

Assessing the efficacy of different semen extenders on post-thaw quality of Bangladeshi buffalo sperm under a manual cryopreservation protocol

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ABSTRACT

Cryopreservation has been used extensively for cattle in Bangladesh, however no study was conducted on the cryopreservation of buffalo sperm in Bangladesh. Thus, the current study aimed to evaluate the suitability of different semen extenders for improving the post-thaw quality of Bangladeshi buffalo sperm under a manual cryopreservation protocol. The manual cryopreservation protocol was compared and optimized with a commercial biofreezer protocol. Then, the efficacy of different diluters was evaluated using the optimized manual cryopreservation protocol. Meanwhile, the post-thaw sperm quality in terms of motility and morphology was evaluated by computer assisted sperm analyzer during the optimization process. During manual cryopreservation, the first cooling from 37 °C to 5 °C was done in an equilibration chamber and the second cooling from 5 °C to -120 °C in a Styrofoam box using liquid nitrogen vapor from different distances (0.5, 1.5, 1.6, 2 and 3 inches). Simultaneously, another batch of sperm was cryopreserved using a programmable freezer. The highest number of motile sperms (62.67 ± 1.12 ; $P < 0.01$) and progressive motility (38.97 ± 1.10 ; $P < 0.001$) was observed at 1.6 inches above liquid nitrogen, which were similar to the results obtained from automated biofreezer protocol (65.94 ± 4.65 and 45.54 ± 3.64 , respectively). To evaluate the semen extenders' efficacy, one locally developed Tris-fructose-egg yolk-based diluter and two commercial diluters (Andromed, and Triladyl) were used in the freezing of buffalo sperm. The highest recovery and conception rates were observed in sperm diluted with tris-fructose-egg yolk-based (TFE) (82.4% and 80%, respectively). Therefore, it is suggested that this manual cryopreservation protocol and the TFE diluter could be a suitable and inexpensive alternative for Bangladeshi buffalo sperm considering post-thaw sperm quality and fertility.

INTRODUCTION

Bangladesh, a South Asian country, relies heavily on agriculture, with a significant portion of its rural population engaged in farming and rearing of livestock and poultry. The contribution of livestock and poultry to Bangladesh's gross domestic product (GDP) was approximately 1.85% in 2023 [1]. Buffalo rearing has also been widespread in the country, particularly in drought-prone regions and impoverished rural areas for plowing. Despite the country's limited buffalo population (1.5 million), these animals play a substantial role in the national economy by contributing to meat and milk production. However, the most remarkable advantage of buffalo is their efficient digestion of less digestible feeds. Unlike cattle, they can process crude protein, dry matter, and coarse feed more effectively, transforming these resources into high-quality milk and meat [2]. Moreover, buffaloes exhibit more excellent disease resistance, with



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less care and management inputs, thus saving costs and time [3]. Consequently, implementing long-term strategies is imperative to harness the potential of buffalo resources in Bangladesh. The buffalo breeds present in the country include indigenous breeds, non-descript water buffaloes, and crossbred varieties such as Murrah, Nili-Ravi, and Jaffrabadi [4].

To enhance buffalo production and develop superior breeds, the availability of high-quality sperm is crucial. Regularly producing good-quality sperm is challenging due to buffaloes' genetic traits, feed, and fodder variations and agroecological conditions. As a result, assisted reproductive technologies, particularly artificial insemination (AI) using cryopreserved sperm, have gained popularity. These technologies aim to transfer improved genetic potentialities and increase milk and meat production capacities [5]. The choice of cryopreservation techniques, cryoprotectants, and semen extenders heavily influences the success of AI. Semen cryopreservation, which involves freezing at extremely low temperatures (-198°C), is a complex process. The steps involved in cryopreservation can potentially compromise semen quality by damaging the acrosome and plasma membrane [6]. During this process, up to 50% of sperm cells can be harmed by stress caused by ice crystal formation, chemical toxicity, and osmotic stress [7, 8]. Another factor influencing sperm freezability is the composition of semen extenders. The commonly used diluters for cattle and buffalo are Tris-based, enriched with sugars and various compositions of glycerol [9], the primary cryoprotectant. Although glycerol is toxic, its toxicity can be managed through optimized mixing steps (two steps) and concentrations (3-8%) [10]. The freezing process is commonly done using nitrogen vapor in an automatic control-rate biofreezer or a homemade box. While the latter method is cost-effective, it yields less accurate results compared to automated freezing due to a lower cell recovery rate [11]. Hence, most commercial AI centers opt for programmable freezers that maintain temperature gradients and minimize ice crystal formation, ensuring consistent post-thaw cell recovery [12]. Nevertheless, these commercial machines are expensive and require specialized facilities and skilled operators. Yet, many countries are developing or are least developed and thus most farms in these countries cannot afford these high-cost freezers. Consequently, there is a pressing need to select appropriate cryopreservation techniques that would provide acceptable rates of viable post-thawed semen under typical production settings.

Therefore, this study aims to evaluate the efficacy of some semen extenders for a manual cryopreservation technique for freezing Bangladeshi buffalo sperm that would enhance freezing capacity. As far as the authors are concerned, this is the first study examining the effects of semen diluters and cryopreservation techniques for buffalo rearing in Bangladeshi conditions. The findings would enable AI centers, research facilities, and medium-scale farmers to obtain more benefits from the buffalo industry, especially in developing and least-developed nations.

MATERIALS AND METHODS

Ethical approval

The study was conducted according to the declaration of Helsinki and approved by the Institutional Ethics Committee of Bangladesh Livestock Research Institute (BLRI/16/12/21).

Collection and evaluation of semen

Semen was collected from five native × Murrah buffalo bulls twice a week using an artificial vagina maintaining a temperature of 37° C. Semen characteristics (color, volume, concentration) were evaluated before freezing following the methods of Jha et al [13]. A hemocytometer was used to measure the sperm concentration rate (>500 ×10⁶/ml). Sperm motility was assessed by Computer Assisted Sperm Analyzer (CASA) (AndroVision, Minitube, USA). Briefly, 10 uL aliquots of semen were placed into glass slides and covered with a glass cover slide. The processed aliquots were loaded into CASA slide chambers and sperm parameters were observed using real-time video capture and analyzed with the CASA software. Spermac stain test was performed to assess sperm morphological abnormality. Briefly, the smear was prepared from 10 uL aliquot of semen and air dried for 5 minutes. Then, the glass slide with the sample was stained by dipping 7 times in stains A, B and C and washing in distilled water. Finally, slides were observed under a light microscope (100 × magnification) using oil immersion.

Dilution of semen

Semen samples having motility >70%, normal morphology (no abnormality in >70%) and normal concentration (>500×10⁶/ml) were diluted with a locally developed Tris-fructose-egg yolk-based (TFE) diluter to obtain spermatozoa concentration of 20×10⁶/straw. The TFE diluter was prepared as described [13]. Briefly, the diluent with all components was split into two fractions; one did not contain glycerol and the other contained 12.8% glycerol. After mixing gradually, the final concentration of glycerol was 6.4%, which was then aliquoted into test tubes (Table 1).

Table 1. The composition of the Tris- fructose-egg yolk-based diluter.

Ingredients	Fraction 1	Fraction 2
Tris	2.24 g	2.24 g
Citric acid	1.48 g	1.48 g
Fructose	1 g	1 g
Penicillin-streptomycin	500000 IU	500000 IU
Egg yolk	20%	20%
Glycerol	-	12.8%
Distill Water	100 ml	100 ml

Freezing procedures and determining the straw position in the nitrogen vapor technique

The diluted semen was gradually cooled in a BLRI-developed equilibration chamber from 37 °C to 5 °C and takes around 30 minutes (1 °C decrease/minute) for temperature equilibration followed by filling and sealing of semen into 0.25 ml straws. The equilibration chamber is a biofreezer made of steel, plant sheet and cocksheets. It has two compressors which reduce the temperature gradually. Moreover, the UV light installed in the chamber is used to eradicate external contamination. A Styrofoam box of 20 inches in length, 13 inches in height and 12 inches in width was used for cooling from 5 °C to -120 °C using liquid nitrogen vapor for 15 min. First, liquid nitrogen was poured into the box. A purposefully designed steel rack was used to place the straws on the Styrofoam box which enabled the straws to remain in place and allowed the nitrogen vapor to spread easily. The rack was placed at different distances (1.3 to 7.6 cm)

over the liquid nitrogen in the box. Finally, the straws were plunged into liquid nitrogen at -196 °C and stored.

At the same time, another batch of straw was cryopreserved using a minitube programmable automated freezer. In brief, semen straw was transferred into a freezer and the temperature was reduced from +5°C to -140°C (+5°C to -5°C (20°C/min); -5°C to -110°C (55°C/min); -110°C to -140°C (35°C min)). Finally, the straws were plunged into liquid nitrogen at -196°C and stored. After 24 hours of storage, straws were thawed at 37 °C for 30-60 seconds and examined by CASA.

The efficacy of the TFE diluter with some commercial extenders were compared based on sperm characteristics. Hereupon, two commercial diluters (Andromed and Trilady1) were used. Following dilution, semen was frozen following the above-mentioned protocol and assessed by CASA.

Fertility test via artificial insemination

Finally, the fertility was assessed, and the conception rate was determined 90 days after insemination by rectal palpation. For this purpose, forty-five buffaloes (fifteen in each group) were selected randomly; heated buffalo was determined by teaser bull in the early morning and two times insemination was performed 12 hours and 14 hours intervals after heat detection by the recto-vaginal method [12]. During artificial insemination, cryopreserved sperm was thawed at 37°C for 25 seconds and used within 5 minutes.

Statistical analysis

Descriptive statistics were performed to present the mean and standard error of the mean. Next, One-way ANOVA followed by Duncan's post-hoc test was performed to present significant differences in means. A statistical significance was considered at $p < 0.05$. All the data was managed by Excel 2019 and SPSS (Version 25.0). Moreover, the sperm recovery rate was calculated by the following formula:

$$\text{Sperm Recovery Rate (\%)} = (\text{post-thaw motility/pre-frozen motility}) \times 100$$

RESULTS

Optimizing the distance of straws for semen freezing by nitrogen vapor

The motility and morphology of the cryopreserved sperm stored at different distances over liquid nitrogen using nitrogen vapor is shown in Table 2. As expected, there were significant differences in various distances regarding sperm motility. Both overall motility and progressive motility (PM) increased with the rise of distance for a certain point and then significantly decreased. The highest number of motile sperms ($62.67 \pm 1.12\%$; $p < 0.01$) was observed when semen was placed 1.6 inches above liquid nitrogen (Figure 1A). Moreover, PM was also the highest ($38.97 \pm 1.10\%$; $p < 0.001$) at the same distance. However, there was no significant difference in the morphology of cryopreserved semen at varying distances except for that in the bent tail. The bent tail was significantly lower ($4.30 \pm 0.85\%$; $p < 0.05$) in semen cryopreserved at 1.6 inches above liquid nitrogen.

Table 2. Optimizing the distance of semen straws over liquid nitrogen.

Distance	Motility (%)		Morphology (%)				
	Motile sperm	Progressive motility	Bent tail	Coiled tail	Distal midpiece reflex	Distal Droplet	Proximal Droplet
Pre-frozen semen	90.40 ^a ± 2.50	51.23 ^a ± 8.79	9.60 ^a ± 2.50	0.33 ± 0.24	1.27± 0.50	0.07 ± 0.07	1.17 ± 0.24
0.5 inch	38.47 ^c ± 7.34	21.43 ^b ± 2.80	7.37 ^b ± 1.80	0.83 ± 0.47	1.43 ± 0.15	3.33± 1.23	13.97 ± 9.51
1.5 inch	47.13 ^c ± 3.37	24.50 ^b ± 1.29	4.63 ^a ± 0.43	0.27 ± 0.07	1.43 ± 0.20	2.30 ± 0.30	4.30 ± 0.36
1.6 inch	62.67 ^b ± 1.12	38.97 ^a ± 1.10	4.30 ^a ± 0.85	0.13 ± 0.03	1.37 ± 0.23	3.20 ± 1.06	18.17 ± 12.38
2.0 inch	38.10 ^c ± 1.97	23.10 ^b ± 3.95	7.83 ^b ± 2.03	0.30± 0.10	1.67 ± 0.18	3.70 ± 0.61	6.53 ± 0.76
3.0 inch	35.03 ^c ± 8.25	18.07 ^b ± 4.20	8.13 ^b ± 1.38	0.43 ± 0.20	1.60 ± 0.25	4.20 ± 1.27	5.53 ± 0.99
p-value	0.000	0.001	0.020	0.228	0.633	0.204	0.726

n=5, results are presented in mean±SEM. Different letters (a, b, c) indicate differences among freezing groups (p<0.05).

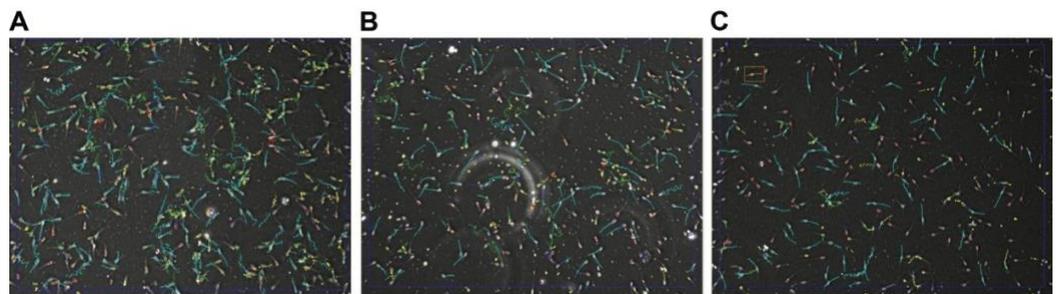


Figure 1. Comparison of motility and morphology of cryopreserved sperm analyzed by CASA (200x magnification). A) Pre-frozen semen, B) Nitrogen vapor technique (4cm above nitrogen), and C) Commercial freezing technique. The green lines indicated the motility of sperm. Sperms with light blue tracks are progressively motile. The red dots indicated static sperms while the yellow color indicated sperms that entered or left too early within the analysis frame.

Comparison of motility and morphology of cryopreserved sperm under the nitrogen vapor and a commercial biofreezer protocol

Results from the present study suggest that there were no significant differences in sperm motility cryopreserved in the nitrogen vapor technique and the commercial freezing technique. However, considerably less motility was observed in both cases than in pre-frozen semen (Table 3).

Table 3. Comparison of cryopreserved sperm motility and morphology in nitrogen vapor and commercial freezer protocols.

Variables	Motility (%)			Morphology (%)				
	Motile sperm	Progressive motility	Slow motility	Bent tail	Coiled tail	Distal midpiece reflex	Distal Droplet	Proximal Droplet
Pre-frozen semen	97.84 ^(a) ± 1.94	71.62 ^(a) ± 3.98	0.26 ^(a) ± 0.08	3.78±1.78	0.24±0.10	2.12±0.50	1.98±0.48	23.10±9.23
Minitube	65.94 ^(b) ± 4.65	45.54 ^(b) ± 3.64	2.43 ^(b) ± 0.36	6.62± 0.85	0.33±0.06	3.36±0.38	2.60±2.69	11.10±2.69
Nitrogen vapor technique	57.49 ^(b) ± 5.67	38.70 ^(b) ± 4.04	3.83 ^(b) ± 0.63	7.55±1.05	0.51±0.10	2.97±0.33	2.92±0.37	9.30±2.74
p-value	0.044	0.005	0.012	0.285	0.188	0.344	0.517	0.170

Minitube-TurboFreezer, (n=5, results are presented in Mean±SEM). Different letters (a, b) indicate differences among freezing groups (p<0.05).

The percentage of motile sperm in the commercial freezing technique was $65.94 \pm 4.65\%$ which was not statistically different from sperm motility in the nitrogen vapor technique ($57.49 \pm 5.67\%$). Moreover, there was no difference among the morphological features of pre-frozen sperm and sperm cryopreserved by a commercial or nitrogen vapor technique (Figure 1 B-C).

Effect of different diluters on semen motility and morphology after using the nitrogen vapor technique

We also assessed the efficacy of different semen extenders for sperm motility and morphology, and the results are compiled in Table 4. It was observed that sperm characteristics, both the motility and morphology of sperm, varied with the extender used. Briefly, lower motility of sperm ($27.5 \pm 19.24\%$, $p < 0.05$) was observed while cryopreserved with Andromed (motile sperm) compared to those with TFE ($72.69 \pm 15.92\%$) and Triladyl ($70.07 \pm 16.40\%$). Besides, no significant difference was observed between PM in TFE ($49.13 \pm 17.16\%$) and Triladyl ($70.07 \pm 16.40\%$), but both are significantly different from Andromed.

Furthermore, significant morphological differences were observed among sperm cryopreserved with different diluters. Bent tail, coiled tail and distal droplet were found highest in Andromed, while these were the same in the other diluters (Figure 2).

Table 4. Sperm motility and morphology with different diluters in the nitrogen vapor technique.

Diluters	Motility (%)			Morphology (%)			
	Motile sperm	Progressive motility	Slow motility	Bent tail	Coiled tail	DMRI	Distal Droplet
Pre-frozen	88.17 ± 15.80^c	67.92 ± 8.55^c	0.49 ± 0.64^a	4.39 ± 1.82^a	0.29 ± 0.31^a	1.35 ± 0.84^a	1.20 ± 0.73^a
TFE	72.69 ± 15.92^b	49.13 ± 17.16^b	5.89 ± 9.23^b	5.82 ± 3.97^a	0.39 ± 0.25^a	2.93 ± 1.48^b	2.40 ± 2.17^a
Andromed	27.5 ± 19.24^a	11.76 ± 10.76^a	1.19 ± 1.33^a	13.49 ± 5.97^b	0.82 ± 0.60^b	1.59 ± 0.50^a	4.87 ± 1.77^c
Triladyl	70.07 ± 16.40^b	39.54 ± 13.40^b	5.38 ± 3.13^a	5.80 ± 3.26^a	0.55 ± 0.60^a	3.40 ± 1.66^b	3.18 ± 1.57^b
p-value	0.00	0.00	0.06	0.00	0.11	0.00	0.00

N=5, results are presented in mean \pm SEM. Different letters (a, b, c) indicate differences among freezing groups ($p < 0.05$).

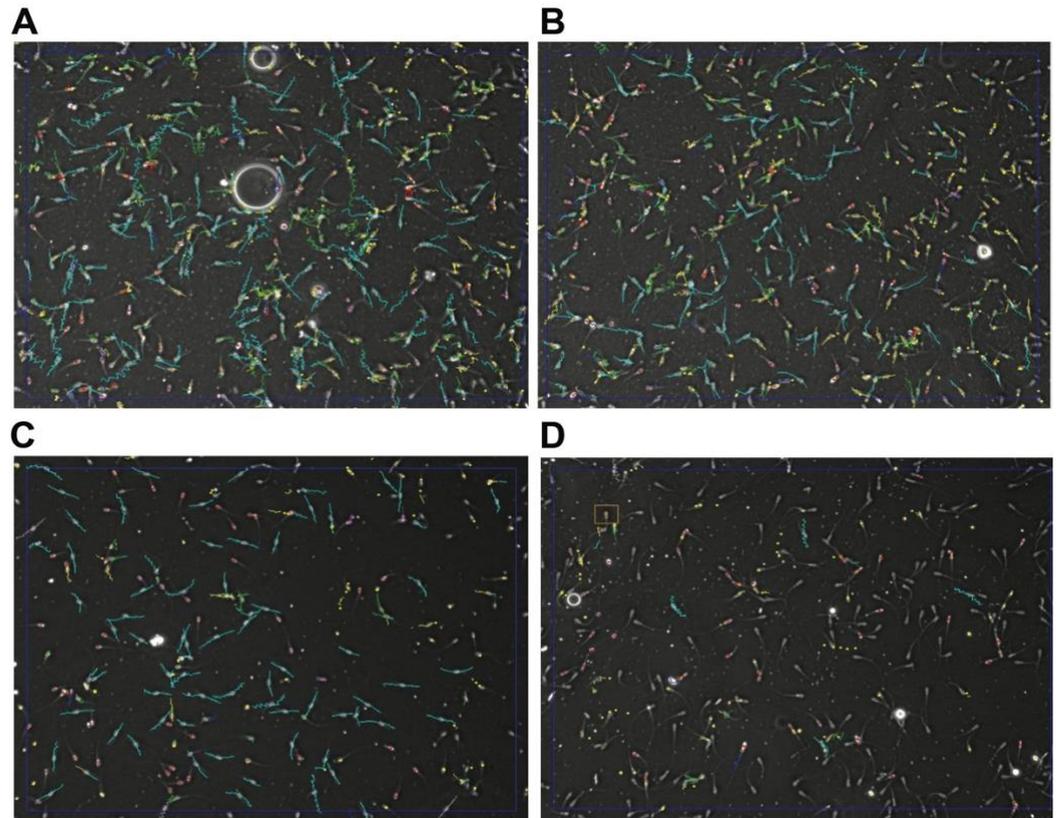


Figure 2. Effect of different diluters on sperm motility and morphology analyzed by CASA (200×magnification). A) Pre-frozen, B) TFE, C) Triladyl, and D) Andromed. The green lines indicated the motility of sperm. Sperms with light blue tracks are progressively motile. The red dots indicated static sperms, while the yellow color indicated sperms that entered or were left too early within the analysis frame.

The recovery rate after cryopreservation is depicted in Figure 3. Similar to sperm motility and morphology, the recovery rate was significantly higher in sperm frozen with TFE (82.4%) followed by Triladyl (79.5%) compared to Andromed ($p < 0.05$). In the case of PM, sperm cryopreserved with TFE had a significantly high recovery rate (72.3%, $p < 0.05$). Moreover, the number of abnormal sperm in TFE is lower than in other diluters.

Furthermore, the conception rates of sperm cryopreserved with different diluters are compiled in Table 5. It was observed that the conception rate was higher in buffalo inseminated using semen diluted with both TFE and Triladyl, while there was a significant difference in the conception rate (CR) among semen diluted with TFE, Triladyl and Andromed ($p < 0.05$).

Table 5. Conception rate of diluted semen.

Name	Conception rate, n= 15
Tris-fructose-egg yolk	80% (12) ^a
Triladyl	80% (12) ^a
Andromed	40.0% (6) ^b

N=15, Different letters (a, b) indicate differences among groups

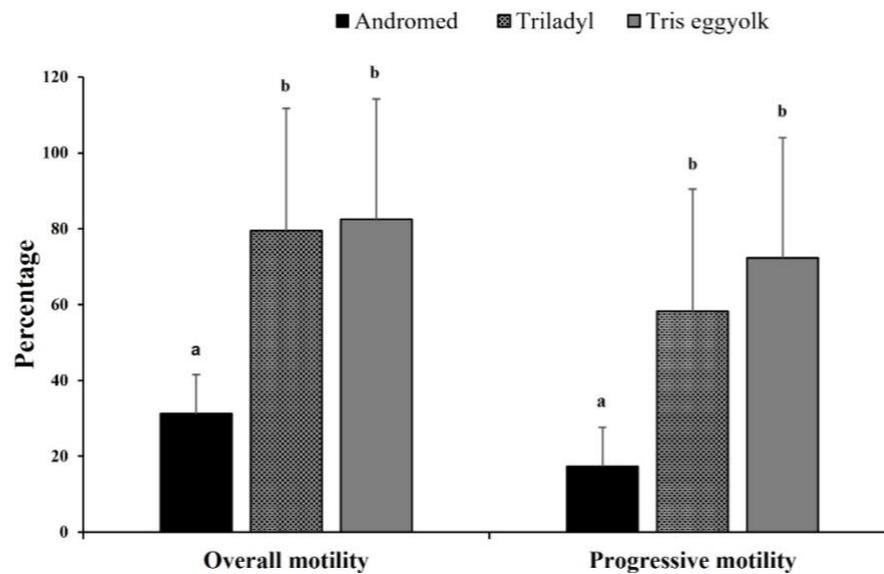


Figure 3. The recovery rate of cryopreserved semen with different diluters. Different letters (a, b) indicate differences among freezing groups ($p < 0.05$).

DISCUSSION

This is the first study investigating the cryopreservation efficacy of some diluters under a manual freezing technique using nitrogen vapor for Bangladeshi buffalo semen. Numerous cryopreservation methods have emerged, varying between cost-effective options yielding lower-quality semen and expensive methods limited to established and specialized facilities. Therefore, we have provided experimental proof for the efficacy of some easily available semen extenders using the inexpensive nitrogen vapor technique to cryopreserve buffalo semen under Bangladeshi conditions. This method can function without any established conditions and yields similar results to commercial biofreezers.

The findings of this study clinched that manual freezing by a gradual reduction of temperature in an affordable equilibration chamber, followed by nitrogen vapor, is a more cost-effective and efficient method for buffalo semen cryopreservation. The highest number of motile and progressive sperms and lower bent tails were found when semen was positioned 1.6 inches above the liquid nitrogen level. Furthermore, although motility was reduced after freezing, no significant differences were observed in motility and morphological features between semen cryopreserved using the commercial technique and those utilizing nitrogen vapor. Although motility experienced a reduction post-freezing, there was no significant difference in motility between the nitrogen vapor protocol and the commercial freezing technique. Previous studies have demonstrated that semen freezing in the nitrogen vapor by placing straws 4 to 5 cm above the LN (liquid nitrogen) for 10 to 15 minutes yielded positive results for cattle and ram semen [5, 14]. Placing semen straws in proximity to nitrogen causes a rapid temperature drop, while placing them at a distance result in gradual cooling, potentially leading to damage due to inconsistent temperature changes. It is evident that cold shock is comparably more pronounced during slow or rapid cooling [8]. Cryopreservation in nitrogen mist, a well-utilized method, entails placing semen straws in a liquid nitrogen tank to rapidly cool semen to a temperature of -196°C . However, a growing body of evidence suggests that rapid freezing can lead to the formation of ice

crystals around sperm, ultimately causing cell death [9]. Additionally, temperature fluctuations during the freezing and thawing process can impact early capacitation and damage acrosome, plasma membrane integrity, and sperm motility [15]. Hence, a gradual temperature reduction is recommended. We employed the nitrogen vapor technique where the temperature is lowered from 37°C to 5°C (at a rate of 1°C per minute) over 30 minutes in a semen equilibration chamber and then from 5°C to -120°C in 15 minutes within a Styrofoam box. This method induces less cytotoxic damage [16] and yields results similar to those achieved with expensive biofreezers. Therefore, this technique proves suitable for freezing buffalo semen.

This study also found that the highest semen motility, morphology, and recovery could be achieved using the locally developed Tris-fructose-egg yolk diluent, followed by Triladyl. Triladyl, a commercially available extender, is typically formulated under stringent laboratory conditions with precise concentrations of medium components. However, the improved performance of the TFE diluter could be attributed to the slightly higher glycerol content [14]. Glycerol, widely used in the freeze-thaw process, is a widely used cryoprotectant in freezing-thawing that works by reducing the formation of intracellular ice crystals through penetration into the cells and binding with water content [17]. Besides this, intracellular cryoprotectant also minimizes the difference in osmotic pressure [18]. However, several studies have indicated that optimal glycerol level is different for different species. For most species, the recommended glycerol concentration ranges between 3% and 5% [19], albeit a higher concentration of glycerol (6 to 7%) is recommended for buffalo. Nonetheless, glycerol's toxic effects have been described, with concentrations exceeding 6% showing negative impacts on post-thaw semen motility and morphology. This phenomenon is attributed to the slower action of glycerol, which struggles to replace intracellular water efficiently, thus disturbing the balance of osmotic pressure changes [20]. Therefore, the addition of glycerol in two-step is advisable. For instance, a study employed a two-step dilution strategy at an equilibrated temperature of 4°C to 5°C [21].

Regarding buffalo semen cryopreservation, a 6% to 7% glycerol concentration is recommended [22]. Nonetheless, glycerol's toxic effects have been documented, with concentrations exceeding 6% showing negative impacts on post-thaw semen motility and morphology [5]. This phenomenon is attributed to the slower action of glycerol that cannot replace intracellular water efficiently, thus disturbing the balance of osmotic pressure changes [21]. Therefore, adopting a two-step glycerol addition process is advisable. For instance, two studies reported adding glycerol gradually in two fractions, similar to this study, after cooling to 5°C [13,22]. It's noteworthy that the addition of glycerol before equilibration, as observed by Rosato et al., can lead to membrane instability, exacerbating changes in osmotic pressure, sperm concentrations, and calcium influx [23]. We added 6.4% glycerol in the TFE diluent in two steps, which may mitigate detrimental effects and achieved a high sperm recovery rate of 82.4%.

Moreover, both motility and morphology were higher in the TFE diluter. A study found that using 7% glycerol for freezing swamp buffalo semen resulted in the highest number of viable sperm and preserved plasma membrane integrity [24, 25]. However, the quality of bulls and semen is a crucial factor during cryopreservation. Another significant factor contributing to the enhanced efficacy of the TFE diluent is egg yolk. Most extenders consist of 20% egg yolk [19], and a previous study indicated that reducing its proportion to 10% significantly compromised semen quality in animals [26]. The presence of low-density lipoproteins (LDL) in egg yolk renders protection to the sperms by forming a layer around the plasma membrane. A study showed that utilizing 9% of LDL instead of whole egg yolk led to reduced sperm damage in frozen-

thawed pig semen [27]. However, it's worth noting that animal-origin cryoprotectants like egg yolk can have detrimental effects on semen quality due to compositional variations and the presence of contaminants that damage cellular integrity and reduce freezing capacity [28]. Therefore, plant-based cryoprotectants like soy lecithin could be considered, although the effectiveness of soy lecithin-based semen extenders has shown inconsistent results in some studies. Nevertheless, egg yolk prevails as the primary non-permeable cryoprotectant [18]. Furthermore, the suitability of the nitrogen vapor technique and the TFE diluent for buffalo semen is supported by the fertility test. Our study demonstrated that using the TFE diluent resulted in an 80% conception rate in buffalo. Cryopreservation has been reported to render approximately 50% of sperm immotile and damage the remaining sperm, leading to lower conception rates [28]. Moreover, it's important to note that good-quality semen doesn't always guarantee successful animal conception. For instance, a wealth of studies revealed that semen frozen using automatic freezing machines exhibited higher acrosomal integrity and post-thawing motility [29, 30]. Therefore, ensuring the insemination of buffalo with quality semen each time is essential for achieving higher conception rates. Our findings are particularly important given the challenges in heat detection in buffalo, which is hindered by delayed puberty, less distinct estrous behavior, and prolonged postpartum ovarian inactivity [31, 32]. As semen frozen using the nitrogen vapor technique and TFE diluent exhibited higher motility and superior morphology, it holds promise for the cryopreservation of indigenous buffalo in Bangladesh.

The most significant finding of this study was the higher sperm recovery achieved through the use of a cost-effective manual cryopreservation technique by nitrogen vapor with the locally developed Tris-fructose-egg-based diluent. These findings are particularly relevant for a developing country like Bangladesh. However, further research is needed to validate these findings and develop more efficient techniques that can be applied on a broader scale, especially at the farm level. Additionally, more field data is required regarding artificial insemination to assess the quality of cryopreserved semen in real-world settings accurately.

CONCLUSIONS

Freezing of Bangladeshi buffalo semen by a locally developed semen extender following a manual cryopreservation technique could be a potentially cost-effective option for the small-level farm and even for the individual. This study found no significant difference in motility and morphology of sperm cryopreserved with a commercial biofreezer protocol and the manual nitrogen vapor protocol. Moreover, the locally developed Tris-fructose-egg yolk diluter produced superior-quality semen. Efficient semen freezing is a critical factor for obtaining the desired rate of conception. Thus, this low-cost and easily available nitrogen vapor technique would allow moderate to large-scale farmers, artificial insemination centers and various research institutes to easily preserve semen of economically important native cattle breeds and provide it when necessary.

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AUTHOR CONTRIBUTIONS

MAK and SMJ conceptualized, acquired funding acquisition, and supervised the study. MAK, SFS and DD performed experiments and data analysis. AAN wrote the draft, created figures, and validated the study. All authors contributed to editing and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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