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Assessment of Semen Quality of Two Ram Breeds at Pre-freeze Stage of Cryopreservation

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Abstract

Pre-freeze semen quality is a prediction for an efficient and successful freezing in cryopreservation. This study was designed to assess the semen quality of two ram breeds at pre-freeze stage of cryopreservation. A total of 30 ejaculates (5 ejaculates per ram) were collected using artificial vagina twice a week for a period of two months from two sexually active ram breeds; indigenous (n=3), and Muzaffarnagari crossbred (n=3), and evaluated for semen characteristics. Ejaculate volume, color, mass activity, and sperm concentration were evaluated for fresh semen while motility, viability, morphologically normal sperm, intact membrane, intact acrosome values were evaluated in both fresh (37^{0}C) and glycerol equilibrated (5^{0}C, pre-freeze) stage. Sperm concentration was found significantly ($p<0.05$) higher in Muzaffarnagari crossbred rams. Sperm motility, viability, morphologically normal, intact membrane, and intact acrosome values have not differed significantly ($p>0.05$) at the fresh stage but varied significantly ($p<0.05$) at pre-freezing stage. Therefore, it can be predicted that the selected breeding rams are of good quality freezer for frozen semen production.

Keywords: Indigenous, Muzaffarnagari, Pre-freezing, Semen Evaluation

Introduction

The ram's potentiality and efficiency in breeding or semen production for artificial insemination (AI) are determined by physical examination, inspection of reproductive organs, semen collection, and evaluation (Pezzanite *et al.*, 2017). Semen volume, sperm concentration, motility, and morphology allow detection and elimination of clear-cut cases of male infertility or subfertility (Verstegen *et al.*, 2002). The color indicates damage or infection in reproductive tract. Sperm motility, viability, and membrane integrity are sturdy indicators of sperm functionality (Pena *et al.*, 2005). In artificial insemination, many researchers recommended up to 15% morphologically abnormal sperm for fertility (Fernandez *et al*., 2004; Malama *et al*., 2013). The lost acrosome, membrane damaged, and de-capacitated sperm cells cannot fertilize oocytes (Mazur, 1984). Hence, the intact acrosome is crucially important for transit, penetration, acrosome reaction and fertilization (Neild *et al*., 2005; Juyena, 2011; Partyka *et al*., 2012).

During cryopreservation process, semen from certain males can be frozen with less cryoinjury than that of others, which implies males as good or bad freezers (Watson, 2000). The addition of glycerol has toxic effect on spermatozoa (Holt, 2000) and exerts osmotic damage (Purdy, 2006), which causes structural damage and decreases sperm motility. The glycerol equilibration time and freezing protocol affect semen quality and fertility (Edwin and Ulaganathan, 1988; Leboeuf *et al.*, 2000). The quality of seminal traits is important indices of quality semen production and correlated significantly with freezability and/or fertility (Fiaz *et al.*, 2010). The motility, livability, and acrosomal integrity are practically applied in routine semen evaluation to predict freezability, preservability, and fertility of spermatozoa (Chikhaliya *et al*., 2018). Pre freeze poor quality spermatozoa are the indicator of a poor freezable semen sample. Knowing the score of one variable, the researchers can ideally predict the score on the second variable (Ho, 2006; Gupta and Singh, 2018). There are few studies on pre-freeze semen qualities in Bangladeshi native ram, and no study is obtainable for Muzaffarnagari crossbred ram semen. Therefore, the current study was aimed to assess semen quality at the pre-freezing stage to predict the good or bad semen freezer ram for quality frozen semen production. Moreover, the study would be important for the efficient use of time, labor, and liquid nitrogen in freezing procedure.

Materials and Methods

Study Location

The study was conducted at the reproduction laboratory at Bangladesh Agricultural University (BAU), Bangladesh, from September to December 2018. The study site is located from 24.730 N latitude to 90.440 E longitude, and receives on a mean 174 mm of rainfall with the mean annual minimum to maximum temperatures vary from 16.46 to 29.13ºC, respectively.

Animals and Their Management

Two ram breeds- indigenous and Muzaffarnagari crossbreds maintained at Sheep Research Farm, Bangladesh Agricultural University, Mymensingh were utilized. Rams were grazed on natural grazing land daily for 6 to 7 hours, supplemented with close to 300 g of mixture feeds per head, containing maize grit, wheat bran with di-calcium phosphate, and salt (NaCl). Rams were provided free excess to safe drinking water ad-libitum thrice a day and dewormed routinely.

Reagents and Diluent Preparation

Eosin-Negrosin (Sigma-Aldrich Chemie Steinheim, Germany) stain was prepared as per formula of Evans and Salamon, (1987). The hypo-osmotic solution was prepared as per formula of Jeyendran *et al*. (1992) using sodium citrate (Merck, Germany) and fructose (Research -lab Fine Chem Industries, Mumbai, India). Sodium citrate (2.9%) buffer was prepared by tri-sodium citrate dehydrates (Merck, Germany) with phosphate buffer saline. Giemsa stain was prepared as per description of Mohteshamuddin and Tandle (2015). Tris-citric acid egg yolk glycerol extender was prepared according to Acharya (2017) using Tris (hydroxymethyl) aminomethane, citric acid, fructose (Research-lab Fine Chem Industries, Mumbai, India), Glycerol (Merck, Germany), Gentamycin (The ACME Laboratories Ltd. Dhaka, Bangladesh) and egg yolk.

Study Procedure

Six breeding rams (indigenous, n=3 and Muzaffarnagari crossbred, n=3) were randomly assigned for semen analysis at the initial and pre-freeze stage of cryopreservation. Seminal traits of 30 ejaculates (15 ejaculates from every breed) were assessed for the prediction regarding freezer rams. All the equipment used for semen collection was maintained in sterile and dry conditions until use. The semen samples were collected by artificial vagina (AV) prepared following the guideline of Vishal (2014). The ejaculate volume was measured directly from the graduated collecting tube (Elsharif, 2010; Mafolo, 2018). The colour of ejaculate was assessed directly by visual observation (Elsharif, 2010) and was graded as per Jha *et al*. (2013). Mass activity, sperm motility and sperm concentration were studied according to procedure of Avdi (2004), Moghaddam *et al.* (2012) and Jha *et al.* (2018), respectively. The viability (live-dead) was studied using eosin-nigrosin stain as per standard procedure (Sitali *et al.*, 2017). Sperm morphology was assessed by screening morphological abnormalities using the same stained slide (Vishal, 2014, Sitali *et al.*, 2017, Rai *et al.*, 2020). Plasma membrane integrity was evaluated following the procedures of Jeyendran *et al.* (1984); Nalley and Arifiantini (2013); Ahmad *et al.* (2014). The sperm acrosomal damage was studied in Giemsa-stained smears following the studied of Watson, (1975), Barth and Oko (1989), Mohteshamuddin and Tandle (2015), and Mir *et al.* (2012).

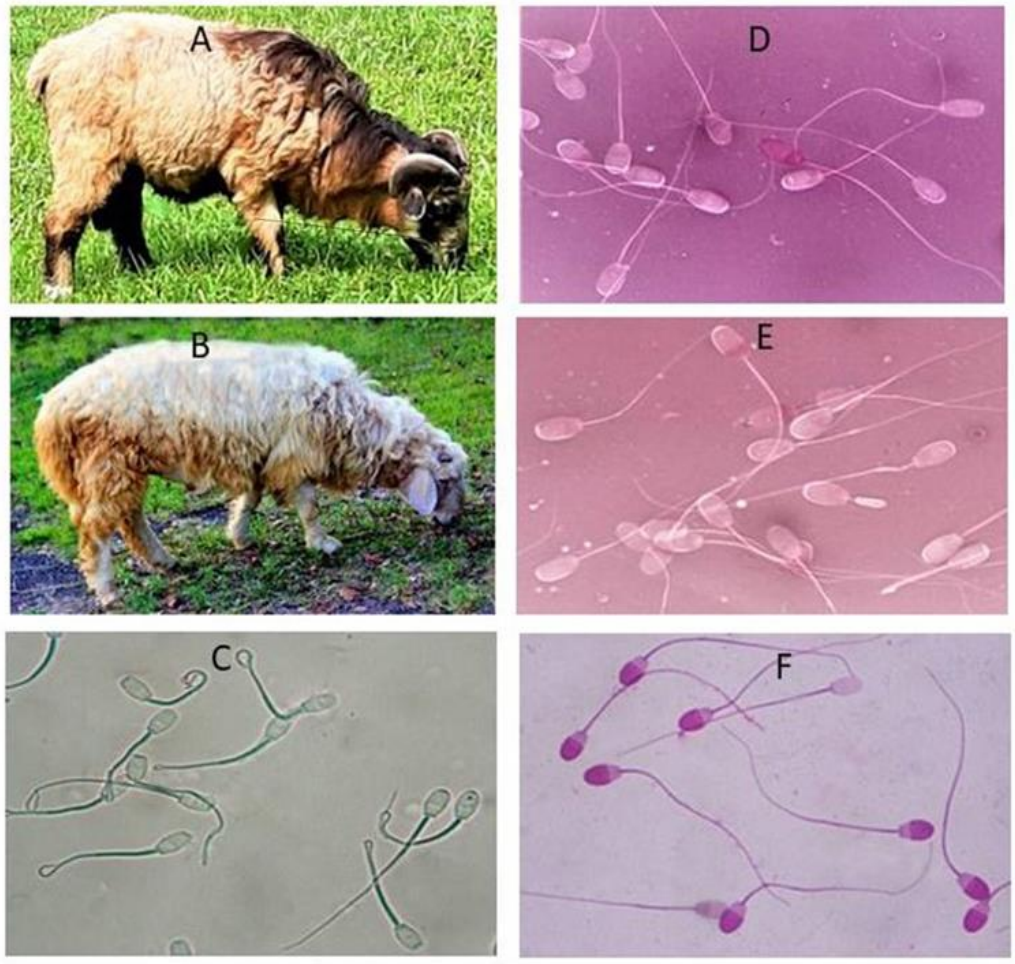

Figure 1: (A) Indigenous ram, (B) Muzaffarnagari crossbred ram, (C) Sperm membrane integrity (curled and coiled tail), (D) Sperm viability (live-dead), (E) Sperm morphology (normal and abnormal), (F) Sperm acrosome integrity (Intact, complete and partial loss)

Before semen collection, fresh well churned egg yolk was added @ 15% of both parts (A and B) of diluents and placed in a water bath at 37°C.After collection and short evaluation, the semen sample was diluted with half of the calculated volume of diluent from part A (v/v), and the rest half of the diluent from part B in another tube was cooled in a refrigerator for two hours at 5°C.After two hours of chilling, the second step dilution was made by adding refrigerated part B diluent. After two hours of equilibration in a refrigerator at 5°C, diluted semen underwent pre-freeze evaluation for motility, viability, morphology, plasma membrane integrity, and acrosome integrity. The

data was analyzed with a statistical procedure using Microsoft Excel 2010 software. The means (± SE) values were determined using descriptive statistics; and One-Way ANOVA (Single Factor) was used to compare seminal attributes of two breeds of ram. The result was presented as mean ± standard error (Snedecor and Cochran, 1994). Significant difference was considered at ($p < 0.05$).

Results and Discussion

Ejaculate Volume

The ejaculate volume had no significant ($p>0.05$) difference between breeds (Table 1). The semen volume recorded in indigenous rams was in agreement with Mahmuda *et al*. (2015) and Jha *et al*. (2018) were to be 0.6 ± 1.0 to 0.8 ± 0.2 and 0.6 ± 0.3 to 0.9 ± 0.3 ml, respectively. The recorded ejaculate volume in Muzaffarnagari crossbreed ram was in corroborates with the observations of Boediono (2004), Tejaswi *et al.* (2016) and Rajashri *et al.* (2017) were to be 0.7 ± 0.01, 0.71 ±.15 and 0.75 ± 0.07 ml. in Garut, Nari Suvarna and Deccani rams, respectively.

Table 1: Fresh semen characteristics in Bangladeshi indigenous and Muzaffarnagari crossbred rams

Seminal characteristics	Breeds of Ram		
	Indigenous (n = 15)	Muzaffarnagari cross (n = 15)	F values
Volume (ml)	0.77 ± 0.04	0.7 ± 0.04	0.217^{ns}
Color (1-4 grades)	3.73 ± 0.118	3.87 ±0.091	0.800^{ns}
Mass activity (1-5 grades)	3.6 ± 0.11	3.73 ± 0.12	0.571^{ns}
Sperm concentration (10^6/ml)	364.47 ± 7.363	392.60 ± 5.707	9.121^{*}

*ns: Not significant, *Significant, p<0.05*

Semen Colour

The color observed in almost all semen samples were not significantly ($p> 0.05$) different in either of the breeds (Table 1). The studied values were in line with the observations of Boediono (2004), Elsharif (2010), Moghaddam and Pourseif (2014), Jha *et al. (*2018) were found to be 3.75 ± 0.00, 3.75 ± 0.00, 3.61 ± 0.41, and 3.5 ± 0.8 - 4.0 ± 0.0 grades in Garut, Hamari, Ghezelx Baluchi, and Bangladeshi native ram, respectively

Sperm Mass Activity

The mass activity showed no significant ($p>0.05$) difference between breeds (Table 1). The current findings in both breeds were in agreement with the observations of Moghaddam and Pourseif (2014) in Arkhar Merino x Ghezel cross ram, and Rahman *et al.* (2015) in Bangladeshi native rams.

Sperm Concentration

The average sperm concentration of Muzaffarnagari crossbred ram semen was found to be significantly ($P<0.05$) higher than indigenous rams (Table 1). The significant difference in concentration recorded in the present study may be due to variation in the age and weights of rams (Salhab *et al.,* 2003) and breed characteristics. However, the findings of Muzaffarnagari crossbred ram were comparable with the observation of Azubuike *et al.* (2017) in Yankasa rams. The sperm concentration of indigenous rams in the current study was in agreement with the observed values as Zel ram, Bapedi ram, and Bangladeshi native ram reported by Ahmadi Hamedani *et al.* (2016), Mafolo (2018) and Jha *et al.* (2018), respectively.

Sperm Motility

The sperm motility in fresh semen of indigenous and Muzaffarnagari crossbred rams were not significant ($p>0.05$) between breeds (Table 2). The observed motility in both breeds was comparable with Mahmuda *et al.*, (2015), Jha *et al.,* (2018) in Bangladeshi native ram and Rajashri *et al.* (2017) in Deccani sheep. The pre-freeze sperm motility in both breeds was decreased significantly ($p<0.05$) from the initial stage and was not significant between breeds. However, the present pre-freeze findings were in line with Mahmuda *et al.* (2015), Ahmadi Hamedani *et al.* (2016), Khalil *et al.* (2020) in Bangladeshi native, Zel, and Ossimi ram.

Table 2: Seminal attributes in Bangladeshi indigenous and Muzaffarnagari crossbred rams at neat and pre-freeze stages

Seminal attributes	Ram types	Fresh semen	Pre-freeze	F value
Sperm motility (%)	Indigenous	80 ± 1.09	74.67 ± 1.333	9.582*
	Muzaffarnagari cross	81.67 ± 0.93	77.33 ± 0.826	12.071*
	F value	1.346 ns	2.890ns	
Live sperm (%)	Indigenous	90.93 ± 0.74	80.73 ± 1.22	50.826**
	Muzaffarnagari cross	91.27 ± 0.57	73.67 ± 0.86	290.054**
	F value	0.126 ns	22.299a	
Normal sperm (%)	Indigenous	85.33 ± 0.60	82.66 ± 0.52	11.256*
	Muzaffarnagari cross	85.27 ± 0.64	83.13 ± 0.74	4.763*
	F value	0.005 ns	0.267 ns	
Membrane intact (%)	Indigenous	81.93 ± 0.77	75.07±0.87	35.129**
	Muzaffarnagari cross	82.4 ± 0.77	69.73 ± .92	111.370**
	F value	0.184 ns	17.785a	
Acrosome intact (%)	Indigenous	94.73 ± 0.75	92.6 ± 0.53	5.413*
	Muzaffarnagari cross	95.8 ± 0.39	91.27 ± 0.37	70.365**
	F value	0.216 ns	4.217b	

*a,b different superscripts in the same column indicate significant differences (p<0.05), ns: Not significant, * significant at 5%. ** significant at 1%*

Live Sperm Percentage

The average live sperm percentage in fresh semen of indigenous and Muzaffarnagari crossbred rams were not significant (p>0.05) between the breeds (Table 2). The observed live percentage in both breeds corroborated with Mahmuda *et al.* (2015), Hossain *et al.* (2016), and Jha *et al.* (2018) in Bangladeshi native rams. Furthermore, the current findings were also agreed with Boediono (2004) and Azubuike *et al.* (2017) in Garut and Yankasa rams. The live spermatozoa decreased significantly (p<0.01) at the pre-freeze stage than that of the initial stage and between the breeds. These differences may be due to cold shock and breed characteristics. However, live spermatozoa of the pre-freeze semen were consensus with Mahmuda *et al.* (2015), Ahmadi Hamedani *et al.* (2016), Kurmi *et al.* (2018) in Bangladeshi native, Zel and Chhotanagpuri rams, respectively.

Normal Sperm Percentage

The normal sperm percentage had no significant (p>0.05) difference between breeds at the initial stage of fresh semen evaluation (Table 2). The current findings were in line with Mahmuda *et al.* (2015), Hossain *et al.* (2016), Jha *et al.* (2018) in Bangladeshi native rams. There also no significant (p>0.05) differences in normal sperm percentage at the pre-freeze stage between breeds, but a significant (p<0.05) decrease was observed at the pre-freeze stage from the initial stage of evaluation in both breeds. These findings were in agreement with Mahmuda *et al.* (2015), Bangladeshi native ram, and Ahmadi Hamedani *et al.* (2016) in Zel ram.

Sperm Membrane Integrity

The statistical analysis revealed no significant difference (p>0.05) in the sperm membrane intactness of fresh semen between the two breeds (Table 2). The current findings were in close agreement with Hossain *et al.* (2016) and Jha *et al.* (2018) in Bangladeshi native ram, and Valente *et al.* (2010) in Portuguese and Salvia rams. The sperm membrane intactness of the pre-freeze stage decreased significantly (p<0.01) than that of the initial stage and between the breeds. These differences may be due to cold shock and breed characteristics. However, the pre-freeze sperm membrane intactness was in line with Ahmadi Hamedani *et al.* (2016), Kurmi *et al.* (2018), and Khalil *et al.* (2020) in Zel, Chhotanagpuri, and Ossimi rams, respectively.

Acrosome Integrity

The acrosome intactness in the fresh semen had no significant (p>0.05) difference between breeds (Table 2). The

present findings were in line with Jha *et al.* (2018) in Bangladeshi native ram, comparatively higher to Boediono (2004) and Mohlomi (2015) in Garut, and South African Mutton Merino ram, respectively. These differences may be due to semen additives and breed characteristics. There was a significant ($p<0.05$) decrease in the percentage of acrosome intact between breeds and also at the pre-freeze stage (Table 2). A decrease in acrosome intactness was due to the damage caused to the acrosome during dilution and cooling (Tasseron *et al.*, 1977). However, the present findings were in line with the observation of Banday *et al.* (2017) in crossbred ram.

Conclusion

The semen samples of our selected two ram breeds were good at the pre-freezing stage of cryo-freezing. The observed motility, viability, and intact membrane were to be >70%, and normal morphology and acrosome integrity >90%. Therefore, it could be concluded and predicted that the selected breeding rams were good quality rams for efficient frozen semen production.

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Conflict of Interests

There is no conflict of interest.

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