a water bath at 5°C for further equilibriation inside the cold cabinet for about 1 hour. Straws were held in bundles tabbed on the bottom of the water bath to remove excess powder, rolled and dried in absorbant towels. These straws were then kept in racks, made of stainless steel with a support, where the straws could be dried exposing all the surface and kept for half an hour at 5°C. Once the straws were dried they were exposed to liquid nitrogen vapour (temperature, -120°C) at the junction between the neck and the body of the freezing tank. After seven minutes of exposure the straws were lowered into liquid nitrogen.

2.7 Assessment of frozen semen

After freezing the straws were transferred to a small liquid nitrogen tank and taken to the Department of Animal Husbandry Laboratory. Test straws were removed at 24 hrs and 96 hrs after freezing and thawed in warm water at 37°C for 12 seconds

and assessed for motility and live/dead sperm percentage. In a separate study the motility and live/dead sperm percentage of thawed semen samples were also determined at 0, 2, 4, 6, 8, 10 and 12 hrs after thawing.

3. Results and Discussion

A total of 86 collections were made from 8 bulls during the experimental period. However almost half of the collections could not be frozen due to the insufficient volume, low initial motility ($\leq$65%), high dead sperm count ($\geq$ 20%) and due to other technical difficulties. In all 47 collections were frozen in TRIS and CAW diluents. The semen characteristics of the freshly collected semen are shown in Table 1. The motility value observed in our study is in agreement with the value of 65% reported by Gill *et al*,[3] However, others[4] have reported a higher initial motility of 81.5% in Murrah breeds. There was no significant breed effect on motility. Murrah breed gave the highest volume of semen. The value obtained for Murrah breed is in agreement with other reports.[3],[5] The low value obtained in Surti and local buffalo can be due to the difference in age and body size. There was no difference in the concentration of sperms in Murrah and Surti semen. However, semen of local buffalo was watery, light in colour and recorded the lowest concentration. The concentration observed in this study was much higher than those reported by others.[3],[5]

TABLE 1. Percentage of motile sperm, dead sperm percentage concentration and volume of freshly ejaculated semen

Breed	*No. of samples*	*Motility (in %)*	*Dead sperms (in %)*	*Concentration (in billion/ml)*	*Volume (in ml)*
Murrah	23	70.1 ± 1.8^a	17.6 ± 1.2^a	$4.47 \pm 0\ 3^a$	2.82 ± 0.53^a
Surti	17	68.1 ± 2.6^a	17.2 ± 1.3^a	3.58 ± 0.4^a	1.97 ± 0.68^b
Local	07	72.5 ± 4.8^a	25.7 ± 4.5^b	3.17 ± 0.5^b	1.98 ± 0.34^b

Means bearing different superscripts within column are different ($P < 0.05$)

The percentage of motile sperms and dead sperms after dilution in TRIS and CAW diluent are shown in Table 2. There was no dilution or breed effect and again no difference in motility and dead sperm percentage were observed even after 6 hours of equilibriation in TRIS and CAW diluents (Table 3). However, there was a significant reduction in motility and increase in dead sperm percent after equilibriation. In this study the diluted semen samples after equilibriation were transported to a distant place (16 kilo metres away) for freezing. Motility and dead sperm percentage before and after transport of the diluted samples indicates no significant differences in these two parameters. This is of practical importance, as semen could be collected in one place, equilibriated and could be transported to another place in ice and freezing could be done without affecting the semen quality.

TABLE 2. Percentage of motile sperm and dead sperm after dilution of buffalo semen in TRIS and CAW diluent

Breed	*Motility (%)*		*Dead sperm (%)*	
	TRIS	*CAW*	*TRIS*	*CAW*
Murrah	75±2.1	70±1.8	17.5±3.7	18.3±4.6
Surti	76±2.4	77±2.2	14.5±1.7	18.0±2.0
Local	75±1.7	75±1.4	22.8±2.0	26.1±2.1

Data statistically not significant ($P < 0.05$)

TABLE 3. Percentage of motile sperm and dead sperm after equilibriation of buffalo semen in TRIS and CAW diluents

Breed	*Motility (%)*		*Dead sperm (%)*	
	TRIS	*CAW*	*TRIS*	*CAW*
Murrah	60.7±2.9	56.7±4.9	20.4±1.2	22.5±0.8
Surti	60.8±4.1	61.8±3.1	19.4±1.7	24.2±1.0
Local	62.8±4.2	60.2±3.2	21.4±1.4	22.8±1.1

Data statistically not significant ($P < 0.05$)

The motility and dead sperm percentage of sperm in TRIS and CAW diluents after freezing are shown in Table 4. There was significant reduction in sperm motility and elevation in percent dead sperms in both the diluents after freezing and thawing. However in TRIS diluent the reduction in motility was 27% compared to CAW where it was found to be 66%. This observation is in agreement with the finding of Sharma *et al.*[8] Therefore from this study it can be said that TRIS is a better diluent than CAW for the freezing of buffalo semen under Sri Lankan conditions. The effect of time on post thaw motility of buffalo sperm frozen in TRIS diluent is shown in Table 5. According to this observation frozen semen after thawing can be kept for a period of 6 hours under conditions prevailing in our country.

The fertility rate following insemination with frozen semen was tested in post-partum cows, in another study. The maximum fertility rate observed was only 35% and this low fertility was attributed to most of the animals not ovulating after hormonal treatment rather than to the quality of frozen semen.

TABLE 4. Percentage of motile sperm and dead sperm, 24 hours and 96 hours after freezing in TRIS and CAW diluent

Breed	*24 hrs post freezing*				*96 hrs after freezing*			
	Motility (%)		*Dead sperm (%)*		*Motility (%)*		*Dead sperm (%)*	
	TRIS	*CAW*	*TRIS*	*CAW*	*TRIS*	*CAW*	*TRIS*	*CAW*
Murrah	50.2 ± 4.1^{a}	19.6 ± 2.4^{b}	$43.6 \pm 3.5^{a^1}$	$58.5 \pm 5.5^{b^1}$	50.4 ± 4.4^{a}	18.8 ± 2.4^{b}	$43.1 \pm 3.4^{a^1}$	$60.6 \pm 2.8^{b^1}$
Surti	40.8 ± 5.7^{a}	23.4 ± 3.8^{b}	$47.4 \pm 6.3^{a^1}$	$55.6 \pm 4.9^{b^1}$	40.9 ± 5.1^{a}	23.2 ± 3.8^{b}	$59.0 \pm 6.6^{a^1}$	$54.8 \pm 5.1^{a^1}$
Local	50.0 ± 7.5^{a}	17.5 ± 3.0^{b}	$47.0 \pm 5.5^{a^1}$	$56.8 \pm 4.6^{b^1}$	46.1 ± 7.1^{a}	17.6 ± 4.2^{b}	$43.3 \pm 8.1^{a^1}$	$60.8 \pm 4.1^{b^1}$

Mean bearing different superscripts along rows are different (P 0.05)

a-b indicates motility % a^1 - b^1 indicates dead sperm %

TABLE 5. Effect of time on post thaw motility percentage of buffalo semen frozen in TRIS diluent

Breed	*0 hrs*	*2 hrs*	*4 hrs*	*6 hrs*	*8 hrs*	*10 hrs*	*12 hrs*
Murrah	44*	44	42	37	34	7	4
Surti	46	53	55	49	28	1	6
Local	47	43	45	36	28	8	4
Mean	45	46	47	40	30	8	5

* number of observations = 10

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