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ORIGINAL ARTICLE

Impact of chilling duration on sperm quality of indigenous buck semen in the Coastal Area of Bangladesh

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Abstract

Background: Assessing semen characteristics is an important step before semen preservation. This study aimed to collect, evaluate, and preserve indigenous buck semen in the coastal area of Bangladesh.

Methods: The study was conducted at the Theriogenology and Animal Reproductive Biotechnology Laboratory, Department of Medicine, Surgery and Obstetrics, Patuakhali Science and Technology University, from July 2021 to June 2022. We selected four bucks based on non-return rate, pregnancy rate of does, and various parameters including age, body weight, scrotal circumference, and testicular epididymal length. These selected animals received a regimen of vitamins, minerals, anthelmintics, and concentrated feed. Semen was collected using the artificial vagina method, and data on color, odor, volume, viscosity, mass activity, consistency, concentration, and individual sperm motility were recorded and analyzed after collection.

Results: The semen of bucks exhibited a creamy white color and a fishy odor. The mean (±SD) of scrotal circumference (SC) of four bucks was 20.43±0.52, while the testicular epididymal length (TEL) was 11.03±0.59. Buck-2 (B-2) and buck-1 (B-1) showed significantly ($p<0.05$) higher volumes than buck-3 (B-3), and buck (B-4). B-1 also displayed significantly ($p<0.05$) higher mass activity compared to B-2, B-3, and B-4. In addition, B-2 and B-1 showed significantly ($p<0.05$) greater consistency than B-3, and B-4. The highest concentrations were observed in B-2 and B-1 compared to B-3 and B-4. In terms of individual sperm motility, B-1 showed significantly ($p<0.05$) higher motility (81.0±05.16) than B-2 (71.0±03.94), B-3 (66.0±03.94), and B-4 (80.0±07.45). Regarding chilling duration, a significant reduction in sperm motility was observed during storage at 4°C. This indicated that sperm motility varied significantly ($p \leq 0.000$) with varying chilling durations.

Conclusions: The study suggests that the individual progressive sperm motility at 4°C gradually decreases but remains suitable for artificial insemination up to 72 h (≥50±7.65). B-1 and B-2 demonstrated relatively promising results in semen evaluation and preservation. Further study is necessary to determine the pregnancy rate of does after insemination with chilled semen.

Key Words: Artificial vagina, scrotal circumference, mass activity, motility.

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Introduction

Agriculture remains as the principal sector in Bangladesh, offering a profitable avenue for livestock rearing, catering to the increasing demand for meat and yielding high-quality hides and skins essential for leather and leather goods industry (BER, 2022). Goat farming is experiencing substantial growth on both small and medium scales due to the high demand for meat in Bangladesh. This expansion is often integrated with other farming systems and, in coastal Bangladesh, represents a traditional practice, often tended to by rural women alongside their household responsibilities (Paul *et al*., 2021). The Black Bengal goat breed, unique to Bangladesh, dominates the landscape, comprising approximately 90% of the goat population. This breed is renowned for its prolificacy, fertility, early sexual maturity, adaptability to hot and humid conditions, and its ability to produce superior quality meat and skin. (Hussain, 1999; Amin *et al*., 2001). These indigenous goats exhibit remarkable resilience, including tolerance to saline conditions, grazing in rainy environments, and navigating stagnant water fields (Paul *et al*., 2021). Nevertheless, haphazard breeding practices threaten these distinctive genetic traits, underscoring the critical importance of conserving this local breed.

Now a day, artificial insemination (AI) in doe has gained popularity over natural service among farmers due to its rapid genetic merit. The success of semen preservation markedly depends on the semen quality such as color, odor, consistency, viscosity, concentration, mass activity, individual motility, and viability. The Artificial Vagina (AV) method is a proven technique for collecting semen from bucks, allowing for analysis and preservation (Wulster-Radcliffe *et al*., 2001). Our prior studies have been pivotal in selecting bucks based on fertility criteria (Paul *et al*., 2021) and in establishing routine semen evaluation protocols (Swarna *et al*., 2022).

However, the storage of semen, particularly in a frozen state, poses challenges, resulting in ultra-structural, biochemical, and functional damage to the spermatozoa. This damage manifests as reduced motility, viability, impaired transport, and fertility. Cooled semen is not immune to such issues, experiencing declines in motility and structural integrity, coupled with reduced survival in the female reproductive tract, diminished fertility, and increased embryonic loss (Paulenz *et al*., 2002).

However, while the fertility of stored semen is typically lower than that of fresh semen, it tends to perform better than semen that has undergone freezing and thawing.

Specifically, semen stored at room temperature or cooled at 5-8 ºC exhibits higher fertility compared to frozen semen (Paulenz *et al*., 2005). Especially, goat semen preservation has encountered a unique challenge related to the detrimental effect of seminal plasma on sperm viability when egg yolk or milk-based diluents are used. There is no published data on buck semen preservation, particularly concerning indigenous breed in the coastal region of Bangladesh. Therefore, this study aims to investigate the impact of chilling duration on sperm motility of indigenous buck semen in the study region.

Materials and Methods

Materials

All the ingredients for making stock solutions and extenders were purchased from different companies (Table 1).

Study place

The study was conducted at the Theriogenology and Animal Reproductive Biotechnology Laboratory, Department of Medicine, Surgery and Obstetrics, Faculty of Animal Science and Veterinary Medicine, Patuakhali Science and Technology University. This laboratory is situated in the outer campus in Babugonj, Barishal.

Selection and management of bucks

Four bucks were selected based on fertility rates and observations from our previous study (Paul *et al*., 2021). These bucks, aged between 2 and 3 years and weighing 15 to 20 kg with good body condition, were maintained on natural grazing, with supplemented feeding. The supplementary diet included a concentrate mixture consisting of 25% crushed maize, 50% wheat bran, 20% soybean meal, 1% fish meal, 2% DCP Powder, 1.5% salt, and 0.5% vitamin-mineral premix, provided at 250 g/head/day.

Table 1: The composition and list of various ingredients used in preparing the stock solution

Ingredients	Composition	Company
Tris (500g)	$C_4H_{11}NO_3$ MW=121.14g/mol (2-amino-2-hydroxymethyl-propane-1,3-diol)	Genaxxon bioscience GmbH – soflinger str. 100- 89077.
Citric acid (500g)	$C_6H_8O_7$ M= 192.12g/mol	Merk Specialities Private Ltd. Mumbai-400 018.
Fructose (100g)	$C_6H_{12}O_6$ MW= 180.16g/mol	Sisco Research Laboratories Pvt. Ltd. Maharastra, India.
Glycerol (100 ml)	$C_3H_8O_3$ MW=92.09g/mol	Sisco Research Laboratories Pvt. Ltd. Mumbai, India.

They had access to water and a mineral salt lick ad libitum and were reared under the same management system. Prior to semen collection, all goats were dewormed using Renadex® (Renata Animal Health, Dhaka, Bangladesh) and received three injections of 3 ml vitamin ADE (Renasol AD3E® Renata Animal Health, Dhaka, Bangladesh) and B-complex (Multivit® vet, Square Pharmaceuticals Limited, Dhaka, Bangladesh) at 5-day intervals one month before the collection. Mounting practice in a dummy occurred at two-day intervals. Four bucks were employed to collect the semen sample. Each buck underwent semen collection ten times for evaluation and five times for preservation. Semen collection using the Artificial Vagina (AV) method involved mounting with an estrus doe once a week. During the collection process, various characteristics were assessed, such as the creamy-white color of semen and the presence of a distinct fishy fragrance in the collected semen.

Preparation of Artificial Vagina (AV set) and semen collection

The preparation of the AV set and the collection of buck semen were carried out following established techniques as previously described (Pradhan *et al.*, 2013). Semen was collected using the Artificial Vagina (AV set, Minitube, Germany, Gentech International Ltd.) method at a frequency of once a week. Donor bucks were usually allowed at least 2 false mounts before the collection of final ejaculates. The bucks were trained previously to ejaculate in AV.

During the collection process, the penis end of the AV was lubricated with non-spermicidal gel (Sterile Lubricating Jelly®, First Priority, Inc. USA). A plastic cone was attached to the other end of the AV, connected to a calibrated collecting plastic tube. Prior to collection, the buck's prepuce was cleaned with normal saline water to ensure cleanliness and prevent semen contamination. The AV was held in the right hand along the buck's flank during collection, with the open end facing downward at a 45° angle. When the buck mounted, the erect penis was carefully directed toward the open end of the AV to allow vigorous upward and forward thrusts, stimulating ejaculation. The buck was allowed to withdraw its penis immediately after ejaculation into the AV. The graduated collecting tube was then separated from the cone, its open end was sealed with a plastic cap, and it was labeled accordingly. After collection, the semen was maintained at 37°C in a water bath until the necessary media and reagents were added.

Semen evaluation

The routine evaluation of fresh semen (volume, color, odor, sperm concentration, viscosity, mass activity and individual motility) was performed following the procedure described by Swarna *et al*. (2022).

Processing of semen

The extender used in the study was prepared following the method described by Paul and Biswas (2021). The composition of the extender is shown in Table 2. Two types of semen extenders were used: extender A (without glycerol) and extender B (with glycerol). After preparation, the extender solution was stored at 4°C in a refrigerator for a maximum period of 1 to 2 weeks.

On the day of semen collection, 100 ml of the final extender was prepared by adding 25% egg yolk to the stock solution. The egg yolk citrate diluents is used for preserving semen at chilling temperature of 4-5°C, was mixed at a 1:5 ratios for 4 days. The egg yolk extender was freshly prepared for use according to the method

described by Herman and Madeen (1963) with certain modifications (Table 2).

To create a stock solution for tris-glucose-citrate diluents, tris, glucose, and citrate were dissolved in 84 ml of distilled water and the pH of the extender was adjusted to 7.4. The extenders were labeled as A and B. Additionally, 8 ml of glycerol was added to extender B. This processing step was done at room temperature within 5 to10 minutes.

The processed semen was preserved in test tubes at 4°C in a kit box, kept in a dark environment and finally stored in the refrigerator at a temperature of 4-5°C throughout the study period.

Table 2: Compositions of the extender

Agents	Amount/100ml
Tris	3.028g
Citric acid	1.675g
Fructose	1.25gm
Alpha amylase	100mg
Glycerol	8.0ml
Lactose	6.0gm
Distilled water	92ml
Egg yolk	25ml
Penicillin	1000IU/ml
Streptomycin	1mg/ml

Preparation of chilled semen

The preparation of chilled semen followed the procedures outlined in the study by Rekha *et al.* (2016). First, all materials were autoclaved for sterilization. Strict hygiene measures were maintained to ensure the health of live sperm on the day of semen collection. Then solutions previously prepared were brought from freezing temperatures to room temperature (approximately 25°C). Extender-A (egg yolk) was added to the solutions at room temperature, and the mixture was thoroughly stirred to achieve complete mixing. The mixture was then frozen at 4ºC for a duration of 90 minutes. The artificial vagina was prepared, and both the buck and the teaser doe were properly cleaned in preparation for semen collection. After the initial 90 minutes of freezing, the mixture was removed, and Extender-B (containing 8% glycerol) was added. Thorough stirring was performed with a sterile stirrer to ensure proper mixing. The mixture was again frozen at 4ºC, this time for 20 minutes. Then semen collection was performed, and the collected semen was promptly transported to the laboratory. In the laboratory, calculations were made to determine the doses and concentration of the fresh semen. The motility rate was also assessed under a microscope at 10x and 40x magnifications. Following the second chilling period, the extender mixture was removed from the refrigerator. The semen was diluted, and the diluted semen was loaded into vials at a ratio of 1:5 (semen: extender). This dilution process was conducted at room temperature (25°C). The filling and sealing of vials were also performed at room temperature. The vials containing the diluted semen were stored at 4ºC. A total of five sterile vials were used, with each vial containing 1 ml of diluted semen volume. The motility of sperm was assessed at 24-hour intervals for a maximum of 96 hours.

Statistical Analysis

The collected semen was evaluated, and individual data were recorded. These recorded data were entered in the Microsoft Excel spreadsheet for further analysis. The semen volume, consistency, concentration of sperm, and mass activity were calculated and expressed as mean values along with their respective standard deviations (mean ±SD). Sperm motility was expressed as an average percentage. To ensure robustness, the tests were repeated 10 times for evaluation and 5 times for preservation for each buck. The descriptive analysis and correlation coefficients calculations were performed using the Statistical Program for Social Science (SPSS® Version 22.0). In all tests, statistical significance was defined as p-value of ≤ 0.05. Comparative analysis of sperm motility was conducted among the different bucks by t-test.

Results and Discussion

In this study, we found that the mean ±SD of scrotal circumference (SC) of four bucks was 20.43±0.52 and the TEL was 11.03±0.59. The measurement of the SC is an integral part of assessing the breeding soundness of animals, especially in bulls with pendulous scrotums (Goyal and Memon, 2007). Our findings showed similarities with those of Gemeda and Workalemahu (2017), who also reported an overall mean SC value of 20.8±1.94 and testicular length (TL) of 4.97±0.79. Additionally, Raji *et al*. (2008) reported SC measurements of 23.99 ± 0.17 and 20.75 ± 0.25 cm in Red Sokoto and Borno White bucks, respectively, in Nigeria. These results align with our findings. Furthermore, the mean SC values obtained for the three breeds in our study were higher than the reported values of 15.73 cm and 17.15 ± 1.14 cm for African Dwarf bucks (Abu *et al*., 2016) and Shale bucks (Oyeyemi, 2012), respectively. However, our measurements were lower than the range of 28 to 39cm reported for Nubian buck (Goyal and Memon, 2007). These differences could be attributed to genetic or breed variations; as similar differences among breeds have been reported in goats (Raji *et al*., 2008).

The NRR of B-1, B-2, B-3 and B-4 were 80%, 70%, 90% and 70%, respectively (Table 3). Our findings are consistent with those of Sultana *et al*. (2013), who reported NRRs in different Black Bengal bucks as follows: B5 (85.71 ± 7.99%), B2 (80.00 ± 6.02%), B3 (76.00 ± 10.43%), B4 (63.26 ± 6.98%), and B1 (62.96 ± 6.72%). Paulenez *et al*. (2003) observed a 25-day NRR of 87% in Norwegian dairy goats. This variation in reported conception rates may arise from differences in the method of pregnancy diagnosis. In our study, the conception rate was determined on a non-return basis.

Table 3: Buck performance based on pregnancy rate of does

Buck No.	Non-Return Rate (%)
B-1	80
B-2	70
B-3	90
B-4	70

Effect of different parameters on semen characteristics

The semen parameters of the four bucks are shown in table 4. The semen volume showed significant (P=0.000) differences among the four bucks in logistic regression analysis. Our findings are in accordance with previous studies (Sultana *et al*., 2013; Das *et al*., 2006; Barbas *et al*., 2006). Sultana *et al*. (2013) also reported significant ($p < 0.05$) individual variations in the semen volume in Black Bengal bucks.

In our study, the highest ejaculate volume obtained was 0.98 ml, which was slightly lower than the values of 1.28 ml and 1.54 ml reported by Haro *et al*. (2019) for Sahelian bucks in Burkina Faso and Singh *et al*. (2019) for Beetal goats in India, respectively. However, it was higher than the 0.51 ml reported by Souley (2013) for Sahel goats in Niger. Pradhan *et al*. (2013), who also worked with four Black Bengal bucks, found significant variation (P>0.05) in volume per ejaculate.
In terms of semen viscosity, among the four bucks, B-2 exhibited the highest viscosity (3.80 ± 0.42), followed by B-1 (3.20 ± 0.79), B-3 (2.60 ± 0.52), and B-4 (3.00 ± 0.67). The differences in semen viscosity among the bucks were significant (P=0.001).

Similar findings were reported by Haro *et al*. (2019), who observed a majority percentage (69.2%) of milky white semen in Sahelian goats in Burkina Faso. The high viscosity of semen, coupled with its whitish color, is indicative of the normal appearance of semen.
The good mass activity of fresh semen is the key factor to having good motility of preserved semen. Among the four bucks, B1 showed the highest mass activity (3.60±0.52) with the other bucks showing values depending on their SC and TEL. Similar results were also reported by other authors (Singh *et al*., 2019; Haro *et al*., 2019; Sacko *et al*., 2022). Raji *et al*. (2015) found variability in mass activity among bucks.

B-1 had the highest consistency (4.20±0.79) among all bucks except for B-2. The differences in semen consistency among the bucks were significant (P=0.000). These consistency values can be compared with those reported by Atara *et al*. (2018).

Regarding sperm concentration, B-2 (1.94±0.13×109) and B-1 (1.58±0.46×109) showed the highest concentration per ml of semen volume compared to other bucks.

Table 4: Evaluation of buck semen characteristics

Characteristics	Buck	Mean ± SD	95% Confidence Interval for mean		P value (Within and Between Groups)
			Lower Bound	Upper Bound	
Volume (ml)	1	0.74±0.18	0.609	0.871	
	2	0.98±0.29	0.770	1.190	
	3	0.42±0.12	0.332	0.508	0.000
	4	0.60±0.15	0.493	0.707	
	Average	0.68±0.28	0.595	0.775	
Viscosity	1	3.20±0.79	2.64	3.76	
	2	3.80±0.42	3.50	4.10	
	3	2.60±0.52	2.23	2.97	0.001
	4	3.00±0.67	2.52	3.48	
	Average	3.15±0.74	2.91	3.39	
Mass activity	1	3.60±0.52	3.23	3.97	
	2	3.20±0.42	2.90	3.50	
	3	2.40±0.52	2.03	2.77	0.000
	4	3.40±0.84	2.80	4.00	
	Average	3.15±0.74	2.91	3.39	
Consistency	1	4.20±0.79	3.64	4.76	
	2	4.80±0.42	4.50	5.10	
	3	2.80±0.42	2.50	3.10	0.000
	4	4.00±0.67	3.52	4.48	
	Average	3.95±0.93	3.65	4.25	
Concentration (10^9/ml)	1	1.58±0.46	1.248	1.91	
	2	1.94±0.13	1.850	2.030	
	3	0.62±0.18	0.490	0.750	0.000
	4	1.54±0.43	1.232	1.848	
	Average	1.42±0.59	1.232	1.608	
Individual sperm motility (%)	1	81.0±05.16	77.31	84.69	
	2	80.0±07.45	74.67	85.33	
	3	66.0±03.94	63.18	68.82	0.000
	4	71.0±03.94	68.18	73.82	
	Average	74.5±08.15	71.89	77.11	

For fresh semen, B-1 showed the highest individual motility (81.0±05.16%) among all selected bucks. The differences in individual motility among the four breeding bucks were significant (P=0.000). Sperm motility is the first and foremost criterion of semen preservation whether it would be selected or discarded. It is advisable to assess motility as soon as possible, as it plays a crucial role in successful conception (Anand *et al*., 2016). These findings are consistent with the observations of Siddiqua *et al*. (2016), who reported fresh sperm motility of 80.83 ± 3.53%. Our results also partially align with the findings of Dhar *et al*. (2010). Motility is a key seminal attribute that facilitates the timely transport of spermatozoa to the site of fertilization and is highly correlated with successful conception (Anand *et al*., 2016).

Effect of chilling duration on sperm motility

Our study aimed to explore the relationship and differences in sperm motility before and after the preservation of buck semen across various preservation durations. We observed that the highest individual motility of sperm was obtained on the day of freezing (0 hours) for B-1 and B-4, with values of

80 ± 4.12% and 80 ± 4.95%, respectively (Table 5). In contrast, the individual motility of sperm obtained at 0 hours of freezing for B-2 and B-3 was slightly lower, with values of 75 ± 4.64% and 75 ± 6.04%, respectively.

As we extended the preservation duration, the motility of sperm at 24 hours of freezing varied. The highest values were obtained from B-1 and B-4, with 70 ± 4.95% and 70 ± 4.85%, respectively, while the lowest values were observed for B-2 and B-3. At 48 hours of freezing, B-1 exhibited the highest motility at 65 ± 4.85%, outperforming the other bucks. Moving to 72 hours of freezing, B-1 still maintained relatively high motility at 60 ± 3.67%, followed by B-2 at 55 ± 6.96%. B-3 and B-4 exhibited slightly lower motility at 50 ± 7.04% and 50 ± 7.65%, respectively. Finally, at 96 hours of freezing, B-1 and B-2 showed the highest values at 55 ± 5.96% and 50 ± 7.21%, respectively.

We observed that, B-1 displayed 80 ± 4.12% sperm motility at 0 hours, but this gradually declined to 55 ± 5.96% at 96 hours. On the other hand, the other three bucks (B-2, B-3, B-4) exhibited a similar pattern of decreasing sperm motility over time. This suggests that the quality of chilled semen deteriorates significantly after four days of preservation. Our conclusions align with the findings of other authors (Pradhan *et al*., 2013; Rekha *et al*., 2016), who reported a reduction in sperm motility with increasing preservation time in rams. Sperm motility is an important factor in successful pregnancies, as it influences the ability of sperm to travel towards an ovum for fertilization (Quill and Garbers, 2002). The motility of sperm is associated with a series of biochemical changes, including capacitation, binding to the zona pellucida of the egg, and the acrosome reaction (Yanagimachi, 1994). Non-motile or abnormally motile sperm are less likely to fertilize the oocyte. Therefore, assessing the proportion of motile sperm is a widely used measure for evaluating semen quality (Farrell *et al*., 1998).

It is worth noting that the four bucks in this study belonged to the same breed and were of similar age, with similar management, nutritional status, and general health conditions. However, differences in semen volume might reflect their varying genetic potential, as genetically superior bucks have been shown to produce a higher volume of semen in previous studies (Swarna *et al*., 2022). Our results are consistent with previous findings reported by other authors (Sultana *et al*., 2013; Das *et al*., 2006; Barbas *et al*., 2006; Dhar, 2010).

Table 5: Effect of chilling duration on sperm motility

Buck No.	**Sperm motility (**Mean ± SD **of %)**					**P value**
	0 h	**24 h**	**48 h**	**72 h**	**96 h**	
B-1	80±4.12^{a}	70±4.95^{a}	65±4.85^{a}	60±3.67^{b}	55±5.96^{b}	0.000
B-2	75±4.64^{a}	65±5.34^{a}	60±5.66^{b}	55±6.96^{b}	50±7.21^{bc}	0.000
B-3	75±6.04^{a}	65±6.04^{a}	65±6.04^{a}	50±7.04^{bc}	40±8.12^{c}	0.000
B-4	80±4.95^{a}	70±4.85^{a}	60±7.78^{b}	50±7.65^{bc}	30±6.82^{c}	0.000

No. of replication 5 times/buck, a,b,c indicates significant (P ≤0.000) difference in the same rows within the buck.

Limitations

The method used in the experiment was a routine semen evaluation procedure as described by different researchers. While this method provided a rapid analysis of semen, it was limited in its ability to explore the molecular characterization of sperm. One of the advantages of this approach was its efficiency in quickly assessing semen quality. However, it did not provide a complete assessment of sperm quality. This method did not incorporate Computer Assisted Semen Analysis (CASA), which would have enabled the quantification of live and dead sperm.

Conclusions

In conclusion, the progressive motility of sperm at 4°C freezing gradually decreased and remained suitable for artificial insemination up to 72 hours. Variations were observed in semen analysis parameters among the four bucks. B-1 and B-2 showed relatively favorable results in semen evaluation and preservation. However, the overall data suggests that the bucks' semen can be assessed for viability and motility before and after freezing when used as liquid semen. Chilled semen should not be utilized after 72 hours of preservation.

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Conflict of interest

The authors have declared no conflict of interest.

Authorship Contribution Statement

AK Paul designed the experiment, supervised the study, analyzed the data, and revised the final draft of the manuscript. M Swarna and S Biswas were directly involved to do the experiment, collecting data, and reviewing the literature and tabulating the data, and writing the draft of this manuscript.

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