Article

Comparison between Manual and Automated Cryopreservation Techniques and Effects of Different Semen Extenders on Post-Thaw Semen Quality of Buffalo

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Simple Summary: Buffalo plays an important role in the economy and protein and nutritional requirements of Bangladesh. However, no study has investigated the cryopreservation techniques of Buffalo. Therefore, this study aimed to evaluate a manual freezing technique and some semen extenders suitable for buffalo semen. This study found that no statistical difference was observed in motility and morphology between semen cryopreserved in the nitrogen vapor technique and the automated technique. Moreover, the locally developed Tris-fructose-egg yolk-based diluter produced more post-thaw viable sperms than commercial diluters. Therefore, this technique and TFE diluent can be used for the cryopreservation of buffalo semen that can be used for artificial insemination.

Abstract: Cryopreservation has been used extensively for cattle in Bangladesh, albeit no study was conducted on the cryopreservation techniques of buffalo. This study compares two freezing methods and the effects of diluters on the semen quality of buffalo. In the first freezing protocol, semen was frozen in two-step: from 37 °C to 5 °C for 30 minutes in a BLRI-developed equilibration chamber and 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). At the same time semen was frozen in a programmable freezer in three steps. The semen samples were then evaluated for motility and morphological quality by CASA. In another experiment, the efficacy of one locally developed diluter-Tris-fructose-egg yolk-based (TFE) and three commercial diluters (Andromed, Triladyl and Steridyl) were evaluated. 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. There was no statistical difference in overall motility, progressive and slow motility between semen cryopreserved in the nitrogen vapor technique (57.49±5.67, 38.70± 4.04 and 3.83± 0.63, respectively) and automated technique (65.94±4.65, 45.54 ± 3.64 and 2.43± 0.36, respectively). The highest recovery rate and conception rate were observed in semen diluted with TFE (82.4% and 80%, respectively). Hence, the cryopreservation technique using nitrogen vapor and TFE diluent is suitable for freezing buffalo semen that would produce superior semen for artificial insemination.

Keywords: Cryopreservation; Nitrogen vapor; Buffalo semen; Bangladeshi buffalo; Diluents and extenders

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1. Introduction

Bangladesh, a South Asian country heavily depends on agriculture. The majority of the rural people engaged in farming including livestock and poultry rearing. Livestock and poultry account for about 1.44% of the gross domestic product (GDP) of Bangladesh in 2021 [1]. The livestock population of the country is around 56.33 million, of which, the buffalo population is around 1.5 million [1]. Although their population remains limited, buffalo contribute significantly to the national economy by producing meat and milk and by aiding agricultural production. The buffalo breeds in Bangladesh are categorized into indigenous, non-descript water buffaloes and migrated or cross-breeds such as Murrah, Nili-Ravi and Jaffrabadi [2]. The most beneficial and profitable feature of water buffalo is its efficient utilization of less digestible feeds. They can digest crude protein, dry matter and coarse feed more efficiently than cattle and convert the feeds into high-quality milk and meat [3]. Moreover, buffalo are more disease resistant and demand less attention and management for production [2]. Thus, long-term schemes are needed to utilize these beneficial resources.

However, for increasing buffalo production and developing improved breeds, high-quality semen is a must. Having good-quality semen regularly is quite challenging due to the poor genetic traits of buffalo, variability of feed and fodder and agroecological conditions. Hence, assisted reproductive technologies, predominantly artificial insemination (AI) using cryopreserved semen have received widespread popularity for transferring improved genetic potentialities and improving milk and meat production capacities [4]. The success of AI depends largely on the utilization of frozen semen and the techniques used for providing fertile frozen semen. Semen cryopreservation is a complex technique due to freezing them at a very low temperature (-198°C). The main steps are extension, equilibration or cooling, freezing, storage and thawing which may compromise semen quality by causing damage to the acrosome and plasma membrane [5]. Several semen freezing techniques such as freezing with liquid nitrogen in an automatic control-rate biofreezer or a hand-made box have been used extensively. The latter method is inexpensive but provides less accurate results because of low cell recovery caused by the formation of ice crystals in sperm cells [6]. During cryopreservation, up to 50% of sperm cells can be damaged by stresses that occur due to ice crystal formation, chemical toxicity and osmotic stress which ultimately result in a lower conception rate [7, 8]. Therefore, most commercial AI centers use commercial programmable freezers that can maintain temperature gradient and minimize ice crystal formation which renders consistent post-cell recovery [9]. However, these commercial machines are very expensive, can be used in specialized facilities and demand experienced users to operate. Nevertheless, most of the agriculture-producing countries are developing or least developed where the majority of farms can hardly afford the high-cost freezers. Hence, it has created a need to develop a system that would be economical and provide an acceptable rate of viable post-thawed semen in typical production settings.

Therefore, this study aims to assess a cost-effective cryopreservation system for buffalo as well as other cattle that appears to provide results similar to a commercial biofreezer. It involves a self-developed semen equilibration chamber and nitrogen vapor

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in a Styrofoam box for semen preservation. If corroborated, this new economical freezing system will allow AI centers, research centers and medium-holding farmers to obtain more benefits from AI that uses frozen semen and help improve the cattle industry in developing and least-developed countries.

2. Materials and Methods

2.1. Experimental Animals

Semen was collected from five animals of the Murrah buffalo breed. This research was conducted from February 2022 to August 2022.

2.2. Collection and evaluation of semen

Semen was collected twice a week using an artificial vagina and kept in a water bath to maintain the temperature of 35° C to 37° C. Before freezing, different characteristics of semen (color, volume, concentration) were evaluated following the methods of Jha et al [10]. Haemocytometer was used to measure the sperm concentration rate (>500*106/ml). Sperm motility and morphological features were assessed by Computer Assisted Sperm Analyzer (CASA). Then semen was cryopreserved using two methods; one using the BLRI-developed equilibration chamber and a Styrofoam box and another using a commercial biofreezer (TurboFreezer M, minitube, Germany)

2.3. Dilution of Semen

Semen samples having motility>70%, normal morphology and concentration (>500x106/ml) were diluted with a locally developed Tris-fructose-egg yolk-based semen extender medium for getting spermatozoa concentration of 20 x 106 per straw. The TFE diluter was prepared following Baiee et al [11]. The composition is described in Table 1. Briefly, the diluent was split into two fractions with all the components; one had no glycerol and the other contained 12.8% of glycerol. After mixing gradually, the final concentration of glycerol was 6.4% which was then aliquoted into test tubes.

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

2.4. Freezing procedures and determining the straw position on nitrogen vapor

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. 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 from different distances (0.5, 1.5, 1.6, 2 and 3 inches) in 15 min. 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. Finally, the straws were plunged into liquid

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nitrogen at -196 °C and stored. At the same time, buffalo semen straw was cryopreserved using a minitube programmable automated freezer. In a brief, semen straw was transferred into the semen 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, semen straws were thawed at 37 °C for 30-60 seconds and examined by CASA.

2.5. Effects of different diluters cryopreservation using nitrogen vapor technique

We compare the efficacy of the TFE semen extender with some other extenders on sperm characteristics. Hereupon, three commercial diluters (Andromed, Triladyl and Steridyl) were used for buffalo semen. Following dilution, semen was cryopreserved in the above-mentioned protocol and quality was assessed by CASA.

2.6. Data Analysis

Descriptive statistics were performed first. 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 SPPS (Version 25.0) Moreover, the sperm recovery rate was calculated by the following formula:

Sperm Recovery Rate (%) = (post-thaw motility/pre-frozen motility) × 100

3. Results

3.1. Optimizing the distance for semen cryopreservation by nitrogen vapor

Table 2 describes the optimum distance for semen cryopreservation using nitrogen vapor. As expected, there were significant differences in different distances regarding sperm motility. Both motile sperm and progressive motility (PM) were increased with the increase of distance for a certain point and then significantly decreased. The highest number of motile sperms (62.67±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±1.10; $P < 0.001$) at the same distance. However, there was no significant difference in the morphology of cryopreserved semen at different distances except for that in the bent tail. The bent tail was significantly lower (4.30±0.85 $P < 0.05$) in semen cryopreserved at 1.6 inches above liquid nitrogen.

Table 2: Optimizing the distance for semen cryopreservation by nitrogen vapor technique

Distance	Motility (%)		Morphology (%)				
	Motile sperm	Progressive motility	Bent tail	Coiled tail	DMRI	Distal Droplet	Proximal Droplet
Fresh 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
1.6 inches	$62.67^{(b)}$ ± 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
1.5 inches	$47.13^{(c)}$ ± 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

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2 inches	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 inches	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
Significance	0.000	0.001	0.020	0.228	0.633	0.204	0.726

3.2. Comparison of motility and morphology of cryopreserved semen in nitrogen vapor and a commercial biofreezer protocol

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

Table 3: Comparison of cryopreserved semen motility and morphology on nitrogen vapor technique and a commercial freezer (minitube- TurboFreezer)

Variables	Motility (%)			Morphology (%)				
	Motile sperm	Progressive motility	Slow motility	Bent tail	Coiled tail	DMRI	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
Significance	0.044	0.005	0.012	0.285	0.188	0.344	0.517	0.170

The number of motile sperm after cryopreservation in the commercial freezing technique was 65.94±4.65 which is not statistically different from semen motility in the nitrogen vapor technique (57.49±5.67). The data also revealed that PM and the number of slow sperm were similar in semen cryopreserved by both techniques. Moreover, there was no difference among the morphological features of pre-frozen semen and semen cryopreserved by a commercial or nitrogen vapor technique (Figure 1 B-D).

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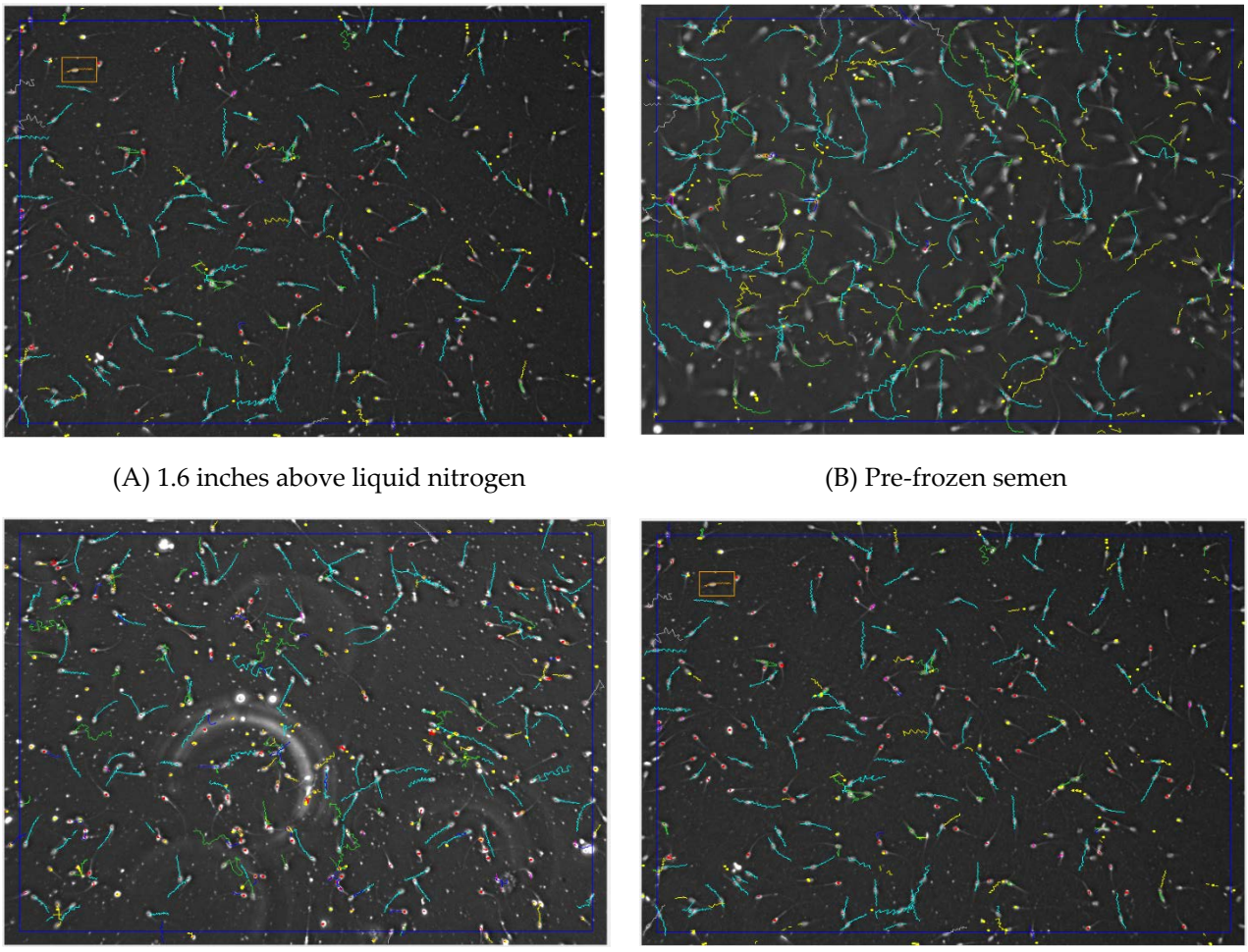

(A) 1.6 inches above liquid nitrogen

(B) Pre-frozen semen

(C) Minitube

(D) Nitrogen vapor technique

Figure 1. Motility and Morphology of cryopreserved semen analyzed by CASA

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

After determining the height of cryopreservation by nitrogen vapor, we next assessed whether the diluters had any effect on sperm motility and morphology. It can be observed from Table 4 that there are variations in sperm characteristics of semen cryopreserved with Andromed, TFE and Triladyl. Briefly, sperm quality was significantly low in semen cryopreserved with Andromed (27.5±19.24% motile sperm) while motile sperms were higher in both TFE (72.69±15.92%) and Triladyl (70.07±16.40%). Besides, no significant difference was observed between PM in TFE (49.13±17.16%) and Triladyl (70.07±16.40%) but both of these are significantly different from Andromed.

Table 4: Semen motility and morphology after using different diluters in the nitrogen vapor technique

Diluters	Motility (%)			Morphology (%)			
	Motile sperm	Progressive motility	Slow motility	Bent tail	Coiled tail	DMRI	Distal Droplet
Fresh	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}

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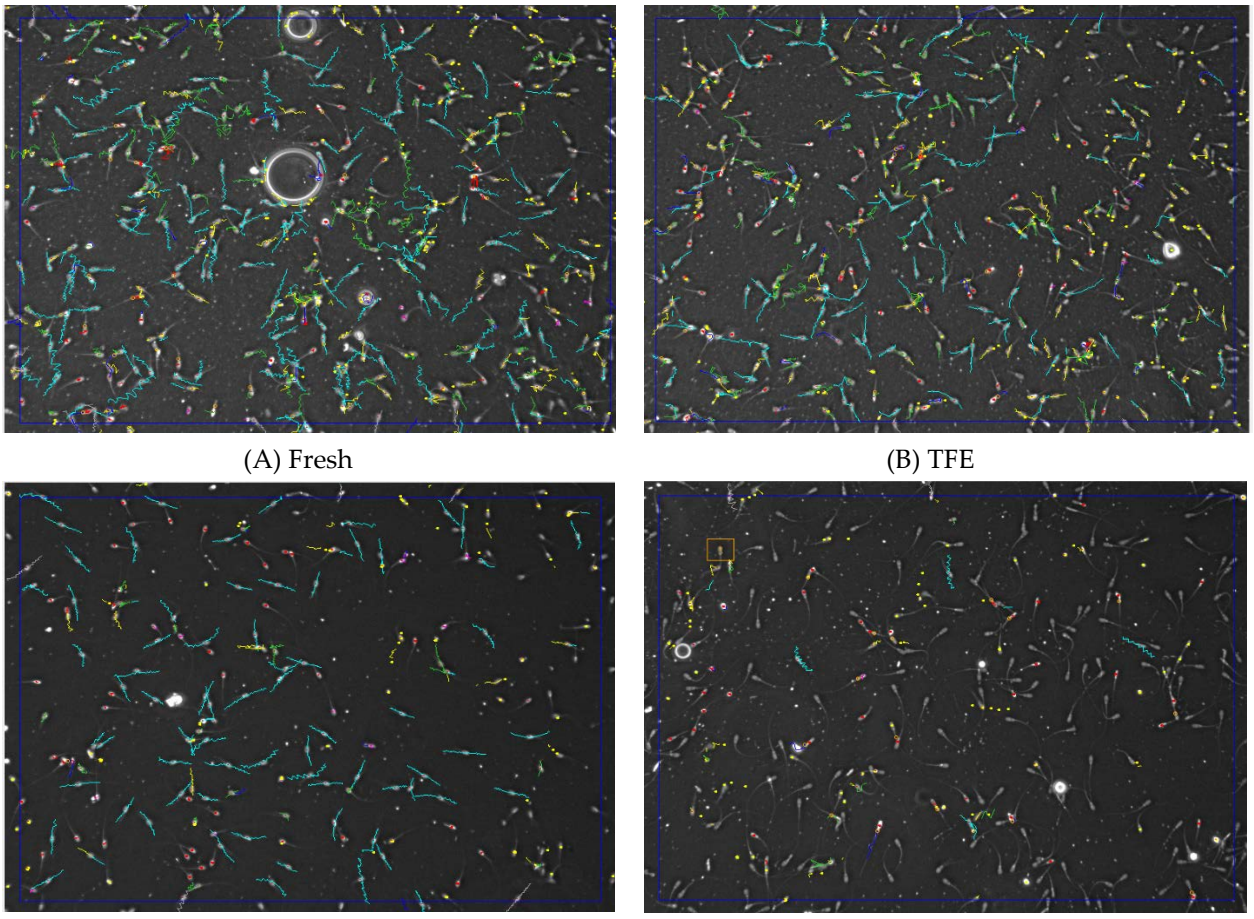
TFE	72.69±15.92^{b}	49.13±17.16^{b}	5.89±9.23^{b}	5.82±3.97^{a}	0.39±0.25^{a}	2.93±1.48^{b}	2.40±2.17^{a}
Andromed	27.5±19.24^{a}	11.76±10.76^{a}	1.19±1.33^{a}	13.49±5.97^{b}	0.82±0.60^{b}	1.59±0.50^{a}	4.87±1.77^{c}
Triladyl	70.07±16.40^{b}	39.54±13.40^{b}	5.38±3.13^{a}	5.80±3.26^{a}	0.55±0.60^{a}	3.40±1.66^{b}	3.18±1.57^{b}
Significance	0.00	0.00	0.06	0.00	0.11	0.00	0.00

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



(A) Fresh (B) TFE

(C) Triladyl (D) Andromed

Figure 2. Effect of different diluters on semen motility and morphology analyzed by CASA

The recovery rate after cryopreservation of semen is depicted in Figure 1. Similar to semen motility and morphology, the recovery rate was highest in sperm frozen with TFE (82.4%) followed by Triladyl (79.5%). In the case of PM, semen cryopreserved with TFE had a significantly high recovery rate (72.3%). Moreover, the number of abnormal semen in TFE is lower than in other diluters.

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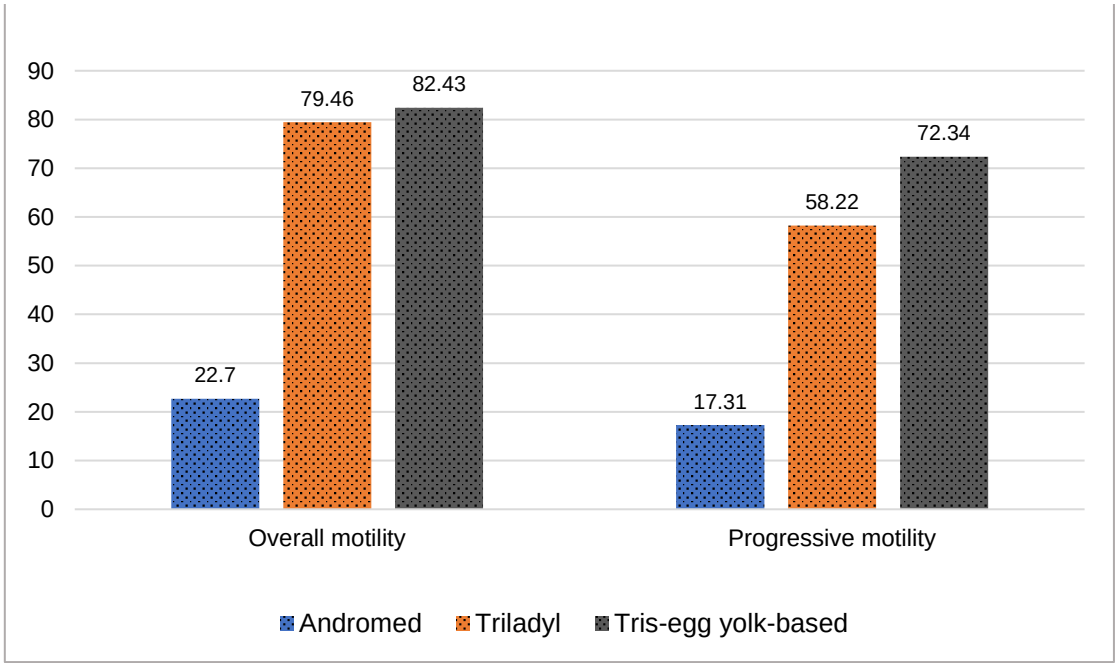

Figure 3. Recovery rate of cryopreserved semen with different diluters

Table 5 depicts that CR was higher in buffalo inseminated using semen diluted with both TFE and Triladyl while there was a significant difference in the CR among semen diluted with TFE, Trildyl and Andromed.

Table 5: Conception rate of diluted semen

Name	Conception rate (N)
Tris-fructose-egg yolk	80% (12)
Triladyl	80% (12)
Andromed	40.0% (6)

Discussion

This is the first study on the cryopreservation of buffalo semen by nitrogen vapor. Since the discovery of the cryopreservation technique, it has been a widely used assisted reproductive technology in livestock sectors. Many methods of cryopreservation have been developed which are either inexpensive and provide low-quality semen or very expensive and can be used in established and selected facilities. Therefore, we established a cryopreservation technique by inexpensive equilibration chamber and nitrogen vapor for freezing buffalo semen that can work without any established conditions and produces similar results to a commercial biofreezer.

The findings of this study clinched that manual freezing by reducing the temperature in an inexpensive equilibration chamber followed by nitrogen vapor is a cheaper and more effective process for buffalo semen cryopreservation. The highest number of motile and

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progressive sperms and lower bent tails were found when semen was placed 1.6 inches above liquid nitrogen. Furthermore, there was no difference among the motility and morphological features of semen cryopreserved by a commercial and nitrogen vapor technique. In this study, although motility was reduced after freezing, there was no significant difference in motility between the nitrogen vapor technique protocol and the commercial freezing technique. Previous studies demonstrated that manual freezing of semen by placing the straws 4 to 5 cm above the LN for 10 - 15 min yield good results for cattle and ram [5, 12].

However, no studies have been conducted on buffalo semen. When semen straws are placed close to nitrogen, the temperature drops sharply while placing straws at a long distance would cool the straws at a very slow rate which could lead to potential damage. It is evident that cold shock is comparatively higher during slow or fast cooling [8]. Cryopreservation in nitrogen mist has been a widely used method where semen straws are placed in a tank containing liquid nitrogen until reaching a temperature of -196°C to cool semen very quickly. But a growing body of evidence revealed that rapid freezing of semen can escalate the formation of ice crystals around the sperm that would ultimately result in cell death [9]. Moreover, during the freezing and thawing process, changes in temperatures affect early capacitation and damage acrosome, plasma membrane integrity and sperm motility [13]. Therefore, a gradual reduction in the temperature is recommended. By the nitrogen vapor technique, the temperature is decreased from 37 °C to 5 °C, (1 °C decrease/minute) in 30 minutes in a semen equilibration chamber and 5 °C to -120 °C in 15 min in a Styrofoam box containing liquid nitrogen which induces less cytotoxic damage [14] and produces results similar to an expensive biofreezer. Therefore, this technique can be used for freezing buffalo semen.

In the present study, semen motility, morphology and recovery rates were best with locally-developed Tris-fructose-egg yolk diluent followed by Triladyl. Triladyl is a commercial extender that might be developed in a highly controlled laboratory environment and precise concentrations of medium components. However, the better performance of TFE diluter might be due to the use of glycerol to a slightly higher percentage [14,28]. Glycerol is a widely used cryoprotectant in freeze-thawing that reduce the formation of intracellular ice crystal by entering the cells and binding with water content [15]. Besides this intracellular cryoprotectant also minimizes the difference in osmotic pressure. However, a large number of studies implicated that differences exist regarding the optimum level of glycerol during cryopreservation in different species. For most species, the recommended level of glycerol is 3–5%[16]. In the case of buffalo semen cryopreservation, 6 to 7% of glycerol concentration is recommended [17]. However, glycerol has toxic effects and studies depict that glycerol concentration >6% had negative effects on post-thaw semen motility and morphology [5]. This is attributed to the slow action of glycerol that cannot replace intracellular water efficiently and leads to the impaired balance of osmotic pressure changes [18]. Therefore, adding glycerol in two steps is recommended. For instance, Anel et al. used glycerol in two-step dilution at equilibrated temperature (4°C–5 °C) [19]. However, Valente et al. [20] and Baiee et al. [11] added glycerol gradually in two fractions like this study after cooling to 5 °C. Moreover, Rosato

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et al. found that adding glycerol before equilibration can render membrane instability and aggravate changes in osmotic pressure changes, sperm concentrations and calcium influx [21]. Our TFE diluent had 6.4% of glycerol that was added gradually in two steps, therefore, reducing the detrimental effect and providing the highest sperm recovery rate (82.4%). Additionally, motility and morphology were also superior in TFE diluter. Herbowo et al. found that the use of 7% glycerol for freezing swamp buffalo semen produced the highest number of viable sperm and plasma membrane integrity [20, 22]. However, the quality of bulls and semen needs to be considered during cryopreservation and our study demonstrated that the nitrogen vapor technique and TFE diluent can render 57-70% viable sperms after freezing and post-thaw. Another notable constituent that contributed to the increased efficacy of TFE is egg yolk. Most diluters are composed of 20% egg yolk [16] and a previous study reported that reducing its proportion to 10% considerably vitiated the semen quality of animals [23]. The presence of low-density lipoproteins in the egg yolk mainly renders protection to the sperms by forming a layer around the plasma membrane. The use of 9% of LDL instead of whole egg yolk causes less sperm damage and produced high-quality sperm in frozen-thawed pig semen [24]. However, cryoprotectants of animal origin like egg yolk have detrimental effects on semen quality due to variations in composition and the presence of bacterial and other contaminants which damage cellular integrity and reduce freezing capacity [25]. Hence, plant-based cryoprotectants such as soy lecithin can be used albeit some studies reported inconsistent results regarding the efficacy of soy-lecithin-based semen extenders and egg yolk continues to be used as the predominant non-permeable cryoprotectant [16].

The suitability of the nitrogen vapor technique and TFE diluent is also supported by the conception rate. It has been reported that cryopreservation rendered about 50% of sperm immotile and damaged the remaining sperm which ultimately leads to low CR [25]. Interestingly, several studies found no difference in semen quality of cryopreserved semen in nitrogen vapor and automatic, programmable freezing [26]. Moreover, good-quality semen cannot always guarantee conception in animals. For instance, Almquist et al. and Landa et al. showed that semen frozen in automatic freezing machines had higher acrosomal integrity and post-thawing motility [26, 27]. However, the conception rate of cattle with semen cryopreserved in automatic freezing machines was lower than that with semen cryopreserved in nitrogen vapor. Several studies in Bangladesh reported lower CR in cattle due to variance in breeds and lack of appropriate semen diluents and freezing techniques. Hossain et al showed that the overall CR was only 59.29% among 224 cattle [28]. Therefore, inseminating buffalo with quality semen every time is necessary to obtain a higher conception rate. We showed that semen frozen with TFE diluent produced 80% of conception in buffalo. Heat detection in buffalo is very difficult owing to delayed puberty, less pronounced estrous, and prolonged postpartum inactivity of the ovary [29, 30]. Since semen frozen by nitrogen vapor technique and TFE demonstrated higher motility and retain greater morphology, this can be used for cryopreserving indigenous buffalo of Bangladesh.

The most significant finding of this study was a higher sperm recovery using a BLRI-developed equilibration chamber and nitrogen vapor in a Styrofoam cryobox and locally-

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produced TFE diluter which are suitable for a developing country like Bangladesh. However, these findings need further research to corroborate and develop a more efficient machine and technique that can be generalized at the farm level. More field data is needed regarding artificial insemination to ascertain semen quality cryopreserved in the laboratory setting.

5. Conclusions

The cryopreservation technique by BLRI-developed low-cost equilibration chamber and nitrogen vapor is suitable for the cryopreservation of native buffalo semen that can be used for artificial insemination. This study found no significant difference in cryopreserved semen motility and morphology between a commercial biofreezer and the nitrogen vapor technique. Moreover, the locally-developed Tris-fructose-egg yolk diluter produced superior-quality semen. Thus, this technique would allow moderate to large-scale farmers, AI centers and various research institutes to easily preserve semen of economically important native cattle breeds and provide it when necessary. Efficient semen freezing is a critical factor in AI for obtaining the desired rate of conception. Nitrogen vapor technique is expected to play an important role in the preservation of cattle breeds as well as new breed development.

Abbreviations: AI: Artificial insemination; CASA: Computer Assisted Sperm Analyzer; TFE: Tris-fructose-egg yolk; PM: Progressive motility; CR: Conception rate

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Informed Consent Statement: Not applicable

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Conflicts of Interest: The authors declare no conflict of interest.

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