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Duration of Preservation Affect the Quality of Chilled Black Bengal Buck Semen

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ABSTRACT

The study focuses on the quality assessment of Black Bengal buck semen preserved at chilled condition. In this *in vitro* trial, collected semen from Black Bengal bucks was preserved at chilling temperature (4～5℃) in tris-glucose-citrate yolk medium of 1:5 ratios for four days. Artificial Vagina (AV) method was utilized to collect semen from buck. General evaluation of semen includes the color, mass activity and density were measured by direct visual examination. However, computer-assisted sperm analysis (CASA) and phase contrast microscopy were used to figure out the motility (%), hyper-activated (HYP) motility (%) and number of abnormal spermatozoa (%) initially, and at every 24 h intervals. The result revealed that spermatozoa preserved at chilling temperature showed significantly (P<0.05) lower motility and HYP motility with the progression of preservation. The number of phenotypically abnormal spermatozoa significantly (P<0.05) increased following preservation. Although significant positive correlation (r=0.945; P<0.05) was existed between % motile and % HYP motile spermatozoa however, the % of morphologically abnormal spermatozoa was negatively correlated with % motile (r=−0.997; P<0.05) and % HYP motile spermatozoa (r=−0.946; P<0.01). Therefore, we concluded that the quality of chilled semen progressively losses its viability and doesn't remain useable after certain period of preservation with respect to its motility and morphology.

(Key words : Black Bengal buck, semen, chilled, preservation, spermatozoa)

INTRODUCTION

Artificial insemination (AI) has gain widespread acceptance in the cattle industries of most developed countries but it has not yet received such widespread acceptance in the goat breeding industries (Leboeuf *et al.*, 2000). The preservation of semen and its quality assessment based on sperm features including total and progressive motility, morphology and DNA quality, has been studied extensively in males of different species including the bull, pig, ram and human (Love, 2011). However, similar reports on buck semen are not available in literature. On the other hand, other has reported that the buck semen represents less persistency during preservation in relation to the semen of other domestic animal species (Apu, 2012). In that case the suggested causes for poor preservation competence of buck semen have been ranged from detrimental effects of buck seminal plasma on the subsequent viability of spermatozoa *in vitro* (Leboeuf *et al.*, 2000) to improper handling of semen during processing of semen for preservation (Sullivan, 1978). However, Zamfirescu and Nadolu (2009) reported that the buck semen has high probability to preserve and can be used for AI.

After preservation of fresh buck semen it become very important to maintain its quality specially the percentage of motile and normal/abnormal spermatozoa before using it for AI because of only the good quality and motile spermatozoa is responsible for the fertilization of the oocyte in the female reproductive tract through capacitation (Yanagimachi, 1994). Several researchers were studied on buck semen preservation at different conditions including both frozen and chilled. Although, preservation of semen in frozen condition provide satisfactory viability over time (Zamfirescu and Nadolu, 2009) but viability in chilled condition is remain questionable. Zhao *et al.* (2009), reported the 48% motility of spermatozoa even after 13 days of chilling (21-fold dilution with egg yolk-extender) through renewed of the extender in every 48 h during of preservation. However, to date, there has been no publication of detailed the survival rate of Black Bengal buck semen preserved in chilled condition. As such, a comprehensive *in vitro*

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study is needed, and therefore, the current research was designed to investigate the effect of preservation time on sperm viability based on sperm quantitative features include total and HYP motility and morphology of spermatozoa preserved at chilled (4～5℃) condition.

MATERIALS AND METHODS

1. Selection and Management of Bucks

Four Black Bengal bucks age ranged from 34～45 months with similar body condition scores (BCS) were selected from Bangladesh Agricultural University (BAU) veterinary clinic goat farm. All of the buck were able to produce semen, containing >80% morphologically normal spermatozoa with a satisfactory motility and concentration. The bucks were reared in a separate shed and kept isolated from does during study period. They were reared in semi-intensive system and allowed grazing under supervision of an attendance for 7～8 h daily. Each buck was fed with rice grain (100 gm/buck/day), wheat bran (200 gm/buck/day), and oil cake (50 gm/buck /day) with salt. Clean and safe water was made available at all time. The bucks were dewormed routinely with oral administration of morantel citrate (Deminth®, Reneta Ltd. Bangladesh) at the dose rate of 15 mg/kg body weight after examining fecal samples for helminthes eggs.

2. Experimental Design

The entire experiment was performed following four steps i.e. 1. the preparation of Artificial Vagina (AV) and collection of semen, 2. evaluation of fresh semen, 3. extension and preservation of the collected semen and 4. re-examination of preserved semen at the interval of 24, 48, 72 and 96 h of preservation to observed the percentage of motile and morphological abnormalities if any.

1) Preparation of AV and Collection of Semen

The semen was collected through Artificial Vagina (AV) method at a frequency of once in a week. Donor bucks were allowed usually at least 2 times false mounts before collection of final ejaculates. The bucks were trained previously to ejaculate in AV. The penis-end of the AV was lubricated with non-spermicidal gel (Sterile Lubricating Jelly®, First Priority, Inc. USA). At the other end of the AV a plastic cone was attached with a calibrated collecting plastic tube. Before collection, the prepuce of the buck was wiped by normal saline water to clean and prevent the contamination of semen. During collection the AV was held in right hand along the buck's flank. The open end was facing towards the penis and downwards at an angle of 45°. When buck mounted, the erected penis was carefully directed to the open end of the AV to permit vigorous upward and forward thrusts, which stimulate the ejaculation. The buck was allowed to withdraw its penis immediately after ejaculation into the AV. The graduated collecting tube was separated from the cone and its open end was closed with a plastic cap and labeled. After collection, semen was kept at 37℃ in water-bath until the media and reagents were added.

2) Evolution of Fresh Semen the Motility and Morphology

The routine evaluation of fresh semen was done immediately after collection as described previously (Suttiyotin and Thwaites, 1993). The volume, color, density, and mass activity of spermatozoa in fresh ejaculate of individual buck were recorded by direct visual examination. A Computer-assisted sperm analysis (CASA, SAIS Plus version 10.1, Medical Supply) was used for analyses of sperm motility (%). Briefly, 10 μl of sample was placed in a Makler chamber (Makler). The filled chamber was then placed on a 37℃ heated stage. Using a 10× objective in phase contrast mode, the image was relayed, digitized, and analyzed by SAIS. The movement of the at least 250 sperm cells was recorded for each sample from random fields (>5). Sperm motility was assessed by recording the percentage of motility (MOT) and non-motile spermatozoa. Motile spermatozoa were classified into spermatozoa with progressive (hyper activated motile) and those with non-progressive motility. To examine the acrosome, mid piece and tail morphology of the spermatozoa, semen samples were fixed in buffered formal-saline at the same temperature (37℃). Formal saline was prepared by dissolving di-sodium hydrogen phosphate with two molecules water (34.7 m mol), Potassium di-hydrogen phosphate (18.7 mmol), Sodium chloride (92.6 mmol) and formaldehyde (1.54 mmol) in 1 liter distilled water. The abnormality found in formal saline fixed spermatozoa was observed under phase contrast microscopy; whereas at least 200 spermatozoa were examined from each replicate each day, at 1,000× magnification. Categorization of morphologic features included percentages of normal sperm and abnormalities (aberrations of the head, acrosomes, and midpiece; detached heads, coiled and bent tails). If multiple abnormalities were identified on individual sperm, all were recorded, thus the actual frequency of

each abnormality in the population was determined.

3) Processing and Preservation of Semen

Egg yolk citrate diluent was used for the preservation of semen at chilling temperature (4～5℃) at 1:5 ratio for 4 days. The egg yolk extender was prepared freshly for use according to Herman and Madeen (1963). A stock solution for trisglucose-citratediluents was prepared by dissolving tris, glucose and citrate in 85 ml distilled water. The pH of the extender was adjusted to 7.4. The processing was done at room temperature within 5～10 minutes. Thus the semen were processed and preserved in test tubes at 4℃ in a kit box holded in a dark environment and finally stored in refrigerator at 4～5℃ temperature during study period.

4) Evaluation of Preserved Semen

Computer-assisted sperm analysis (CASA, SAIS Plus version 10.1, Medical Supply) and phage contrast microscopy were used to check the % motility and morphology of semen at 24, 48, 72, and 96 h hourly during preservation (described previously).

3. Statistical Analysis

The data were analyzed in SPSS (v. 16.0). F-test was used to compare the semen parameter at different time of preservation whereas Pearson's correlation coefficient was used to determine the relationship between sperm motility and abnormality following preservation. $P<0.05$ was considered significantly different. All data are expressed as mean ± SEM.

RESULTS

1. Variation among Freshly Collected Buck Semen Parameter

The semen was collected from the four different bucks with similar body condition but different age, body weight and scrotal circumference. There was no significant difference in the mass activity, motility and concentration of normal spermatozoa in freshly collected semen ($P>0.05$). However, significant variation was noted in volume per ejaculate and concentration of spermatozoa (Table 1) ($P>0.05$).

2. Effect of Preservation on Motility, Heper-activated (HYP) Motility and Abnormality of Spermatozoa

Significant decreases % of motile and heper-activated (HYP) motile spermatozoa were illustrated following preservation of buck semen. Lowered motility of spermatozoa was noted on day 3 and 4 compare with first two days of preservation (day 0 & day 1) (Fig. 1A). However, day 3 and 4 were represented significantly ($P>0.05$) reduced HYP motility of spermatozoa in buck semen in contrast with first day of preservation (Fig. 1 B). Moreover, both parameters were positively correlated following preservation ($P>0.05$, R=0.965) (Fig. 2).

Our study was also demonstrated the increased spermatozoal abnormalities in buck semen following preservation (Fig. 3). The mean percentage of abnormal spermatozoa on first (day 0), second (day 1), third (day 2), forth (day 3) and fifth (day 4) day of preservation were 5.88, 7.16, 8.16, 9.23 and 10.24% respectively. The difference in the percentage of abnormal spermatozoa among preservation times were signifi-

Table 1. Parameter of freshly collected buck semen

Buck No.	Age (m)	Body weight (kg)	Scrotal circumference (cm)	Fresh semen color	Density	Volume/ ejaculate(ml)	Mass activity	Individual motility (%)	Normal sperm (%)	Concentration (million/ml) (mean± SE)
1	46	34.37	21.43	3.3±0.5	3.1±0.5	0.50±0.01	3.2±0.50	79.27±0.63	90.19±0.78	2,852.67±0.88
2	35	22.51	20.65	3.2±1.1	3.4±0.5	0.47±0.01	3.3±0.50	79.28±0.49	90.23±0.13	2,704.33±2.02
3	40	29.58	17.11	4±1.2	3.6±0.5	0.35±0.02	3.2±0.45	77.94±0.47	92.27±0.09	2,350.60±0.88
4	35	22.48	19.68	3.3±0.0	3.5±0.5	0.39±0.01	3.4±0.50	78.42±0.05	91.67±0.09	2,433.33±1.20
F-test						S^*	NS^*	NS^*	NS^*	S^*

The data are the mean of 5 replicates ± SEM. Color of semen has been described as 1= opalescent; 2= milky white; 3= yellowish white and 4= creamy white; Density has been defined as 1= watery; 2= milky; 3=thin creamy and 4= creamy; Mass activity has been characterized as 1= no perceptible motion; 2=few spermatozoa moving without waves; 3= movement with slow waves and 4= vigorously moving with rapid waves. NS*= within same column, mean values are non-significant at the $P>0.05$ level by F-test; S^*= within same column, mean values are significant at the $P>0.05$ level by F-test.

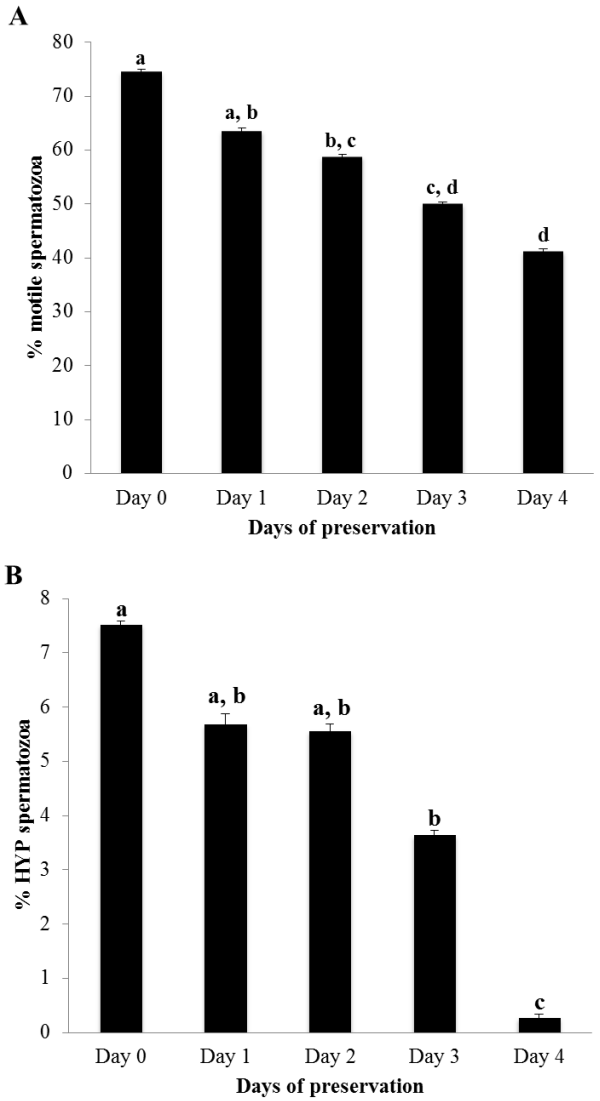

Fig. 1. Percentage of spermatozoa following preservation. (A) Percentage of motile spermatozoa follows preservation. (B) Percentage of hyper-activated motile (HYP) spermatozoa follows preservation. Data are expressed as the mean ± SEM, n=5. Values with different superscripts (a, b, c, d) indicate significant differences between each groups on one-way ANOVA (P<0.05).

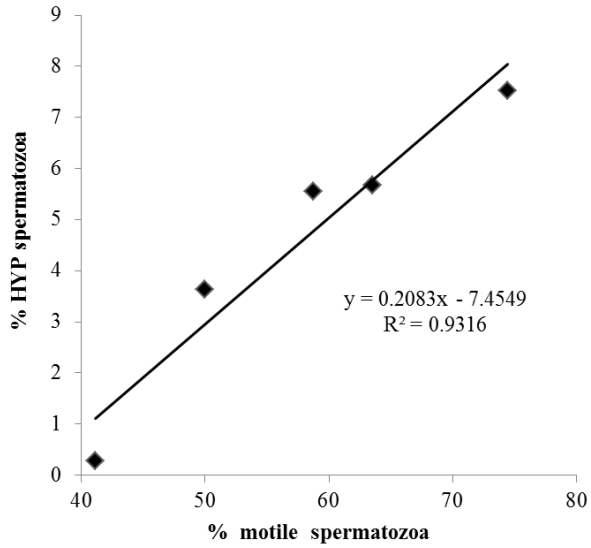

Fig. 2. The correlation between hyper-activated motile (HYP) vs motile spermatozoa. Each datum point represents the mean of the motile and HYP spermatozoa of independent replicates.

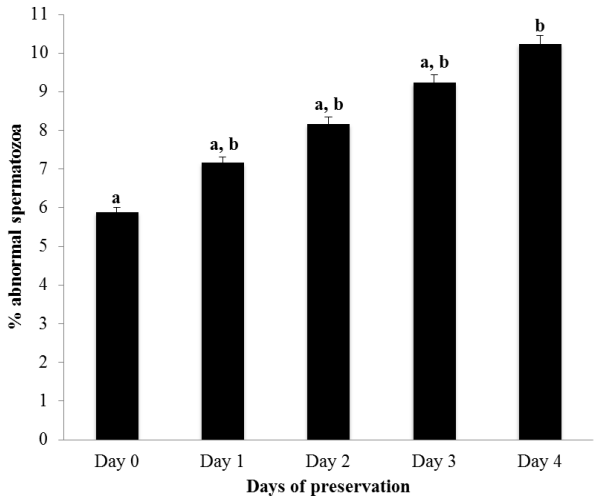

Fig. 3. Percentage of morphologically abnormal spermatozoa in respect to preservation. a, b within each variable, values followed by the same letter are not significantly different according to the one-way ANOVA test P<0.05.

cant (P>0.05). However, height abnormalities were found at the last day of preservation (day 4) in compare with other (Fig. 3).

3. Relationship among Number of Abnormal, Motile and HYP Motile Spermatozoa

It was noteworthy that number of abnormal spermatozoa were significantly increased (P<0.01) as the number of motile and HYP motile spermatozoa decreased following preservation. Moreover, it was not surprising that number of motile spermatozoa were significantly (P<0.001) higher compare with HYP motile spermatozoa. The correlation between abnormal vs motile and HYP motile spermatozoa are shown in Fig. 4. Since, both motile (P<0.01; R=–997, Fig. 4A) and HYP motile (P<0.01; R=–997, Fig. 4B) spermatozoa showed negative correlation with occurrence of abnormal spermatozoa.

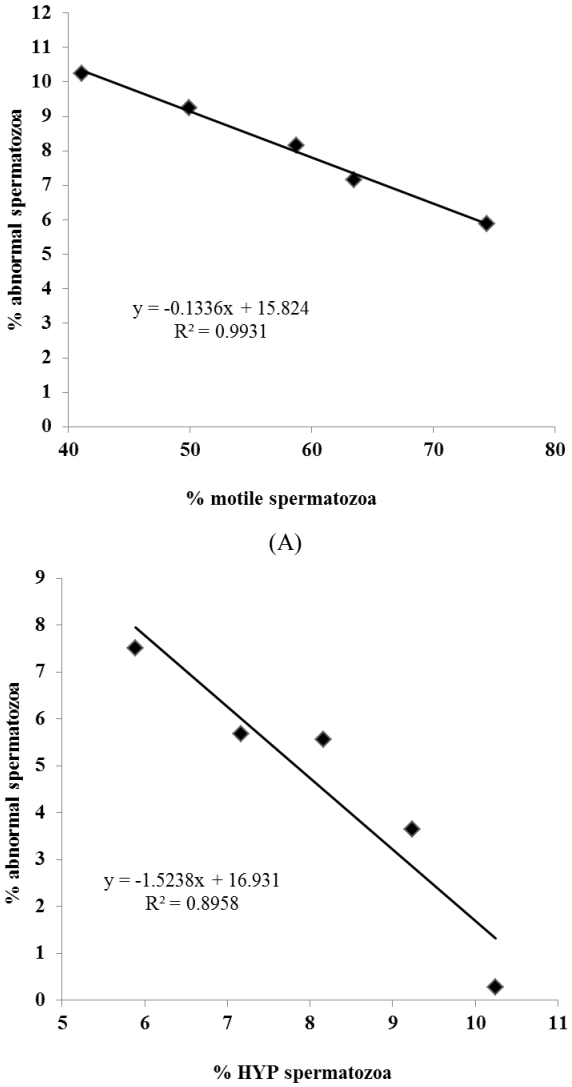

Fig. 4. The correlation of abnormal spermatozoa with motile and hyper-activated motile (HYP) spermatozoa. (A) The correlation between abnormal vs motile spermatozoa. (B) The correlation between abnormal vs HYP spermatozoa. Occurrence of abnormal spermatozoa correlates negatively with both motile and HYP spermatozoa. Each datum point represents the mean of the abnormal motile and HYP spermatozoa of independent replicates.

DISCUSSION

Current study focuses on the relationship between sperm qualitative features i.e. motility, hyper activated motility and spermatozoa abnormality and duration of preservation at chilling condition (4～5℃). Although, *de novo* spermatozoa motion kinematics (motility, morphology) is not always cor- relate to its fertility moreover, to date it is the most widely used technique to access the qualitative value of semen sample in different species of animal worldwide (Wise et al., 2003).

The average volume (ml) and concentration (million/ml) of spermatozoa in semen were higher in buck No. 1 in this present study (Table 1). This variation might due to difference in scrotal circumference of individual buck which was previously demonstrated by Singh et al. (1985); Viller; Salamon 1993 and Bakshi *et al.* (1987). Karatzas *et al.* (1997) and Karagiannidis *et al.* (2000) reported that the volume of buck semen increased during the breeding seasons because the accessory glands become more active during that period. Additionally, the efficiency of semen collector also can affect the total ejaculatory volume of semen (Rahman *et al.*, 2012a & b).

Significant decreases % of motile and HYP motile spermatozoa were illustrated following preservation of buck semen in the current study. The causes of declining of those sperm parameters might related with reduced metabolism, exhaustion of reserved energy in spermatozoa, fluctuation of temperature in refrigerator, effect of metabolic by-products such as spermicidal endotoxins, seasonal difference (Karatzas *et al.*, 1997; Karagiannidis *et al.*, 2000) and detrimental effect of seminal plasma on the subsequent viability of spermatozoa (Leboeuf et al., 2000). In addition, sperm mitochondrial defects after preservation have been mentioned by O'Connell et al. (2002) and a correlation has been found with loss of motility. It is well accepted that motile spermatozoa is the prerequisite for achieving successful fertilization. Itach et al. (2011) reported that insufficient sperm motility is a common cause of subfertility or infertility in domestic animal and human. According to Love (2011), reduced percentage of *in vitro* fertilization (IVF) rate and acrosomal reaction (AR) are due to inseminating the oocyte by the spermatozoa containing lower HYP motility spermatozoa in stallion. On the contrary Wise et al. (2003) stated that *de novo* spermatozoa motion kinematics (motility and morphology) is not always correlate to its fertility. This result might due to considering the different breeds of animal at different climatic condition.

In general, sperm motility describes the ability of sperm to move properly towards an egg or the quality of the sperm, which is a reason in successful pregnancies (Quill and Garbers, 2002). However, a finest motility of spermatozoa is responsible for the fertilization of the oocyte through a sequence of biochemical changes in the female reproductive tract, known

as capacitation. Following capacitation spermatozoon can bind to the zona pellucida of the egg and undergo the acrosome reaction (Yanagimachi, 1994) so far. Without any technological interference, we may assume that a non-motile or abnormally-motile sperm are not going to fertilize the oocyte. Therefore, assessing the portion of a sperm population that is motile is possibly the most widely-used measure of accessing semen quality (Farrell et al., 1998).

Our study was also demonstrated the increased spermatozoal abnormalities in buck semen following preservation (Fig. 3). Three different types of morphological abnormality were considered in this study which includes head abnormality (detached head, acrosomal abnormality, and presence of proximal/ distal cytoplasmic droplet) mid-piece abnormality (broken neck, deformed mid-piece) and tail abnormalities (bent tail, coiled tail, tail coiled around the neck and double folded tail). However, the occurrence of particular defects following preservation was insignificant (data not shown). The most probable reason for this morphological abnormality seems to be the physical and chemical environments to which a spermatozoon is exposed during the preservation. On the other hand, Medeiros et al. (2002) and Ozkavukcu et al. (2008) noted that the spermatozoal cell water exchange during the early stages of the preservation causes cell swellings and shrinkages which may be intolerable for the majority of organelles and might predispose to spermatozoal morphological abnormality.

It was noteworthy that number of abnormal spermatozoa were significantly increased ($P<0.01$) as the number of motile and HYP motile spermatozoa decreased following preservation. Our current result supports the previous study on chilled preserved semen by Shamsuddin and Rodriguez-Martinez (1994) and Sugulle et al. (2006). However, it is also reported that the proportion of morphologically abnormal spermatozoa in semen correlates negatively with fertility estimates (Rodriguez-Martin *et al.*, 1994). According to Herman and Madeen (1963) buck semen is not recommended for AI if the proportion of normal spermatozoa is below 80%.

In conclusion our study highlighted the relationship between motility (computer assisted motility analysis) and the morphology of buck spermatozoa following preservation. We showed that the quality of the preserved semen deteriorate severely largely after 2 days of preservation. Therefore, we concluded that the quality of chilled semen progressively losses its viability and doesn't remain useable after day 2 with respect to its motility and morphology.

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