Semen Quality of the Black Bengal Bucks Used at Commercial Artificial Insemination

Ajoy Chandra Dhar[1], Anup Kumar Talukder[1,2,*], Mohammad Bozlur Rahman[1], Abdullah-Al-Mamun[1] and Mohammed Shamsuddin[1]

[1]Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[2]Department of Medicine and Surgery, Faculty of Animal Science and Veterinary Medicine, Patuakhali Science and Technology University, Barisal 8210, Bangladesh

ABSTRACT

Only an optimum number of viable spermatozoa in a frozen-thawed insemination dose can ensure conception at artificial insemination (AI). We report here the percentages of normal, abnormal and viable spermatozoa present in the frozen-thawed semen of 20 Black Bengal bucks used for commercial AI. Bucks in this experiment were of 19.3~46.1 months old and 25~42 kg body weight. Four semen straws (0.25 ml) from each buck were collected for evaluation of their kinetic parameters. Scrotal circumference was measured by using a scrotal tape, sperm motility was estimated on eye estimation and sperm concentration was determined by using a haemocytometer. Sperm morphology was studied in paraformaldehyde fixed spermatozoa under differential interference contrast (DIC) microscope. To determine the proportion of live (plasma membrane intact) spermatozoa, semen was stained with SYBR-14 and propidium iodide and examined under fluorescent microscope. Scrotal circumference, post-thaw sperm motility, sperm concentration per insemination dose and proportion of normal spermatozoa were 21.5 ± 0.7 cm, 43.5 ± 5.4%, 83.5 ± 6.7 million and 88.3 ± 4.1%, respectively. The percentages of spermatozoa with head shape and acrosome abnormalities were lower (2.7 ± 1.1 and 1.4 ± 1.3, respectively), whereas higher percentages of abnormalities (7.0 ± 1.8) were observed in mid piece and tail portion. The proportion of live spermatozoa was 28.5 ± 5.4. It is concluded that although a good number of morphologically normal spermatozoa are present in the insemination dose, the proportion of live spermatozoa is low, which warrants further improvements of buck semen freezing procedures to ensure good quality at AI.

(Key words : artificial insemination, Black Bengal buck, scrotal circumference, sperm motility, sperm morphology)

INTRODUCTION

The status of normal and abnormal spermatozoa present in semen is important to predict the fertility of stud animals irrespective of natural service or AI. Especially in the case of AI, the number of spermatozoa is many times lower than that of natural service. Moreover, a large number of abnormal spermatozoa in the insemination dose may turn the potential sperm number at a level, which is much lower than the threshold level. AI is the preferred method of introducing superior genes from individuals free from specific diseases and thereby improving the production of offspring. An additional important benefit of AI in goats is the opportunity of breeding them outside their natural breeding seasons (Peterson *et al.*, 2007). Interest in AI in goats has increased because only few bucks of good genetic merits are available for breeding program (Herman *et al.*, 1994). Hence, the utilization of the AI technology with bucks of superior genetic merit would increase the productivity of goat industry many folds in Bangladesh.

Successful AI program depends upon the quality of semen collected from the bucks' of superior genetic merits. Improper management of semen collection, storage/cryopreservation, distribution, field usages and thawing may induce ultrastructural, biochemical and functional damages resulting in a reduction of sperm motility, membrane integrity, viability, longevity and ultimately, in fertilizing capacity (Watson, 2000; Purdy, 2006; Rodriguez-Martinez and Barth, 2007). A number of cryoprotectants are being used to reduce the sperm damage from cryopreservation. Among them egg yolk is still remained as a common component of semen cryopreservation extenders (Salamon and Maxwell, 1995). Goat seminal plasma contain egg yolk-coagulating enzyme (EYCE), recognized as phospholipase A

† This study was supported by the grant (BG-ARS-121) of the United States of Department of Agriculture (USDA), USA.
* Correspondence : E-mail : anupbau@gmail.com

(Ritar and Salamon, 1991). This enzyme acts on egg yolk lecithin contained in the semen extenders and thus produces fatty acid and lysolecithin through lipase activity which is detrimental for spermatozoa. Thus, the addition of egg yolk reduces sperm motility and sperm viability and increase the incidence of acrosomal damage (Holt *et al.*, 1996; Pellicer-Rubio *et al.*, 1997; Aboagla and Terada, 2004). Therefore, it seems rationale to evaluate frozen-thawed sperm motility, viability and morphology to ensure that sufficient number of normal and viable spermatozoa is present in the insemination dose.

Sperm morphology, concentration, motility and longevity are all important criteria in semen evaluation. A decrease in the number of morphologically normal spermatozoa in the ejaculate leads to reduced fertility (Sekoni and Gustafsson, 1987; Chandler *et al.*, 1988; Kruger *et al.*, 1988). Söderquist *et al.* (1991) reported that the frequency of abnormal spermatozoa in an insemination dose was inversely correlated with fertility of the semen. The evaluation of sperm motility and morphology is an essential parameter in the examination of sperm quality and establishment of correlation between semen quality and fertility (Shanis *et al.*, 1989; Evans and Maxwell, 1990; Salamon and Maxwell, 2000). It is, in fact, an indispensable part where a new technology like AI is being introduced for commercial or nationwide use. Therefore, the present study was designed to determine the normal, abnormal and viable spermatozoa in the frozen-thawed insemination dose of buck semen used for commercial AI in goats, which has been very recently undertaken in Bangladesh.

MATERIALS AND METHODS

Unless otherwise stated, all the chemicals, reagents, media, biologics and media constituents were purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA).

1. Animals

Twenty Black Bengal bucks from the BRAC Bull and Buck Station, Mymensingh, Bangladesh were used in this study. The age and body weight of the bucks were 19.3~46.1 months and 25~42 kg, respectively. Each buck was fed approximately 3 kg green grass into three splits, 400 gm concentrate into two splits daily and was free access to water.

2. Scrotal Circumference Measurement

The scrotal circumference was measured as per method recommended by the Society for Theriogenology (Ball *et al.*, 1983). In brief, the testes were first retracted into the lower part of the scrotum. To prevent separation of the two testes, the thumb and the fingers were placed on the sides rather than on the front or back of the scrotum. Then a flexible metal tape (Scrotal tape; Lane Manufactring Co., Denver, USA) was looped and placed around the greatest diameter of the scrotum and pulled snugly so that the tape was firmly in contact with the entire circumference and reading was taken up to nearest 0.5 cm. Repeated measurements were done and the mean of the measures was recorded to ensure the accuracy.

3. Pre-freezing Semen Evaluation

The ejaculate volume, total numbers of spermatozoa/ejaculate and sperm motility in fresh ejaculate of individual bucks were determined immediately after collection.

The volume of semen was recorded for individual bucks by reading the graduation marks of the receptacle.

The concentration of spermatozoa in the semen was determined by using a haemocytometer (Bane, 1952). Semen sample was diluted with water (1:200) to kill and immobilize the spermatozoa. The coverslip was pressed firmly down on the haemocytometer and a 10 μl drop of diluted and uniformly mixed semen was allowed to flow under the coverslip by capillary action. Then the haemocytometer was given 5 minutes to settle down the spermatozoa in a pre-wetted pertidish. The five large squares of the counting chambers (4 corners and the middle one) of the haemocytometer were counted. By convention, spermatozoa lying on the top and the left-hand-side grids of a square were included and those on the bottom and right-hand-side grids were ignored at counting. Sperm concentrations were expressed in million per ml. The total number of spermatozoa per ejaculate was determined by multiplying the ejaculate volume with the sperm concentration per ml of the respective ejaculate.

To evaluate sperm motility, a drop of 10 μl of semen was placed on a pre-warmed (37℃) slide by using a stage heater, and covered with warmed coverslip (18 × 18 mm.). The slide was examined at 400 × objectives under a DIC microscope (BX 51, OLYMPUS, Tokyo, Japan) equipped with warm stage. Focuses were concentrated on spermatozoa. Proportion of spermatozoa with progressive straight forward movement was recorded as sperm motility. Spermatozoa which had slow movement or backward movement or seem to float were not considered in motility estimation. Sperm motility was determined

based on eye estimation and expressed in percentages. Motility estimation was repeated for at least 5 different fields in the same slide and the average estimate was recorded.

4. Post-thaw Semen Evaluation

Four semen straws from individual bucks were thawed in a water bath at 37℃ for one minute and semen was taken into a 1.5 ml eppendorf tube. Spermatozoa were evaluated for motility, concentration, morphology and viability (assessment of live/dead spermatozoa).

The sperm motility and concentration were determined as decribed for pre-freezing semen evaluation.

To evaluate morphology of spermatozoa, dry and wet preparation of semen smear were made. Dry preparation of semen smear was used to examine sperm head morphology. A straw was thawed (0.25 ml) in a water bath at 37℃ for one minute and the semen was taken into a 1.5 ml Eppendorf tube. The thawed semen was transferred and layered on one ml Tris solution (1 M in distilled water) in 15 ml Falcon tube. Then, the layered semen was centrifuged at 500 rpm for 10 minutes. After centrifugation the supernatant was removed by using suction pump. This procedure of centrifugation was repeated for three times. The semen samples were then fixed in 0.1% paraformaldehyde (1:100) for one minute. After taping softly with finger, 6 μl semen samples were taken into a clear glass slide and smeared by using the tip of the micropipette. The smeared glass slide was placed on a stage heater and dried by air blowing with a hair drier within two minutes. After drying, 4.5 μl phosphate buffer saline (PBS) was taken on the slide and a clean coverslip was placed on the smear. The edges of the coverslip were sealed with clear finger nail polish. Sperm abnormalities were observed at 1,000 × magnification under a microscope equipped with DIC optics. At least 200 spermatozoa were evaluated for morphological abnormalities.

Wet preparation was used to examine the acrosome, midpiece and tail of the spermatozoa. Semen sample was fixed in 0.1% paraformaldihyde as described above for dry preparation. To prevent any temperature variation-related damage to the spermatozoa, the semen and paraformaldihyde were mixed at the same temperature. At least 200 spermatozoa were examined at 1,000 × magnification in each sample under a microscope equipped with DIC optics. Sperm head, acrosome, mid piece and tail abnormalities were recorded individually. The proportion of spermatozoa with no abnormalities in the head, acrosome, mid piece and tail were recorded as normal.

Sperm motility and viability were determined by fluorescent staining and image analysis technique. Briefly, 15 μl frozen-thawed semen was diluted in 30 μl pre-warmed BGM-3 (Bovine Gamete Medium-3, air incubation medium for spermatozoa, Parrish *et al.*, 1998). The diluted semen was stained with 4 μl SYBR-14 (1 μM/ml) and 1 μl propidium iodide (2.4 M/ml) (Live-dead kit, Molecular Probes, Eugene, OR, USA) by incubating 15 minutes at 39℃ in the water bath. Ten μl stained semen was placed on a pre-warmed (39℃) slide, covered with a cover glass (18 × 18 mm) and placed immediately under an epifluorescent microscope (BX51, OLYMPUS, Tokyo, Japan) that was equipped with a digital camera (Nikon, Coolpix 5000, Tokyo, Japan) and a stage heater. The DNA fluorochromes were excited by engaging the filter cube U-MWIB2 (excitation: 460~490 nm, emission: 505 nm and barrier filters: 510 nm) in the fluorescent light path and the slide was viewed through a 20 × universal semi apochromat objective (UplanFI, 1.6 numerical apertures). The camera setting include color mode, focal length was set to infinity and exposure time 1/8 second. After proper calibration of the microscope and camera, 20 digital images were taken systematically from different part of slide for individual bucks. The images were saved in a computer hard disc and later evaluated on screen by using the counter plugins (De Vos, 2006) and Image J (Rasband, 2006). The tracks and dots on the images were identified as motile and non motile spermatozoa, while the greens and reds were viable and dead sperm, respectively.

5. Sperm Digital Microphotography

Digital microphotographs of normal and different abnormal spermatozoa were captured using a digital camera attached to DIC microscopy. A TV card and computer monitor is attached with the camera in order to visualize the pictures clearly. The captured photographs are then imported into Adobe Photoshop CS, version 8 for making printable size but no editing was done in the shape of sperm.

6. Statistical Analysis

The data collected from the BRAC Bull and Buck Station and laboratory findings were entered into Microsoft Excel Work Sheet, 2003. The mean and standard deviation (SD) of scrotal circumference, ejaculate volume, total spermatozoa per insemination dose, sperm motility, normal, abnormal and live spermatozoa were figured out from the original data by performing descriptive statistics.

RESULTS

Normal and abnormal spermatozoa of bucks' frozen semen were studied in this experiment and depicted as normal spermatozoa (Fig. 1) and spermatozoa with abnormalities in the head, acrosome, mid piece and tail (Fig. 2~4).

The mean ± SD of scrotal circumference, ejaculate volume, total spermatozoa per ejaculate and fresh sperm motility of 20 bucks are shown in the Table 1. The scrotal circumference, ejaculate volume, total spermatozoa per ejaculate volume and fresh sperm motility were 21.5 ± 0.7 cm, 0.9 ± 0.2 ml, 1,765.95 ± 581.6 million and 75 ± 5.3%, respectively.

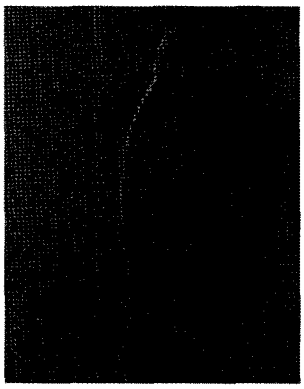

Fig. 1. Digital microphotograph of buck's spermatozoon with normal morphology under differential interference contrast (DIC) optics (1,000 ×).

The mean ± SD of post-thaw sperm motility, sperm concentration, proportion of live spermatozoa and proportion of normal spermatozoa in a frozen insemination dose are shown in the Table 2. The proportion of normal spermatozoa were reached plateau as 88.3 ± 4.1 whereas the proportion of live spermatozoa were only 28.5 ± 5.4.

The mean ± SD of total spermatozoa counted for each buck and different sperm abnormalities are shown in the Table 3. Among 262.6 ± 67.5 spermatozoa counted in each buck, the total percentages of sperm abnormalities were only 11.1 ± 4.2 whereas the percentages of mid piece and tail abnormalities accounted for 7.0 ± 1.8.

DISCUSSION

The results of this study showed that spermatozoa present

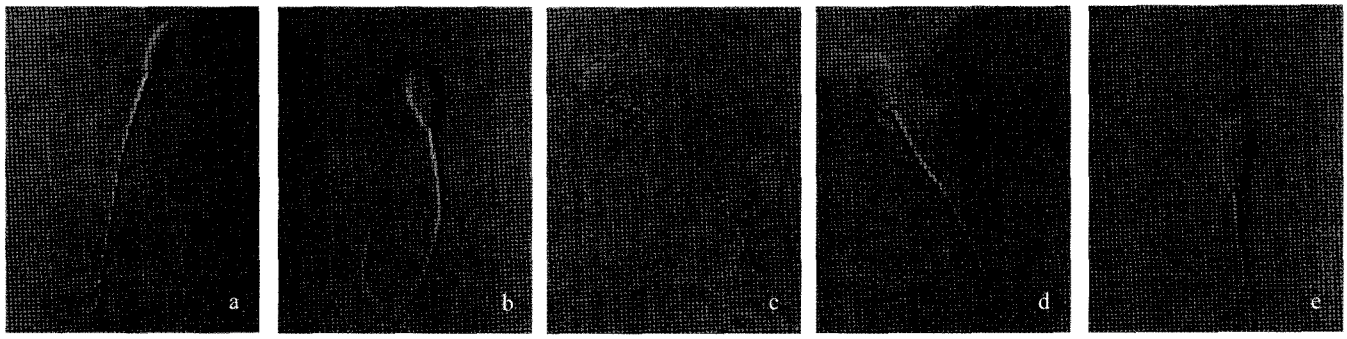

Fig. 2. Digital microphotographs of buck's spermatozoa with head abnormalities under differential interference contrast (DIC) optics (1,000 ×). (a) Narrow head (b) Pear shaped head (c) Broad head (d) Membrane damage (e) Narrow at the base.

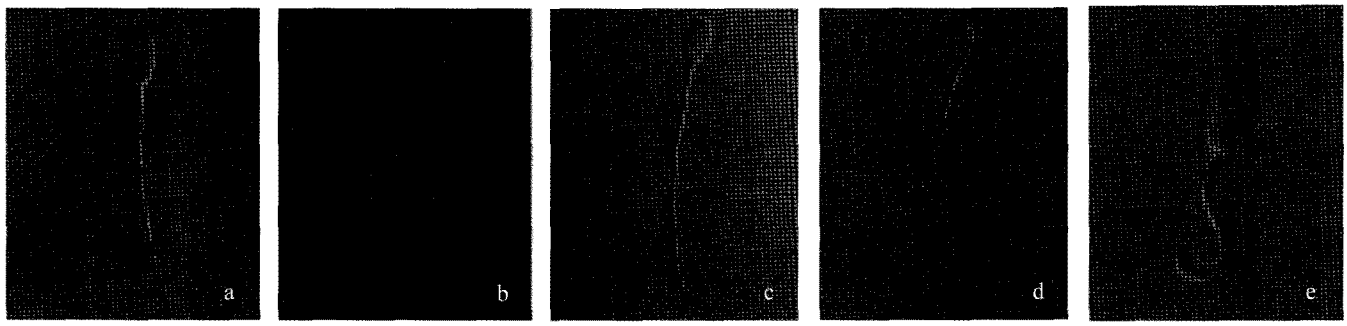

Fig. 3. Digital microphotographs of buck's spermatozoa with different types of acrosomal defects under differential interference contrast (DIC) optics (1,000 ×). (a) Loose acrosome (b) Acrosome reacted spermatozoon (c) Acrosome damage (d) Knobed acrosome (e) Knobed acrosome and coiled tail.

in an insemination dose were of good number and a good proportion of them were of normal morphology; however, the proportion of live spermatozoa was low.

The proportion of normal and abnormal spermatozoa present in semen is an important determinant in selecting semen for natural service or AI. The proportion of morphologically abnormal spermatozoa correlates negatively with fertility (Söderquist, 1991; Shamsuddin *et al.*, 1993). The AI industries worldwide are striving to lessen the number of spermatozoa in the inseminate which are required for maximum conception. In that case, number of normal spermatozoa present in the inseminate is a matter of high emphasis. In this study, about 88% normal spermatozoa were found, which signify its suitability for AI. As sperm morphology is a compensable trait, the fer-

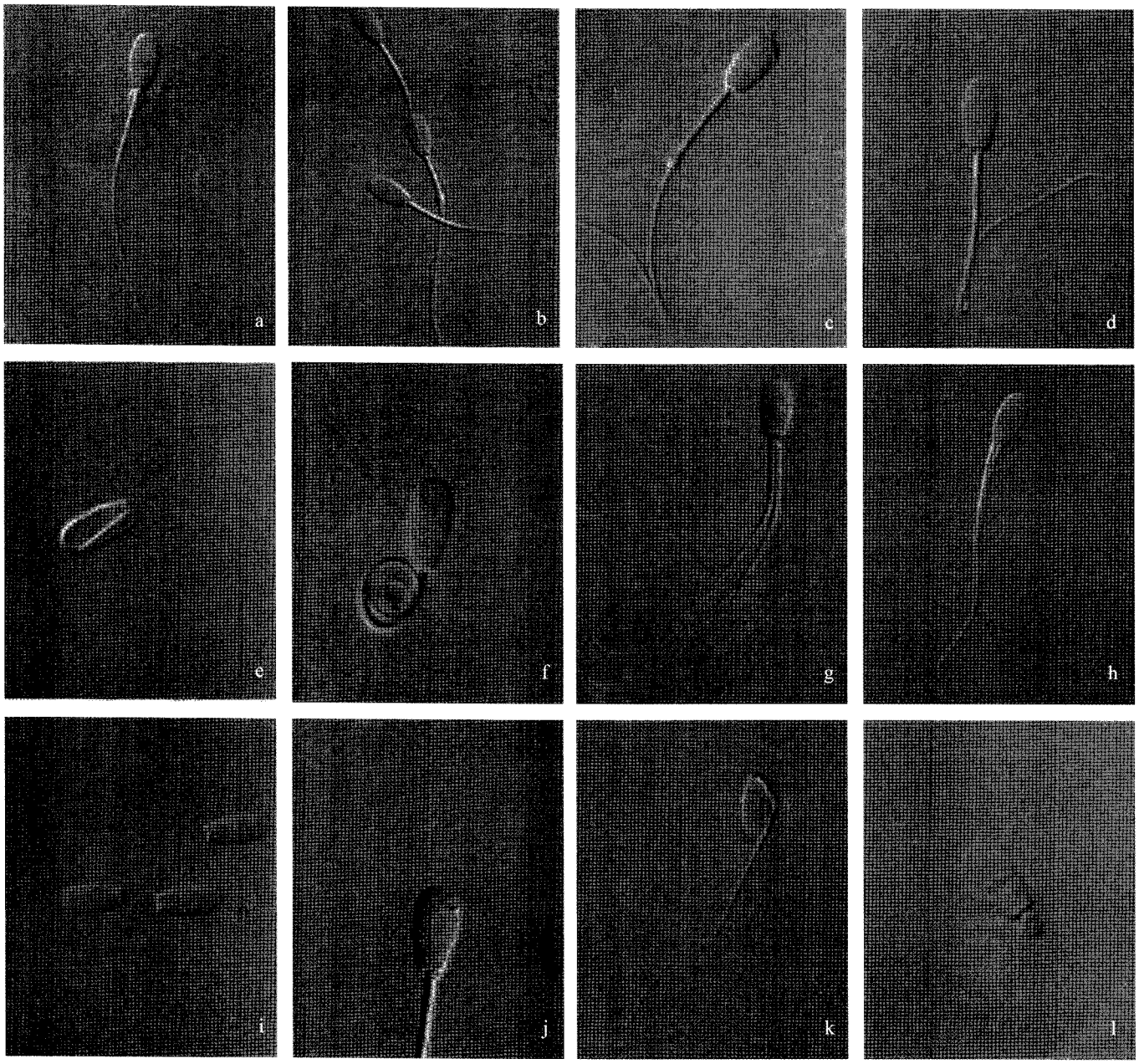

Fig. 4. Digital microphotographs of buck's spermatozoa with different types of mid piece and tail abnormalities under differential interference contrast (DIC) optics (1,000 ×). (a) Proximal cytoplasmic droplet, (b) Distal cytoplasmic droplet, (c) Distal cytoplasmic droplet and simple bent tail, (d) Simple bent tail, (e) Tail coiled around head, (f) Tail coiled below head, (g) Double tail, (h) Double mid piece, (i) Detached head, (j) Abexial connection of tail with the head, (k) Broken neck (l) Stumped.

tility of semen could be improved by increasing the number of spermatozoa in the insemination dose. On the other hand, an uncompensable trait of semen cannot be improved by increasing the number of spermatozoa. Abnormal DNA or chromatin materials are responsible for uncompensable traits of spermatozoa. Spermatozoa that possess abnormal chromatin can reach the appropriate site in the female reproductive tract needed to participate in fertilization and penetrate the zona pellucida (Saacke *et al.*, 1994). However, oocytes fertilized with such spermatozoa may be ineffectual in sustaining zygotic, embryonic or fetal development. So, an uncompensable trait of spermatozoa is a matter of breeders' more concern. Recently, chromatin packaging of spermatozoa, an uncompensable trait, has been studied by using nuclear stains and image analysis and a strong relationship of sperm head shape with fertility was reported (Ostermeier *et al.*, 2001; Siddiqui, 2008).

Table 1. Mean ± SD of scrotal circumference, ejaculate volume, total spermatozoa /ejaculate and fresh sperm motility in Black Bengal Buck (number of bucks=20)

Parameters	Mean ± SD
Scrotal circumference (cm)	21.5 ± 0.7
Ejaculate volume (ml)	0.9 ± 0.2
Total spermatozoa /ejaculate (million)	1,765.95 ± 581.6
Fresh sperm motility (%)	75 ± 5.3

Table 2. Mean ± SD of post-thaw sperm motility, sperm concentration per insemination dose and proportion of live and morphologically normal spermatozoa in frozen buck semen (number of bucks=20)

Parameters	Mean ± SD
Post thaw motility (%)	43.5 ± 5.4
Sperm concentration per insemination dose (million)	83.5 ± 6.7
Proportion of live spermatozoa	28.5 ± 5.4
Proportion of normal spermatozoa	88.3 ± 4.1

In the present study, we found only 1.4% head abnormalities and 2.7% acrosome abnormalities. Further, neck, mid piece and tail abnormalities other than head abnormalities are also responsible for poor fertility, because such spermatozoa cannot reach the site of fertilization to fertilize the oocyte. The proportion of abnormal spermatozoa in an insemination dose, found in this study is quite less than the proportion that would hamper fertility. The increased proportion of morphologically normal spermatozoa in semen is, in fact, accountable for increased fertility (Bhuiyan, 1998). On the other hand, increased proportion of abnormal spermatozoa in semen is liable for decreased fertility (Shamsuddin and Rodriguez-Martinez, 1994). Therefore, the present study on frozen semen with low abnormalities of spermatozoa which accounts for around only 11% should be as good as for AI purposes. The low abnormalities of spermatozoa present in the frozen semen might be due to higher age, body weight, scrotal circumference and nutrition condition of the selected bucks.

Different types of stains and staining procedure have a great influence on sperm morphological evaluation. Staining can either increase or decrease the percentage of morphologically normal spermatozoa in the bull, stallion, boar and buck (Wurgau, 1986). Johnson *et al.* (1991) reported that eosin-nigrosin stain with

Table 3. Proportion of spermatozoa with head, acrosome, mid piece and tail abnormalities (number of buck=20)

Proportions of different abnormalities	Mean ± SD
Total number of spermatozoa counted for each buck	262.6 ± 67.5
Sperm head shape abnormalities (%) [Narrow head, pear shaped, broad head, membrane damage, narrow at the base]	1.4 ± 1.3
Abnormalities in the acrosome (%) [Loose acrosome, acrosome reacted spermatozoon, acrosome damage, knobed acrosome, knobed acrosome and coiled tail]	2.7 ± 1.1
Abnormalities in the mid piece and tail (%) [Proximal cytoplasmic droplet, distal cytoplasmic droplet, simple bent tail, double bent tail, tail coiled around head, tail coiled below head, double tail, double mid piece, detached head, abexial connection of tail with the head broken neck, stumped]	7.0 ± 1.8

osmolality of 135 mOsm and pH of 2.27 increased the percentage of canine spermatozoa with bent tails. The staining methods affected the dimensions of goat spermatozoa significantly (Hidalgo *et al.*, 2006). Williams stain in thin smear was reported to be effective for the evaluation of sperm head morphology (Williams, 1920; Lagerlöf, 1934). It works well in freshly collected semen. The presence of egg yolk in the frozen semen hinders the Williams stain in objectively depicting sperm head abnormalities. Moreover, buck seminal plasma contains an egg yolk coagulating enzyme, Phospholipase-A. It acts on egg yolk lecithin present in the frozen semen and produces fatty acid and lysolecithin which is detrimental both for William stains and spermatozoa as well. Further, to figure out total sperm abnormalities, formol saline-fixed semen (wet preparation) was used in this study and spermatozoa were counted by using a DIC microscope at 1,000 × optics. At least 200 spermatozoa were counted in this study in each buck and evaluated for morphological abnormalities as described by Barth *et al.* (1992). Additionally, to evaluate the sperm abnormalities in thin smear (dry preparation), we used only tris to wash out egg yolk present in the frozen semen, which was later evaluated by DIC microscopy. In this study, we have found clear distinguishable pictures of normal and different types of abnormalities of spermatozoa, for instances, head, neck, mid-piece and tail abnormalities without using any stain. Siddiqui (2008) has used DIC microscopy successfully for evaluation of sperm morphology in the same laboratory. Therefore, the normal and abnormal spermatozoa evaluated in this study would not compromise the accuracy.

Sperm viability test proved to predict fertility of bull semen used in artificial insemination in a retrospective study. Spermatozoa are translucent and virtually invisible by bright field microscopy. Earlier, eosin-nigrosin stain was used to determine the percentage of live spermatozoa. Nigrosin or aniline blue is used to provide background staining and eosin as a vital stain. Eosin penetrates damaged cell membranes and stains injured or nonviable spermatozoa pink which are considered as dead, while, viable cells chase off eosin and appear white that indicates alive. Nevertheless, controversies are not uncommon in determining sperm viability accurately by using eosin-nigrosin staining (Brito *et al.*, 2003; Björndahl *et al.*, 2004). In the present study, we used fluorescent staining technique to determine live-dead spermatozoa. SYBR-14 and propidium iodide were used in this staining technique to stain the spermatozoa. The propidium iodide enters into the membrane damaged spermatozoa and stains the spermatozoa red, which are considered dead. On the other hand, SYBR-14 stained only live spermatozoa, which appeared green. The use of fluorescent staining and digital imaging, used in this study earlier, proved effective in evaluating bull spermatozoa (Siddiqui, 2008). In this study, we have found 28.5% viable spermatozoa which refer low fertility.

Scrotal circumference is the preliminary indicator of how much testicular tissue is present in the animals. It always maintains a strong relationship with the number of spermatozoa produced per ejaculation. The bulls having higher scrotal circumference with larger testes produce higher semen volume with more spermatozoa (Randel, 1994; Sundararaman *et al.*, 2002). Moreover, the smaller testes produce low quality semen and achieve poor calving rate (Randel, 1994). Although such works are limited in bucks, Shamsuddin *et al.* (2000) found that the mean scrotal circumference of pubertal Black Bengal bucks varied from 14 to 16 cm and the produced semen volume varied from 267 to 342 μl. The mean ± SD scrotal circumference and ejaculate volume of matured bucks used in the present study were higher than that reported earlier for young pubertal bucks. Generally, collected semen volume is diluted 6 folds with suitable diluents in buck semen freezing protocols. Hence, the semen volume is an important determinant in selecting bucks for AI programme.

Sperm motility is the first and foremost criteria of a semen sample whether it would be selected or discarded. It is thought that 40~50% sperm motility is needed for successful conception in at AI in goats and cows. In determining motility of goat semen a special attention is required, for instance, the examiner may need a stage heater (37℃) as buck semen is more sensitive to environmental temperature than bull semen (Gardon *et al.*, 2006). It is also advisable to do motility test as soon as thawing of frozen semen. In this study, we have found more than 43% sperm motility in tris extender with 83.5 million spermatozoa per insemination dose (0.25 ml straw) using DIC microscopy equipped with a stage heater (37℃). This finding supports by other reports published elsewhere (Gardon *et al.*, 2006). However, the result of the present study contradicts with the findings in Florida male goat observed by Hidalgo *et al.* (2006) who showed 57.6% motility in tris extenders. These discriminations might be due to delayed observation in this study or use of Black Bengal buck semen. Other explanations for these inconsistencies are perhaps the variations in age, body weight, their feeding management and atmospheric temperature between the studies.

In conclusion, a good number of normal spermatozoa existed in the frozen-thawed insemination dose of buck semen; however, the proportion of viable spermatozoa was low. Since AI in goats with frozen semen has recently been started in Bangladesh, data of the present study would help the AI centre to reexamine their semen processing and freezing protocol to improve the fertility of inseminated goats. Further research including sperm head image analysis by using Fourier harmonic amplitude and determination of conception rate would help improving the goat AI programme in Bangladesh.

REFERENCES

Aboagla E.M.-E and Terada T. 2004. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogenology 62:1160-1172.

Ball L, Ott RS, Mortimer RG and Simons JC. 1983. Manual for breeding soundness examination of bulls. J. Soc. Theriogenology 12:1-65.

Bane A. 1952. A study on the technique of hemocytometric determination of sperm motility and sperm concentration in bull semen. The Corn. Veteri. 42: 518-531.

Barth AD, Brown PA, Gabriel AB and Mapletoft RJ. 1992. Effect of narrow sperm head on fertility in cattle. Canad. Vet. J. 33: 31-39.

Bhuiyan MMU. 1998. The quality of bull semen used in artificial insemination in Bangladesh. MSc. Thesis, Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh.

Björndahl L, Soderlund I, Johansson S, Mohammadieh M, Pourian MR and Kvist U. 2004. Why the WHO recommendations for eosin-nigrosin staining techniques for human sperm vitality assessment must change? J. Androl. 25:671-678.

Brito LF, Barth AD, Biludeau-Goeseels S, Panich PL and Kastelic JP. 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with *in vitro* fertilization rate. Theriogenology 60:1539-1551.

Chandler JE, Painter CL, Adkinson RW, Memon MA and Hoyt PG. 1988. Semen quality characteristics of dairy goats. J. D. Sci. 71:1638-1646.

De Vos, K 2006. http://rsb.info.nih.gov/ij/plugins/cell-counter.html

Evans G and Maxwell CW. 1990. Manejo y valoración del semen. In: Evans G and Maxwell CW (Ed.), Inseminación artificial de ovejas y cabras, Acribia SA, Zaragoza, España pp. 95-107.

Gardon JC, Rodriques JA and Gadea J. 2006. Addition of reduced glutathione to thawing medium improved the sperm motility and reduced ros generation in frozen ovine and caprine spermatozoa. Reprod. Fertil. Dev. 18:155-155.

Herman HA, Mitchell JH and Doak GA. 1994. Extender and extension of semen. In: Herman HA (Ed.), The Artificial Insemination and Embryo Transfer of Dairy and Beef Cattle. Interstate Publishers, Inc. Danville, Illions, pp. 101-116.

Hidalgo M, Rodriguez I and Dorado J. 2006. Influence of staining and sampling procedures on goat sperm morphometry using sperm class analyzer. Theriogenology 66:996-1003.

Holt WV, Abaigar T and Jabbour HN. 1996. Oestrus synchronization, semen preservation and artificial insemination in the Mohor Gazelle (*Gazella dama mhorr*) for the establishment of a Genome Resource Bank Programme. Reprod. Fertil. Dev. 8:1215-1222.

Johnson C, Jacobs J and Walker R. 1991. Diagnosis and control of *Brucella canis* in kennel situations. Morphology-stain induced spermatozoal abnormalities. In: Proc. Soc. Therio. pp. 236-239.

Kruger TF, Acosta AA, Simmonn KF, Swanson RJ, Matta JF and Oehninger S. 1988. Predicting value of abnormal sperm morphology in *in vitro* fertilization. Fertil. Steril. 49:112-117.

Lagerlöf N. 1934. Morphologische Untersuchungen über Veränderungen im Spermabild und in den Hoden bei Bullen mit verminderter oder aufgehobener Fertilität (Researches concernoing morphologic changes in semen picture and the testicles of sterile and sub fertile bulls). Acta Path. Microbiol. Scand. Suppl. 19 thesis, Stockholm.

Ostermeier GC, Sargeant GA, Yandell BS, Evension DP and Parrish JJ. 2001. Relationship of bull fertility to sperm nuclear shape. J. Androl. 22:595-603.

Pellicer-Rubio MT, Magallon T and Combarnous Y. 1997. Deterioration of goat sperm viability in milk extenders is due to a bulbourethral 60-kilodaltonglycoprotein with triglyceride lipase activity. Biol. Reprod. 57:1023-1031.

Peterson K, Kappen MA, Ursem PJ, Nothling JO, Colenbrander B and Gadella BM. 2007. Microscopic and flow cytometric semen assessment of Dutch AI-bucks: Effect of semen processing procedures and their correlation to fertility. Theriogenology 67:863-871.

Purdy PH. 2006. A review on goat sperm cryopreservation. Sm. Rumin. Res. 63:215-225.

Randel RD. 1994. Reproductive characteristics of the Brahman

and Brahman based bulls. In: Fields MJ and Sand RS (Ed.), Factors Affecting Calf Crop, CRC Press Boca Raton, F.L., pp. 331-344.

Rasband WS. 2006. Image J. US National Institute of Health, Bethesda, Maryland, USA 2006; http://rssb.info.nih.gov/ij/.

Ritar AJ and Salamon S. 1991. Effects of month of collection, method of processing, concentration of egg yolk and duration of frozen storage on viability of Angora goat spermatozoa. Sm. Rumin. Res. 4:29-37.

Rodriguez-Martinez H and Barth AD. 2007. *In vitro* evaluation of sperm quality related to *in vivo* function and fertility. Soc. Reprod. Fert. Suppl. 64:39-54.

Saacke RG, Nadir S and Nebel RL. 1994. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. Theriogenology 41:45-50.

Salamon S and Maxwell WM. 2000. Storage of ram semen. Anim. Reprod. Sci. 62:77-111.

Salamon S and Maxwell WMC. 1995. Frozen storage of ram semen: II. Causes of low fertility after cervical insemination and methods of improvement. Anim. Reprod. Sci. 38: 1-36.

Sekoni VO and Gustafsson BK. 1987. Seasonal variation in the incidence of sperm morphological abnormalities in dairy bull regularly used for artificial insemination. Br. Vet. J. 143:312-317.

Shamsuddin M and Rodriguez-Martinez H. 1994. A simple non traumatic culture method for the selection of spermatozoa for *in vitro* fertilization in the bovine. Anim. Reprdo. Sci. 36:51-75.

Shamsuddin M, Amiri Y and Bhuiya MMU. 2000. Characteristics of buck semen with regard to ejaculation number, collection intervals, dilution and preservation periods. Reprod. Dom. Anim. 35:53-57.

Shamsuddin M, Rodrigues-Martinez H and Larson B 1993. Fertilizing capacity of bovine spermatozoa select after swim-up in hyaluronic acid containing medium. Reprod. Fert. Dev. 5:307-315.

Shanis BF, Chek JH and Bollendorf A. 1989. Interpretation and misinterpretation of semen parameters. Arch. Androl. 23: 213- 217.

Siddiqui MAR. 2008. Laboratory tests to predict bull fertility and the value of sperm nuclear shape analysis. Doctoral Thesis, Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh. pp. 1-108.

Söderquist L, Janson L, Larsson K and Einarsson S. 1991. Sperm morphology and fertility in AI bulls. Zent. Vet. A. 38:534-543.

Söderquist L. 1991. Sperm characteristics and fertility in dairy AI bulls. PhD Thesis, Department of Obstetrics and Gynaecology, Faculty Veterinary Medicine, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Sundaramman MN, Thangaraju P and Edwin MJ. 2002. Age related changes in testes size of Jersey bulls and its effects on semen production traits. Ind. J. Anim. Sci. 72:567-568.

Watson PF. 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60:481-492.

Williams WW. 1920. Technique of collecting semen for laboratory examination with a review of several diseased bulls. Corn. Vet. 10:87-94.

Wurgau T. 1986. The significance of tertiary abnormalities for the evaluation of sperm morphology. Thesis: Tierarztliche Fskultet, Ludwing Maximalians Universitat Munchen, German Federal Republic.

(접수: 2010. 9. 22 / 심사: 2010. 9. 27 / 채택: 2010. 10. 5)