Breeding soundness of bulls and the quality of their frozen semen used in cattle artificial insemination in Bangladesh

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Abstract

The present investigation was aimed at evaluating the bulls and their semen from production to insemination in a routine artificial insemination (AI) programme in Bangladesh. Five crossbred bulls were examined for breeding soundness and their semen was preserved and evaluated with respect to sperm motility, and proportion of spermatozoa with normal acrosome, midpiece and tail. Semen was sampled immediately after collection, pre-diluted, cooling down to $+4^0$C, and storage at -196^0C for 1 day, 7 days and 3 months in Central Cattle Breeding Station (CCBS). Semen was transported from CCBS to District AI centre at Mymensingh, and then samples were collected after 7 days, 3 months and immediately before insemination. From the District AI centre, semen was transported to the Sub-centre, Fulbaria and sampled there within 7 days after transportation and immediately before insemination.

The bulls used in this study were regarded as clinically normal and belonged to a homogenous group except one who had leukocytes in semen. The percentage of motile spermatozoa varied from 38±6 to 65±7 depending on the occasions when evaluation was made. The sperm motility dropped due to dilution ($P<0.05$), chilling ($P<0.05$), freezing ($P<0.01$) and storing 3 months in the bull station ($P<0.01$). Three months storage in the District AI centre and transportation from there to the sub-centre also resulted in reduced sperm, motility ($P<0.05$). The percentage of normal spermatozoa with respect to acrosome, midpiece and tail were between 84±6 and 90±5 depending on the occasions of sampling; however, the percentage did not drop significantly among the occasions of sampling.

It is concluded that the freezing protocol and handling of frozen semen during transportation and management during storage need to be improved to ensure at least 50% sperm motility at the time of AI, given 30 million total spermatozoa per cow dose.

Key words: Artificial insemination, breeding soundness, cattle, frozen semen evaluation

Introduction

Artificial insemination (AI) is the single most important technique used for the genetic improvement of cattle. This is possible because a few highly selected bulls produce enough spermatozoa to inseminate thousands of cows per year. The goal of the AI field services is to maximize the number of viable offspring per breeding animal per unit time.

This can be achieved by inseminating cows with sufficient progressively motile spermatozoa from a given ejaculate without reducing their fertilizing capacity. Thus, the small number of frozen-thawed spermatozoa in each insemination dose must be of very high quality to ensure acceptable pregnancy rates (Brinsko and Varner 1993).

A thorough examination of bulls for breeding soundness is a prerequisite for investigating their fertility (Spitzer and Hopkins 1997). Although the outcome of a breeding soundness examination for a bull depends to some extends on the clinical reproductive knowledge and experience of the veterinarian concerned, the test was found good enough to identify diseases and/ or problems which often result in poor quality semen in respect to both freezability and fertilizing capacity (Spitzer et al 1988; Johnson et al1995; Lewis et al1996; Bhuiyan et al1997).

It is important to collect as many ejaculates as possible within a certain period without compromising the quality of semen for a routine AI practice. In case of bulls, sperm motility is a fairly reliable indication of the viability of fresh and frozen semen (Saacke and White 1972; Grahman et al1980). The fertilizing ability of spermatozoa, depends not only on the initial quality of semen, but also on the subsequent laboratory processes that end up with deposition of semen in the genital tract of a cow. The processes of semen dilutions, chilling, freezing, storage, transportation and thawing for insemination invariably result in some reduction of the viability and fertilizing capacity of spermatozoa. Thus the fertilizing capability of spermatozoa in the AI dose is determined not only by the inherent quality of the material produced by the bull but also by man's interaction with the product (Saacke 1983; Serres et al 1997). Moreover, the process of transportation from one tank to another can warm - up the semen straws if is not accomplished quickly and accurately (Hafez 1993). Maintaining optimum nitrogen level in the container is important during prolonged storage and transportation of semen (Sherman 1990; Quintin et al 1997).

The aim of the present investigations was to characterize the physical, sexual and semen characteristics of crossbred bulls (Friesian ´ Sahiwal and Friesian ´ local) and to determine the occasions when the quality of frozen semen deteriorate in a routinely running AI field service.

Materials and methods

The investigation was conducted in the Central Cattle Breeding Station (CCBS), Savar, Dhaka, District Artificial Insemination Centre, Mymensingh and Artificial Insemination Sub-centre, Fulbaria.

Clinical examination of bulls

Five crossbred bulls (Bull ID No. 4025, 5561, 6742, 6816 and 7802) were examined for breeding soundness according to the method described elsewhere (Ott 1986). Bull ID No. 4025, 5561, 6742 and 7802 were crosses of nondescript local with Friesian and bull ID

No. 6816 was cross of Sahiwal with Friesian. The bulls were of 5 to 10 years old. The scrotum of bulls was inspected with respect to size, symmetry and any visible skin diseases. The consistency of testes was assessed on the basis of firmness and resilience into 1-5 scales. Bulls scoring 5, 4, or 3 were considered normal. The nutrition condition of the bulls was scored into 1-5 scale (Nicholson and Butterworth 1986). The bulls were weighted routinely once a month. The bulls were vaccinated against Anthrax, Haemorrhagic Septicemia, Black Quarter, and Foot and Mouth Diseases.

Semen processing, preservation, distribution and transportation

Semen was collected at homosexual mount (using male as a teaser) using artificial vagina. After collection, the ejaculates in receptacles and prepared diluents in conical flasks, were placed in a water bath at 37^0C. After estimation of sperm motility and concentration, semen was diluted with TRIS-fructose-egg yolk (TFEY) extender. Briefly, the basic extender contained TRIS (297.6 mmol/L), fructose (82.6 mmol/L), citric acid (105.3 mmol/L), penicillin G sodium (1000 IU/ml) and streptomycin sulphate (1 mg/ml) in glass-distilled water. Egg yolk was added with the buffer (20%; v/v). The complete extender was divided into two equal parts and 12.8% glycerol was added to one part of the extender. The other part of the diluent was used to make the initial dilution of semen. The equal parts of initially diluted semen and double concentration glycerol-containing extender were mixed together at four steps during a 3 to 4 hrs cooling process. The dilutions were made at $+18^0$C, $+12^0$C, $+8^0$C and $+4^0$C.

Individual insemination doses were filled in 0.25 ml French straws and the open ends of the straws were sealed by using an automatic filling-sealing machine (# YC 250, IMV, Cedex, France). The straws were previously marked with necessary information. After sealing, the straws were left for equilibration at 4^0C for one hour. The cooling and equilibration were performed in a cold handling cabinet (# 036, IMV, Cedex, France). The semen freezing was carried out in a mechanical semen freezer (HN, Mini Tüb, Germany). Semen was cooled from 4^0C to -140^0C during a period of 20 to 30 min and then the straws were directly plunged into liquid nitrogen (-196^0C). After overnight storage in liquid nitrogen, representative straws were thawed (35 to 38^0C for 10 to12 sec) and semen was evaluated for sperm motility. Semen showing at least 40% post thaw sperm motility was selected and distributed for insemination. The CCBS used its own traffic to transport semen to the District AI Centre, Mymensingh within a week. The AI technician of Fulbaria Sub-centre received semen personally from District AI centre by using local traffic.

Semen evaluation

Individual fresh ejaculates were evaluated for colour, volume, density, mass activity, sperm motility, sperm concentration, total spermatozoa per ejaculates, percentage of live spermatozoa, proportions of spermatozoa with normal acrosome, midpiece and tail, and with normal head morphology, and possible presence of somatic cells. Semen was then sample after initial dilution, cooling down to $+4^0$C, deep frozen and stored at -196^0C for 1 day, 7 days and 3 months. Same semen samples were also collected from the District AI

centre, Mymensingh after transportation, and prepared by the technician for AI 3 months after storage. Same semen samples were also collected from the Sub-centre, Fulbaria after transportation from the District AI centre and prepared by the technician for AI. All these samples were evaluated for sperm motility and proportion of spermatozoa with normal acrosome, midpiece and tail.

Semen colour was assessed in the collecting tube. The volume of semen was measured directly from the graduated collecting tube. The density was scored into 1-5 scales. To evaluate mass activity, a drop of undiluted semen was placed on a slide without coverslip and examined under phase contrast microscope(100X) and scored into 1-5 scales. To evaluate sperm motility, a small drop (10 ml) of semen was placed on a prewarmed (37°C) slide, covered by a coverslip and examined under phase contrast microscope (400X). Two investigators scored the sperm motility and the average scores were recorded.

The concentration of the spermatozoa was determined by using haemocytometer (Bane 1952). Semen was diluted with distilled water at the ratio of 1:200 fresh samples. Eosin-nigrosin stain was used to determine the percentage of live spermatozoa. The stain was prepared according to Evans and Maxwell (1990). One drop of semen and one drop of eosin-nigrosin stain were placed closely on a clean slide, and the semen and the stain were mixed with a clean stick, a thin smear was made, dried in air and examined under microscope (400X). At least 200 spermatozoa were examined from each smear.

The abnormalities of the acrosome, midpiece and tail of spermatozoa were evaluated in formol saline-fixed semen using phase contrast optics (1000X). The buffered formol saline was prepared and the formal-saline fixed spermatozoa were classified according to Hancock (1957). The sperm head morphology was evaluated in thin smear stained with William's technique (Williams 1920; Lagerlöf 1934). At least 500 spermatozoa were examined from individual smears. The possible presence of somatic cells in semen was sought in thick smears stained with Hematoxylene and Eosin stain (Luna 1968).

Statistical analysis

The data on the clinical examination of bulls and their semen were presented as mean and standard deviation for individual bull. One-way ANOVA was used to determine individual bull variations with regard to the body condition scores, scrotal circumference, semen volume, sperm concentration per ml, total spermatozoa per ejaculate, sperm motility, proportion of live spermatozoa, proportion of spermatozoa with normal acrosome, midpiece and tail, and with normal head morphology. Repeated measures analysis of variance with different contrast was used to analyze data on sperm motility and proportion of normal spermatozoa with regard to the acrosome, midpiece and tail obtained from fresh ejaculate, and from semen after dilution, chilling and equilibration, freezing, storage at CCBS for 7 days and 3 months, transportation to the District AI Centre and storing there for 3 months, thawing and preparation for insemination at the District AI centre, transportation to an AI Sub-Centre, and after thawing and preparation for insemination at the Sub-Centre using statistical package Systat 6.0 (Anon 1996). The

data were log-transformed to near normality except the proportion values which were transformed by arcsine transformation.

Results

The data on the general and sexual health of crossbred bulls and the results of their libido tests are presented in the Table 1. The bulls were 5.3 to 9.5 years old. Their pulse rate, breathing rate and rectal temperature varied from 54.3 to 64.33/min, 26.0 to 35.0/min, and 38.5 to 38.9ºC, respectively. The bulls' nutritional condition scores varied from 3.1 to 3.8 (1-5 Scale), they weighed between 547 and 725 Kg and their scrotal circumference varied from 36.7 to 40.0 cm. The score of the testicular consistency on the basis of firmness and resilience varied from 3.7 to 4.3 (1-5 scale).

Table 1. Results of the clinical examination and libido test of bulls

Parameters		Identification number of bulls				
		4025	**5561**	**6742**	**6816**	**7802**
Pulse rate/min.		64.3±3.2	57.3±4.7	54.3±1.5	63.3±4.7	59.7 ±4.9
Respiration rate/min		31.0±6.2	31.3±2.5	35.0±4.6	27.7±3.2	26.0±1.7
Rectal temperature, ^{0}C		38.5±0.1	38.9±0.2	38.5±0.2	38.8±0.2	38.7±0.4
Body condition score,(1-5 scale)		3.5±0.5	3.1±0.2	3.8±0.8	3.8±0.6	3.3±0.6
Body weight, kg		612±10	725±15	712±10	705±18	547±9
Scrotal circumference, cm		36.8±0.7	39.7±1.5	38.3±2.3	40.0±1.7	36.7±2.1
Consistency of testes (1-5 scale)*	Left	4.0±1.0	3.7±1.2	4.3±1.2	4.0±1.7	4.0±1.0
	Right	4.0±1.0	3.7±1.2	4.3±1.2	4.0±1.7	4.0±1.0

** Scale 5 was the best and 1 was the worst. The values are mean ± SD*

Table 2 shows the data on the evaluation of fresh semen. A significant bull effect appeared on the sperm concentration ($p<0.05$); Bulls # 5561 and 6742 had lower sperm concentrations (1067 ± 58 and 983 ± 104 million, respectively) than did bulls # 6816 and 7802 (1333 ± 104 and 1483 ± 236 million, respectively). However, the spermatozoa number per ejaculate did not vary depending on bulls. The mean proportion of normal spermatozoa with respect to head morphology was generally high (90.5% to 97.9%); however, bull # 5561 had lower proportion of normal-head spermatozoa (86.7%) than the others did ($p<0.05$). The mean proportion of live spermatozoa was high (80.6 to 89.5%) irrespective of bulls and the differences between bulls were not significant. Leukocytes (Neutrophils) were found in semen of Bull # 5561. Somatic cells were found in the semen of almost all bulls.

Table 2. Results of the evaluation of fresh semen

Parameters	Identification number of bulls				
	4025	**5561**	**6742**	**6816**	**7802**
Ejaculate volume, ml	6.4±0.6^a	6.4±2.0^a	9.2±2.3^a	5.9±0.6^a	4.6±1.1^b
Density of semen (1-5 scale)	3.3±0.6	3.0±0.0	3.0±0.0	3.3±0.6	3.7±0.6

Mass activity (1-5 scale)	3.3±0.6	2.7±0.6	3.3±0.6	3.7±0.6	3.3±0.6
Sperm motility, %	67±6	60±10	65±5	67±6	68±3
Sperm concentration, mill./ml	1400±180^{ab}	1067±58^{b}	983±104^{b}	1333±104^{a}	1483±236^{a}
Total spermatozoa/ejaculate, mill.	8096±3331	6919±2382	8928±1559	7904±1168	6633±734
Spermatozoa with normal acrosome, midpiece and tail, %	89.1±7.4	87.2±2.1	89.9±5.4	89.7±6.3	94.4±3.7
Spermatozoa with normal head morphology, %	92.2±4.1^{ab}	86.7±2.2^{b}	90.5±3.7^{ab}	97.6±1.2^{ab}	97.9±1.3^{a}
Live spermatozoa, %	88.6±6.8	80.6±10.7	92.4±6.3	86.9±9.8	89.5±5.3

The values are mean±SD of 3 observations.
abc: Values with different superscripts in the same row differ significantly from each other (P<0.05).

With respect to the freezing, storage and transportation of semen, the percentage of motile spermatozoa varied from 38% to 65% depending on the occasions when semen evaluation was made (Figure 1). The sperm motility declined due to dilution (P<0.05), chilling (P<0.05), freezing (P<0.001) and storing 3 months in the Bull Station (P<0.01).

Figure 1. Sperm motility in fresh ejaculate, and at different stages of semen freezing, storage, transportation and preparation for AI. 1=fresh ejaculate (65±7%), 2=diluted with tris-egg yolk medium (62±6%), 3=diluted with tris-egg yolk-glycerol medium, cooled down to 4°C and equilibrated for 4 h (58±8%), 4=frozen-thawed semen (48±8%), 5=thawed after 1 week of freezing (47±7%), 6=transported from the Bull Station to the District AI Centre (44±8%), 7=prepared for AI in the District AI Centre (41±6%), 8=stored 3 months at the Bull Station (42±6%), 9=stored 3 months in the District AI Centre (40±7%), 10=transported from the District AI Centre to a Sub Centre (38±8%) and 11=prepared for AI in the Sub Centre (38±6%). Note the values in the parenthesis are the mean±SD of 15 observations

After storing in the District AI Centre for 3 months, sperm motility decreased (P<0.05). Sperm motility declined further (P<0.05) due to transportation of semen from the District AI Centre to the Sub-centre (Figure 1). The proportion of normal spermatozoa with respect to the acrosome, midpiece and tail determined during the process of freezing, storage, transportation and preparation for insemination were between 84 and 90% (Figure 2).

Figure 2. The proportion of spermatozoa with normal acrosome, midpiece and tail in fresh ejaculates, and at different stages of freezing, storage, transportation and preparation for AI. 1=fresh ejaculate (90±5%), 2=diluted with tris-egg yolk medium (89±7%), 3=diluted with tris-egg yolk-glycerol medium, cooled down to 4°C and equilibrated for 4 h (84±6%), 4=frozen-thawed semen (84±7%), 5=thawed after 1 week of freezing (85±5%), 6=transported from the Bull Station to the District AI Centre (85±5%), 7=prepared for AI in the District AI Centre (86±5%), 8=stored 3 months at the Bull Station (85±5%), 9=stored 3 months in the District AI Centre (85±5%), 10=transported from the District AI Centre to a Sub Centre (85±4%) and 11=prepared for AI in the Sub Centre (85±5%). Note the values in the parenthesis are the mean±SD of 15 observations.

However, the difference in proportion normal spermatozoa among different time points of evaluation did not vary significantly (Figure 2).

Discussion

The bulls used for routine AI in the present investigation can be regarded as sound except one that delivered leukocytes in semen. The presence of leucocytes in semen is an indication of infection in the genital tract of the bull (Bhuiyan et al1997). Individual bulls differed with regard to the semen volume and sperm concentration. The differences in semen parameters among bulls may be attributed to the variation in the secretory activities of the accessory sexual glands, scrotal circumference, age and body weight (Graves 1978; Leon et al 1991; Sharma et al 1991; Jainudeen and Hafez 1993). In the present study, although the bulls were considered as a homogeneous group, there was significant ($P<0.05$) variation in proportion of sperm head morphology among bulls. Decreased proportion (86.7%) of normal sperm head morphology in semen indicates pathological conditions in the genital tract of the bull, particularly in the testes. This emphasizes the importance of evaluation of sperm head morphology before selection of bulls for routine AI.

The results of the present investigation indicate that the quality of semen considered good immediately after collection can deteriorates during processing as demonstrated by decreased sperm motility after dilution ($P<0.05$). The quality of semen can be influenced by the extender used (Sullivan 1978). In the present study, tris-fructose-citric acid-egg yolk-glycerol was used to dilute the semen for freezing. There are reports that, frozen semen diluted in skim milk-egg yolk-glycerol or egg yolk-citrate-glycerol had higher viability than that diluted in tris-fructose-citric acid-egg yolk-glycerol extender (Fernandez et al 1989; Belorkar et al 1993; Kommisrud et al 1996; Pramanik and Raina 1998). This may be due to interactions between tris and glycerol in the extender (Pickett et al 1975; Pickett 1993; Kommisrud et al 1996). However, Dhami and Shani (1993) did not find any difference between tris and milk based diluents with regard to the post thaw motility.

It has been demonstrated that glycerol has an adverse effect on post-thaw sperm quality and fertilizing capacity of spermatozoa (Wilmut and Polge 1977**).** Fiser and Fairful 1984; Jeyendran et al 1985). To improve the post-thaw survival rate of spermatozoa, glycerol was recommended to add at $+4^0$C to $+5^0$C (Polge 1953; Miller and Van DeMark 1954). A step-wise addition of glycerol with semen further improved the post-thaw survival of spermatozoa (Pickett and Berndtson 1978; Bhosrekar et al 1986). Accordingly, in the present investigation, glycerol was added with semen in four steps, viz., at $+18^0$, $+12^0$, $+8^0$ and $+4^0$C. In accordance with the standard practice, the present study used to cool down the semen during a period of 3 to 4 hrs from $+37^0$C to $+4^0$C. However, Sullivan (1978) reported that cooling of mammalian spermatozoa at lower temperature (5^0C) can cause cold shock leading to decreased sperm motility and increased proportion of spermatozoa with coiled tail.

Frozen semen needs to be thawed according to the instructions of manufacturer whether it is used for AI or for any kind of evaluation. To minimize ice crystal formation that occurs during slow warming, thawing of straws should be done rapidly (Coulter 1992). Thawing at 35 to 38^0C for 10 to12 sec has been recommended for thawing frozen bull semen (Yilmaz and Yurdadin 1994). Accordingly, in the present study, the semen straws were thawed at 35 to 38^0C for 10 to 12 sec. Moreover, it is harmful to allow the temperature of semen to fluctuate which often happens during the transportation of semen straws from one container to other for distribution of semen among different Centres and Sub-centres. A one-minute exposure of semen straws to ambient temperature can cause significant reduction in post thaw sperm motility (Berndtson et al 1976). This may explain the reason for decreased post-thaw motility of spermatozoa after transportation to the AI Sub-centre from the District AI centre. Because, small number of counted straws were distributed to the Sub-centres by the District AI Centre compared to the distribution of whole goblet (large number of straws) by the Bull station to the District AI centre.

There is a significant inverse relationship between the proportion of spermatozoa with normal acrosome, midpiece and tail, and fertility (Larsson 1988). Insemination performed with increased proportion of abnormal spermatozoa (>20%, all abnormalities) may result in reduced fertility (Larsson 1988). Semen from most males always contains some abnormally formed spermatozoa. However, lower fertility rate is not associated with abnormal spermatozoa until the proportion of the latter exceeds 20% (Hafez 1993). Nevertheless, the proportion of spermatozoa with abnormal acrosome, midpiece and tail, and with abnormal head morphology did not exceed 20% in this investigation.

Visual estimation of the percentage of motile spermatozoa is the most commonly used technique for semen evaluation. Viability of spermatozoa is generally correlated with the percentage of spermatozoa possessing normal structure (Sprecher and Coe 1996). Thus increase in sperm abnormalities are associated with decreases in sperm motility. Most fertility problems encountered in well-managed AI bulls is due to improper handling of frozen semen (Serres et al 1997). Accordingly, in the present study, sperm motility dropped significantly due to dilution of fresh semen, chilling, freezing, storing in the bull station and in the District AI centre and transportation from the AI centre to sub-centre. Similarly, the proportion of normal spermatozoa significantly reduced due to freezing of

semen. As a result, the cow received semen with 38% and 40% average sperm motility in the District AI centre and sub centre, respectively. This means, the cow receives 12.0 million or less motile spermatozoa, given 30 million total spermatozoa per cow dose. The importance of the number of the spermatozoa per cow dose is evident (Larsson 1988). Because, fertility of an AI is dependent upon delivery of sufficient number of quality spermatozoa to the female genital tract at the appropriate time (Saacke et al 1991).

Conclusions

In the present study, the physical and reproductive parameters of crossbred bulls (Friesian ´ Sahiwal and Friesian ´ local) have been characterized to some extent in a selected population.

The freezing protocol and handling of frozen semen during transportation and management during storage need to be improved to ensure at least 50% sperm motility at the time of AI, given 30 million total spermatozoa per cow dose.

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Go to top