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Motility, viability and morphological defects in fresh and liquid preserved semen of indigenous Bengal sheep

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Abstract

Indigenous Bengal sheep is a potential native germplasm of Bangladesh. Evaluation of semen quality is essential to preserve the semen for future use. This study focuses the fresh and short term preserved (at 4°C) liquid semen quality of indigenous Bengal sheep. Thirty ejaculates were evaluated, then diluted using egg yolk-citrate diluter and preserved for 5 days at 4°C. Fresh semen parameters e.g. volume (ml), concentration ($\times 10^6$), progressive motile (%), and live spermatozoa (%) were 0.70 ± 0.04 , 4812.00 ± 92.05 , 72.63 ± 0.58 , and 80.18 ± 0.55 , respectively. Total sperm motility was significantly ($p < 0.05$) highest ($80.42 \pm 0.97\%$) at Day 0 while lowest ($52.67 \pm 2.47\%$) at Day 5 during preservation. Progressive motility percentage was found to be the best at Day 0 ($69.93 \pm 0.32\%$) followed by Day 1 ($64.75 \pm 0.45\%$), Day 2 ($59.87 \pm 0.86\%$), Day 3 ($52.58 \pm 0.64\%$), Day 4 ($40.09 \pm 3.27\%$) and Day 5 ($31.12 \pm 2.96\%$) whereas oscillatory and rotatory motility percentage showed opposite trend. Viable spermatozoa percentage significantly ($p < 0.05$) dropped gradually from 71.63 ± 0.76 to $32.74 \pm 1.78\%$, while morphological defects (head, mid piece and tail) increased with preservation time, highest at Day 5 and lowest at Day 0. The quality of Bengal sheep semen declined as the liquid preservation at 4°C duration was extended, and after three days, progressive motility retention was more than 50%.

Key words: Indigenous ram, semen, short-term preservation, artificial insemination, egg yolk-citrate

Introduction

The sub-tropical climate of Bangladesh is favourable for raising sheep and it is becoming popular because of its adaptability, survivability, and disease resistance. They are comparatively more resistant to stress, environmental (climate) changes and some diseases like parasitosis, coldness and other infective disease like PPR (Flowers, 1997). Sheep are less susceptible to harsh weather and have a lower death rate than goats. The number of sheep increased 2.53 times over the last 12 years in Bangladesh, with an average annual growth rate of 5% and sheep are mostly raised for meat production and rank third in number behind cattle and goat population (Mowsume et al., 2023). Jamuna, Barind, and coastal region sheep are the three major regional groups of indigenous sheep in Bangladesh. The major challenge of sheep raising in Bangladesh is timely inseminate the ewes in heat by natural/artificial means. Though AI in sheep is popular worldwide but the scenario is different in Bangladesh. Same poor quality ram has been used generation after generation thereby increasing the chances of inbreeding. Moreover, good quality ram is not always available in the areas. So, popularize AI in indigenous Bengal sheep using liquid preserved or frozen semen is time demanding. Now the major challenge is to store and supply quality semen among the farmers.

Reduced or stopped spermatozoa metabolism prolongs their fertile life, which is necessary for the preservation of semen. During liquid preservation, motility and sperm morphological integrity are gradually declining which affects fertility (Zlatarev et al., 1988; Maxwell, 1978; Jones and Martin, 1973). Liquid preservation involves storing semen at cool temperatures (around 4-5°C), which minimizes the potential damage caused by ice crystal formation in cryopreservation. Liquid-preserved semen had significantly ($p < 0.05$) higher post-thaw sperm motility and fertilization rates compared to cryopreservation. This could be due to the reduced damage to sperm cells during liquid preservation (Younis et al., 1999).

Since spermatozoa use glucose as a source of energy, more efficient energy metabolism would promote sperm motility during preservation. Alternatively, pyruvate provides metabolic intermediates such as malate, oxaloacetate, and acetyl-CoA (Hereng et al., 2011, Nascimento et al., 2008) and proper functioning of the processes during liquid storage is essential to maintain the motility of sperm (Nascimento et al., 2008). Sperm cells are subjected to artificial

circumstances during the liquid storage procedure, which causes oxidative stress—an imbalance between antioxidants and pro-oxidants following an excess of reactive oxygen species (ROS). Preservation of ram semen is critical due to high PUFA content and high sensitivity of ram sperm to oxidative stress. Additionally, mitochondrial respiration, ATP synthesis, and the decreased ATP level are all inhibited by oxidative stress. Under physiological conditions, low concentrations of ROS, which are typical by products of cell metabolism, are essential for sperm functions such as sperm capacitation, acrosome response, and binding to the zona pellucida (Bansal and Bilaspuri, 2011; Aitken and Curry, 2011; Zini et al., 1995; De Lamirande *et al.*, 1997). High concentrations of ROS are linked to the inhibition of normal sperm function and subsequent peroxidation of polyunsaturated fatty acids present in the sperm membranes, that may change the morphology of sperm (Maia et al., 2010; Peris et al., 2007; Aziz et al., 2004).

Furthermore, ram semen held for one to three days at 4°C or 23°C had a higher AI conception rate than several weeks at the -196° (Wusiman et al., 2012; Kulaksiz et al., 2012). Ram semen has been preserved using a variety of extenders. Typically, glucose phosphate solution, Tris plus egg yolk, egg yolk-citrate solution, lactose solution, fresh and dried skim milk, homogenized whole milk, as well as commercial diluents have been used to dilute semen (Salamon and Maxwell, 2000; Maxwell et al., 1996; Maxwell and Salamon, 1993). Better post-thawed semen characteristics were demonstrated by skim milk and egg yolk in this species, which were comparable to Tris-based buffer (Rota et al., 2001). Researchers found that egg yolk enhanced sperm cell viability and motility after thawing, which led to greater semen preservation (Fioratti et al., 2014). Ram spermatozoa are protected during cold storage by egg yolk inclusion in the extender (Gill et al., 2011). Similar to other livestock species, maintaining the motility, viability, and sperm fertility during preservation is essential to the effectiveness of artificial insemination in sheep. It is reported that extender type and preserved temperature for the storage of spermatozoa play an important role in both frozen and non-frozen sperm survival rate. Therefore, the current research was conducted to explore the fresh semen quality, short term preserved semen quality using egg yolk citrate diluter and the effective time of liquid semen preservation at 4°C in indigenous Bengal sheep.

Materials and methods

Experimental Site

The research was conducted at the Bangladesh Agricultural University Artificial Insemination Centre (BAU AI Centre). This area belongs to sub-tropical climatic zone. The longitude and latitude is 24.73 North 90.42 East, respectively with an average temperature ranges from 23.47°C to 32.49°C.

Management of Experimental Animal

The semen was collected from three selected indigenous Bengal sheep (ram) bearing tag number BBC-121, BBC-222 and BBC-123 and their body weight were 27.3, 26.8 kg and 25.4 kg respectively. All of them were 3.5 years old. Each animal was fed a total of 300 g Vushi Mixed (Charoen Pokphand Group company) and 100 g germinated gram/head at 8 am every day. Green grass was supplied adlibitum throughout the research period. Vaccination against PPR (*Peste des petits ruminants*) was applied in every 6 months, deworming was applied in every 3 months, and necessary medicare were also provided whenever needed.

Preparation of EYC (Egg Yolk - Citrate) Diluter

2.94% sodium citrate solution and 100,000 IU diluted pronapen solution were combined in 100 milliliters of distilled water to prepare the egg yolk-citrate diluter (Herman and Madden, 1963). Then one part egg yolk and four part sodium citrate solution were mixed thoroughly with a stirrer.

Collection of semen:

Semen was collected from each Bengal ram twice a week using artificial vagina. Immediately after the collection, semen was kept in water bath at 37°C until further analysis.

Ejaculate Volume

Using the graduated collection vial, the volume of the ejaculate was quantified in milliliters (Mortimer, 2000).

Fresh Semen Evaluation

Fresh semen was assessed right away after it was collected. Semen color and consistency were assessed on the naked eye to assure the fitness of raw semen for further evaluation.

Mass Motility

A drop of semen was studied using a phase-contrast microscope (10X) to assess the mass motility. The slide was pre-warmed and had no cover slip. It was assessed and graded as a percentage ranging from zero to one hundred depending on the mass movement of the sperm and the existence of waves.

Individual Motility

On a clean, previously warmed (37°C) slide, a tiny drop of semen was applied, and it was covered with a coverslip after being diluted with sodium-citrate buffer media. Eye estimation technique was used to estimate motility and the percentage of sperm moving gradually forward at increased magnification (40X) was recorded separately. In order to increase evaluation accuracy in this instance, at least 200 spermatozoa were counted. Individual motility was divided into three categories such as *progressive motility*- sperms having forward movement, *Rotatory*- sperms having clockwise or anti-clockwise movement and *Oscillatory*- sperms having signs of life without forwarding movement. The same procedure was followed for evaluating semen diluted with EYC extender.

Sperm Concentration

Fresh spermatozoa concentration (million/ml) was determined using haemocytometer. In a standard red cell dilution pipette, semen sample was taken up to 0.5 marks and dilution fluid up to 101 marks. Proper mixing was ensured through eight-knot motion for 3-5 minutes. To get well mixed sperm, first 4 to 5 drops were discarded. After that a drop of semen was allowed to run the cover slip which was placed on counting chamber of haemocytometer. Five large squares were counted under low magnification (25X) (Chowdhury et al., 2023).

Morphological Evaluation

Rose Bengal staining technique was used to evaluate head, midpiece and tail morphology (Chowdhury et al., 2023). A small drop of semen sample was diluted using sodium-citrate buffer solution and made a smear of it. After air drying the smear was dipped into Rose Bengal stain for 5 minutes and after rinsed with water, it was air dried again. At least 200 sperms were randomly counted from the several fields under the phase contrast microscope (40X).

Sperm Viability

The viability of ram sperm was assessed using the Eosin-Nigrosin stain (Agarwal et al., 2006). Two drops of 1% eosin solution were gently combined for 15 seconds with a drop of thawed, thoroughly mixed semen sample that had been placed on a clean, warmed glass slide. Then, it was blended with 2 drops of a 10% aqueous nigrosin solution. As soon as possible, a thin smear was prepared and air dried. After that using a phase contrast microscope (40X), the fixed slides were observed. Spermatozoa that were red or dark pink in color were considered as dead, while those that were white or faint pink were considered as alive. A total of 200 sperms were randomly counted per replication.

Dilution and preservation of semen:

After initial analysis of semen, the selected ejaculates were diluted with EYC (Egg Yolk - Citrate) diluter. After dilution, semen was preserved in a refrigerator at 4° C. Thereafter, semen was evaluated for motility, viability and morphology according to the previously mentioned procedure at 0, 24, 48, 72 and 120 hours of preservation.

Statistical Analysis

Descriptive Statistics of semen parameters were carried out using Graph Pad Prism 5.0 software. The effects of different treatments were assessed using One-way ANOVA, and the DMRT was used to separate the means. 5% level of significance ($p < 0.05$) was considered as significant.

Results

Seminal characteristics in fresh semen of Bengal sheep are presented in the Table 1. This table revealed that the average semen volume was 0.70 ml, concentration 4812.00 million, and progressive motility 72.63% and live spermatozoa 80.18%.

Table 1. Fresh semen parameters of indigenous Bengal sheep

Parameter	Pooled value (Mean±SE)
Volume (ml)	0.70±0.04 (30)
Color (1 to 4 scales)	4.00 (creamy white) (30)

Concentration ($\times 10^6$)	4812.00 $\pm$ 92.05 (30)
Total Sperm/Ejaculate ($\times 10^6$)	3364.20 $\pm$ 226.90 (30)
Mass Motility (%)	86.50 $\pm$ 0.76 (30)
Total Individual Motility (%)	81.83 $\pm$ 0.46 (30)
Progressive (%)	72.63 $\pm$ 0.58 (30)
Oscillatory (%)	6.80 $\pm$ 0.65 (30)
Rotatory (%)	2.49 $\pm$ 0.17 (30)
Live (%)	80.18 $\pm$ 0.55 (30)

Parenthesis indicates number of observations

Sperm Morphology of Fresh Semen

Different types of abnormalities of semen are shown in the Table 2. Overall abnormality was 15.46% in which tail abnormality contributed the maximum (5.76 $\pm$ 0.39%). Among all the three types of deformities, head abnormality was lowest (3.81 $\pm$ 0.48%). The sperm head defects (%) were noted as bent head, knotted head, head detachment, smaller and larger head, acrosome defects (acrosomal swelling and acrosomal detachment). On the other hand, tail defects were noted as tail detachment, tail knotting and bent tail.

Table 2. Morphological defects of indigenous Bengal sheep spermatozoa (Mean $\pm$ SE)

Morphological defects	Pooled value (Mean $\pm$ SE)
Total abnormalities (%)	15.46 $\pm$ 0.61 (30)
Head abnormality (%)	3.81 $\pm$ 0.48 (30)
Mid-piece abnormality (%)	5.29 $\pm$ 0.56 (30)
Tail abnormality (%)	5.76 $\pm$ 0.39 (30)

Parenthesis indicates number of observations

Effects of Preservation Time on Total Individual Motility (%) of Spermatozoa Preserved at 4°C

We compared the quality of spermatozoa up to five days (Table 3). Individual and progressive motility reduced significantly with the passage of preservation time. Reduction of progressive motility decreased significantly ($p<0.05$) while abnormality percentage increased significantly ($p<0.05$).

Table 3. Individual motility percentage of Bengal sheep spermatozoa in different preservation period at 4°C (Mean±SE)

Parameter	Duration of storage					
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Non- motile (%)	19.58 ^d ±0.97	22.13 ^d ±0.88	24.27 ^d ±1.48	31.14 ^c ±0.96	40.09 ^b ±3.36	47.33 ^a ±2.47
Total Motility (%)	80.42 ^a ±0.97	77.87 ^a ±0.88	75.73 ^a ±1.48	68.86 ^b ±0.96	59.91 ^c ±3.36	52.67 ^d ±2.47
Progressive (%)	69.93 ^a ±0.32	64.75 ^{ab} ±0.45	59.87 ^b ±0.86	52.58 ^c ±0.64	40.09 ^d ±3.27	31.12 ^e ±2.96
Oscillatory (%)	7.95 ^c ±0.72	9.29 ^{bc} ±0.60	11.14 ^b ±1.20	11.30 ^b ±0.88	14.38 ^a ±1.36	15.97 ^a ±0.96
Rotatory (%)	2.54 ^c ±0.24	3.82 ^{bc} ±0.42	4.72 ^b ±0.52	4.98 ^{ab} ±0.48	5.44 ^a ±0.66	5.57 ^a ±0.39

^{a,b,c} Values with different superscripts within the same row differed significantly from each other ($p<0.05$). Thirty ejaculates were considered for this study

Effects of Preservation Period on the Morphological Defects with Respect to Head, Mid-piece and Tail

Table 4 displays the comparative study of different types of sperm abnormalities during a five days long preservation period. Head, mid-piece and tail defect percentage was lowest at day 0, then gradually increased with the extension of preservation time and become highest at day 5.

Statistically there was no significant difference between day 0 and day 5 in case of mid-piece defects but had significant difference ($p<0.05$) in sperm head and tail defects among the preservation periods.

Table 4. Effects of preservation periods on the percentage of morphological defective spermatozoa (Mean $\pm$ SE)

Morphological defects	Duration of storage					
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Head %	3.30 ^b $\pm$ 0.29	3.90 ^{ab} $\pm$ 0.47	3.99 ^{ab} $\pm$ 0.36	4.10 ^{ab} $\pm$ 0.47	4.82 ^a $\pm$ 0.38	5.17 ^a $\pm$ 0.52
Mid-piece %	4.96 ^a $\pm$ 0.58	5.09 ^a $\pm$ 0.48	4.81 ^a $\pm$ 0.47	5.90 ^a $\pm$ 0.32	5.88 ^a $\pm$ 0.54	5.23 ^a $\pm$ 0.80
Tail %	4.99 ^b $\pm$ 0.57	5.44 ^{ab} $\pm$ 1.26	5.89 ^{ab} $\pm$ 1.02	5.94 ^{ab} $\pm$ 0.58	7.05 ^{ab} $\pm$ 0.68	7.73 ^a $\pm$ 0.70

^{a, b} Values with different superscripts within the same row differed significantly from each other ($p<0.05$)

Effects of Preservation Time on Viable Sperm (%)

Table 5 shows the live spermatozoa percentage during storage at 4°C from day 0 to 5 days. This percentage varied ($p<0.05$) among the days considered. Highest was observed in day 0 (71.63 $\pm$ 0.76%) while lowest in day 5 (32.74 $\pm$ 1.78%). In Figure 1 also depicts that the dead spermatozoa number significantly ($p<0.05$) increased with the increase of preservation period.

Table 5. Effects of preservation periods on the proportion of viability of spermatozoa percentage (Mean $\pm$ SE)

Duration of storage	Viability (Live Sperm %)
Day 0	71.63 ^a $\pm$ 0.76
Day 1	66.89 ^b $\pm$ 0.62
Day 2	61.34 ^c $\pm$ 1.28

Day 3	56.39 ^d ±1.34
Day 4	42.15 ^e ±2.43
Day 5	32.74 ^f ±1.78

^{a, b, c} Values with different superscripts within the same column differed significantly from each other ($p < 0.05$)

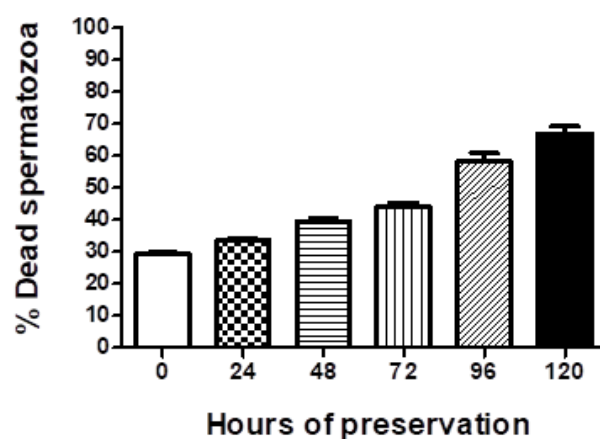


Figure 1: Effects of preservation periods on the percentage of dead spermatozoa

Discussion

The normal ram ejaculate volume ranged from 0.2 to 2.0 ml (Mahmuda et al., 2015; Azizunnesa et al., 2014; Hernandez et al., 2012; Hamidi et al., 2011; Gil et al., 2003; Seremak et al., 1999; Moss et al., 1988). The semen volume depends on the production of seminal fluid mainly. The semen volume of indigenous ram is low due to the size of accessory glands. However, the semen volume varies according to age, breed, frequency of semen collection and collection method. Indigenous ram semen color was usually creamy white to white. According to Bag et al. (2002), ram semen ranges in color from pale creamy to milky-white. Azizunnesa et al. (2014) reported the color of fresh semen was creamy white immediately after collection. According to Hafez and Hafez (2000) semen may turn pink or brownish due to blood, urine, and feces.

In this study, sperm concentration in fresh semen sample of the experimental indigenous Bengal sheep varied from 4.6×10^9 to 4.9×10^9 per ml and the mean value was obtained 4.8×10^9 ml. These results coincides with the previous findings (Pervage et al., 2009; Moss et al., 1988). Carter et al. (1990) reported the mean total number of sperm concentration collected was 5.3×10^9 /ml in ram which is higher than present studied results of indigenous ram. Kulaksiz et al. (2012) recorded spermatozoa concentration of 2.5×10^9 /ml, and Guerrero et al. (2009) recorded spermatozoa concentration of 3.50×10^9 /ml ejaculates were collected from 3 Akkaraman rams and these results are lower than present study. There are a several factors that can affect the concentration of sperm in ejaculates, including breed, age, body weight, season, individual performance variations, and semen collecting frequency.

In the present study, sperm motility of Bengal sheep ranged from 75.39% to 84.6% and the average motility was recorded $81.83 \pm 0.46\%$. Roohalleh and Sirouseythe (2015) reported that motility of 80.0% in ghezel ram semen and Guerrero et al. (2009) found individual sperm motility $87.0 \pm 2.4\%$, these results are almost similar to the present study results. Asaduzzaman et al. (2021) reported that the individual motility of indigenous ram and Muzaffarnagari cross were $80.0 \pm 1.09\%$ and $81.67 \pm 0.93\%$. These outcomes align with the findings of the current investigation. The individual motility of the Karradi and Arrabi rams was reported by Al-Samarrae (2009) to be $60 \pm 1.89\%$. which is less than the current study. The variation in breed, strain, age, and location could be the cause of the wide variability in motility.

In the fresh semen samples from Bengal sheep, the percentage of viable spermatozoa was found to be $80.18 \pm 0.55\%$. Hassan et al. (2009) observed that the spermatozoa viability of local adult sheep was $91.7 \pm 3.6\%$. According to Azizunnesa et al. (2014) and Mahmuda et al. (2015), the viability of native ram semen were and 88.0% and 88.2%. Compared to the current study, these results are higher. Al-Samarrae (2009) reported the viability of Karradi and Arrabi rams was 74.0% and 70.25% which are less than the current investigation. Asaduzzaman et al. (2021) reported that the viability of indigenous ram and Muzaffarnagari cross were 90.93% and 91.27% those are also higher than present study. The differences in the viability of ram spermatozoa may be affected by the many factors such as season, age, collection techniques, and semen collection frequency. The normal head shape of ram spermatozoa was cylindrical, not wide in diameter.

The sperm head defects (%) were noted as acrosome defects (acrosomal swelling and acrosomal detachment), head detachment, bent head, smaller and larger head, knotted head, respectively. The mean value (%) of sperm defect was recorded $3.81 \pm 0.48\%$. The sperm mid-piece defects were recorded as mid-piece detachment, mid-piece knotting, mid-piece bent and mid-piece swelling. The mid-piece defect sperm was observed $5.29 \pm 0.56\%$. The sperm tail defects were recorded as tail knotting, tail detachment and bent tail. The percentage of sperm with defective tail was $5.76 \pm 0.39\%$. In the current study, morphologically normal sperm was 84.54% and total defective sperm was 15.46% where head defects 3.81%, mid-piece defects 5.29%, and tail defects 5.76% in indigenous Bengal sheep semen. Al-Samarrae (2009) reported that total abnormal spermatozoa of Karradi and Arrabi rams was recorded $17.13 \pm 0.72\%$ and $18.0 \pm 0.68\%$. According to Hernández et al. (2012) reported morphologically normal spermatozoa percentages was recorded 94.0% that is higher than present study. Numerous factors, including age, semen collection frequency, and the conditions of management, may be responsible for variations observed in the proportion of morphological abnormalities of spermatozoa in the study.

In the present study, non-motile spermatozoa percentage gradually increased ($p < 0.05$) with the extension of preservation period. On day 5 (120 hours), a significantly ($p < 0.05$) higher percentage (7.73 ± 0.70) of tail defect was found. Defective or abnormal spermatozoa percentage increased gradually from 0 to 5 day. Additionally, Gundogan et al. (2011) reported that storage duration had a significant ($p < 0.01$) effect on the abnormal spermatozoa rate, which was steadily increasing during the storage period. Maksimović et al. (2008) evaluated the spermatozoa abnormality with different diluters and stated that $9.0 \pm 1.57\%$ at 0 hr and $11.33 \pm 2.03\%$ abnormality at 24 hr of liquid preservation of ram semen at 4°C . Significantly ($p < 0.05$) greater percentage ($71.63 \pm 0.76\%$) of live spermatozoa was found on Day 0 (0 h) which gradually decreased day after day. On the contrary, percentage of dead spermatozoa increased with the progress of storage time. Rahman et al. (2008) observed $75.0 \pm 0.5\%$, $62.8 \pm 0.5\%$, $51.8 \pm 0.5\%$ and $44.5 \pm 0.6\%$ viability at 0, 24, 48 and 72 hr, respectively. The number of days in storage and the extenders had a strong correlation with the decline in sperm viability (Rahman et al., 2018). Herdis et al. (2020) conducted an experiment on liquid storage of Garut ram sperm at ($3-5^{\circ}\text{C}$) and found that $85.60 \pm 6.19\%$, $75.20 \pm 3.5\%$, $69.00 \pm 7.31\%$ and $66.25 \pm 3.89\%$ viability at day 1, 2, 3 and 4, respectively. Rather et al. (2017) observed that viability of ram spermatozoa preserved at

4°C were $82.91 \pm 2.29\%$, $78.44 \pm 3.29\%$, $75.94 \pm 4.38\%$ and $70.26 \pm 4.31\%$ at 0, 24, 48, and 72 hr, respectively and viability was significantly affected by preservation period.

When spermatozoa are preserved at 5°C, their metabolism fully stops, de-assimilation products emerge, and temperature effects combine to cause the degeneration of spermatozoa's morphological integrity and reduce the fertility of semen (Menchaca et al., 2005). Some investigators recommend that in order to prevent the decline in semen fertility caused by refrigeration, it is necessary to lower the temperature slowly and uniformly during the equilibration time (Decuadro-Hansen, 2004). Additionally, diluents should be used with the addition of cryoprotectants. Lowering temperature rates, the kind of cryoprotectant agent, its concentration, osmotic pressure, dilution rate, and temperature of extender when semen is diluted are the variables that influence the success of AI using refrigerated stored semen in sheep (Salamon and Maxwell, 2000). The negative effects of temperature decrease on ram spermatozoa are reduced by adding cryoprotectant agents to the diluents at higher concentrations and applying them before the diluted semen attains 5°C (Gil et al., 2003).

Spermatozoa degrade with time in storage, regardless of the diluents, their dilution rate, storage conditions or temperature. According to Qureshi et al. (2013) sperm motility decreased in liquid storage in the first twenty-four hours following collection. On the other hand, Vaughan (2003) noted that sperm motility decreased significantly between 24 and 48 hours of liquid storage. Kulaksiz et al. (2012) recommend that the maximum storage duration is 6 to 12 hours without compromising fertility. According to Maxwell and Salamon (1993) liquid store ram semen for longer than 12 to 24 hours reduces its virility. In contrast, Vishwanath and Shannon (2000) found that the quality of ram liquid semen for more than two to three days without compromising its quality. This coincided with the findings of the present study.

According to Jones and Mann (1973) storing ram semen at 5°C results in the production of organic peroxides. Peroxidation causes irreversible motility loss in ram sperm, inhibits fructolysis and respiration, leaks intracellular enzymes, damages plasma membrane structurally, especially in acrosomal area (Jones and Mann, 1977a). It has also been documented that fatty

acid peroxides cause a motility loss in the sperm of stallion, rat and boar (Jones and Mann, 1977a), bull (Jones and Mann, 1977a), human (Diezel et al., 1980). The peroxidation of membrane lipids has been linked to severe damage to the plasma membrane in sperm of bull (Slaweta et al., 1988), rabbit (Alvarez and Storey, 1982), and rooster (Fujihara and Howarth, 1978). Egg yolk was shown to lower the peroxidation rate in the ram sperm (Jones and Mann, 1977b) while research by Shannon (1972) revealed that increasing proportion of egg yolk increased the dead sperm toxicity in semen held at room temperature (5% vs 20% egg yolk) (Shannon, 1972). When the ram semen is cooled to 5°C, several investigators have discovered that egg yolk at low concentrations has positive effects (Watson and Martin, 1975).

Phospholipids and cholesterol, components of the membranes, play a key role in cold shock, which results in sperm lipid loss. Low cholesterol levels and a high unsaturated:saturated fatty acid ratio in the phospholipids are linked with sperm susceptibility to cold shock. Sperm's high unsaturated fatty acid concentration also makes them susceptible to peroxidation damage, which has a negative impact on their ultrastructure, metabolism, motility, and fertility. Fatty acid peroxidation produces hydroxynonenal, which reduces ram sperm motility and oxygen intake *in vitro* and may interact with the axonemal microtubules -SH groups (White, 1993).

When ram, boar and bull sperm are cold shocked to 0°C and even higher loss occurs after the spermatozoa abruptly freeze to -70°C then thaw, there is a significant loss of total phospholipid likely due to the removal of plasma (Darin-Bennett et al., 1973). This is especially interesting considering the phospholipid-rich egg yolk has long been used as a component of diluents in artificial insemination (Watson and Martin, 1976).

Sperm viability loss following cold shock or freezing surely involves the disruption of sperm plasma membranes. Phospholipids and cholesterol, which are membrane components (Parks and Ehrenwald, 1990), might be crucial to sperm for maintaining the structural integrity of the several highly organized membrane systems. So, changes during the liquid preservation of semen includes the reduction in spermatozoa motility as well as morphological integrity, together with decreased survival in the reproductive tract of female, decreased fertility, and increased the embryonic loss (Maxwell and Salamon, 1993). The use of semen that had been kept for longer

than 24 hours for cervical insemination resulted in a sharp loss in fertility; nevertheless, some spermatozoa were still able to fertilize after 10 days following intrauterine insemination. So, fertility could be improved with laparoscopic intrauterine or transcervical inseminations. These methods may improve the ova fertilization and decrease embryonic loss by resolving the issues of the sperm transport via cervix and spermatozoa aging in the reproductive tract (Maxwell and Salamon, 1993).

Therefore, using ram semen kept at 5°C may be an alternative option rather than frozen semen when storage time is limited and laparoscopy is used for insemination. Compared to cryopreservation, this semen conservation method uses less expensive lab supplies and shorter processing time (López-Sáez et al., 2000).

Conclusions

Motility, morphology and viability in fresh semen of indigenous Bengal sheep are satisfactory. Semen quality decreased with the progress of preservation time and semen diluted with egg-yolk citrate can be preserved up to three days with 50% progressive motility that could be used for AI. However, to improve the indigenous ram semen quality during liquid storage, diluters using antioxidants or antioxidant like compounds may be the good, pertinent strategy.

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