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Effects of semen quality on pregnancy rate in artificially inseminated dairy cows

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Abstract

The study aimed to evaluate the quality of fresh and frozen-thawed semen of five adult Holstein-Friesian crossbred bulls and the pregnancy rate of cows inseminated with frozen semen of those bulls. The fresh semen of breeding bulls collected for artificial insemination (AI) programme in the field was of good quality with volume (6.7 ± 0.2 ml - 8.9 ± 0.5 ml), concentration (904.2 ± 56.4 million/ml), mass activity (3.3 ± 0.2-3.6 ± 0.2), total motility (77.0 ± 1.1% – 92.1 ± 0.6%), progressive motility (67.0 ± 1.2% – 87.4 ± 0.6%) and semen viability (73.0 ± 0.6% to 85.4 ± 0.7%). The computer assisted sperm analysis (CASA) results showed that diluted pre-freezing semen had good sperm total motility (50.1 ± 3.8 % to 59.0 ± 4.7%), progressive motility (30.0 ± 1.2%-39.0 ± 1.6%), the velocity traits of straight linear velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP) of sperm ranged from 48.0 ± 1.3 - 71.3 ± 0.7 µm/s,118.1 ± 2.8-181.3 ± 10.9 µm/s and 68.4 ± 2.5 to 91.0 ± 2.9 µm/s, respectively. Bull 1 showed significantly higher VSL (71.3 ± 0.7 µm/s), VCL (181.3 ± 10.9 µm/s) and VAP (91.0 ± 2.9 µm/s) compared to others. Viability of frozen-thawed semen was lower in Bull 5 (73.0 ± 1.71%) compared to others. Although in frozen-thawed semen these parameters declined, the semen was sufficiently good to be used in AI in the field. The overall pregnancy rate using frozen semen was 55.6% and the highest pregnancy rate (62%) was in cows that were inseminated with frozen semen of Bull 1, but the differences between bulls was not significant. The pregnancy rate had positive correlation with sperm count, total motility, progressive motility, VCL, VSL, VAP, amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN), straightness (STR), sperm viability. In artificially inseminated cows, the intensity of oestrus of cows, timing of AI, site of semen deposition and season had a significant effect on pregnancy rate. In conclusion, the fresh and frozen-thawed semen of breeding bulls supplied in North-East Bangladesh for AI programme were good quality. Heat detection and insemination timing need to be improved to increase the pregnancy rate. (*Bangl. vet*. 2022. Vol. 39, No. 1 - 2, 1 – 15)

Introduction

Cryopreservation of bull semen to improve genetic traits and speeds the artificial insemination (AI) programme for dairy industry (Maxwell, 1984; Rabidas *et al*., 2012;

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Hasan *et al*., 2020). Despite several developments in semen freezing and thawing, approximately 50% of sperm are rendered immotile by cryopreservation, and fertilizing capacity of spermatozoa is decreased (Parisi *et al*., 2000; Celeghini *et al*., 2008; Pini *et al*., 2018) leading to lower pregnancy rates compared to fresh semen. Management and environmental factors also influence the pregnancy rates, prolonging calving interval and decreasing profitability. Evaluation of sperm quality before and after freezing is a good technique, as it can be done under phase contrast microscope (Kumar *et al*., 2015; Patel and Dhami, 2016) or preferably by computer assisted sperm analysis (CASA) (Sundararaman *et al*., 2012; Islam *et al*., 2017).

Moreover, bull fertility can be evaluated by analysing the pregnancy rates. The study was undertaken to evaluate the quality of fresh and frozen-thawed semen and the pregnancy rate of inseminated cows.

Materials and Methods

Experimental animals and ethical issues

Five adult Holstein-Friesian crossbred bulls (1, 2,3, 4, 5) aged between 4 and 7.5 years of age and a mean body weight of 825 ± 83.2 kg were selected. Bulls were physically sound and vaccinated against haemorrhagic septicaemia and foot and mouth disease. Their feeding regimen included good-quality seasonal fodder at the rate of 10% of their body weight, with 2 to 3 kg of concentrates including crushed maize (*Zea mays*), kesari (*Lathyrus sativus*), wheat (*Triticum aestivum*) bran, soybean (*Glycine max*) meal and common salt per bull per day with free access to water. Preventive measures against worm infestation were undertaken thrice a year or whenever necessary. The data regarding bulls' history, feeding, their physical states and preventive measures was from the authority of Central Cattle Breeding Farm taking permission from Department of Livestock Services (DLS). The study was accomplished according to ethical guidelines of Sylhet Agricultural University (SAU) and approved by Animal Experimentation Ethics Committee, SAU, Sylhet; Bangladesh.

Semen collection and evaluation

The semen was collected by artificial vagina (Noakes *et al*. 2018a). Prior to semen collection all the parts of Artificial Vagina (AV, Minitube, Germany) were cleaned, sterilized, assembled accurately and two-thirds of AV set was filled with warm water (50-52ºC) and the remaining with air. The internal temperature was conserved at 45 to 48ºC (Noakes *et al*., 2018a). Good amount of lubricant was applied over inner surface of the artificial vagina with a glass rod. When the bull was hyperactive to mount over the dummy, the penis of the bull was directed toward the artificial vagina grasping the sheath to disperse the ejaculated semen in a graduated tube close to the latex extension cone. Semen was kept in a water bath at 37ºC immediately after collection to prevent cold shock until further handling. The semen was collected twice a week with two ejaculations during each collection session.

The ejaculated semen volume was recorded by reading the graduated mark of the collection tube in millilitres (Alam *et al*., 2005; Mostari *et al.*, 2019; Apu *et al*., 2012). The colour of semen was noted as milky white to thick creamy (Jha *et al*., 2013). The consistency of semen was observed by inclining and moving the semen in collection tube. It was scored in 4 scales, 1= watery, 2 = milky white, 3 = creamy and 4 = creamy-grainy.

Microscopic evaluation (mass activity, sperm concentration and motility) of fresh semen was done to evaluate the sperm characteristics. One drop of fresh semen was placed on pre-heated clean glass slide at 37ºC without a cover slip (Shaha *et al*., 2008; Rabidas *et al*., 2012). The mass activity of semen was evaluated using phase contrast microscope (Nikon Eclipse E100, Japan) with 10x zoom and a heated table (Tomar, 1984). The mass activity was scored from 1 - 4 as follows: no wave motion = 1; slow wave motion = 2; rapid wave motion with formation of eddies at the end of wave = 3 and eddies = 4.

Sperm concentration was measured (millions/ml) using a calibrated spectrophotometer (SDM6, Minitube, Verona, WI, 53593-1821 United States). Motility was evaluated by placing a small drop of semen onto the preheated slide under cover slip with higher magnification (100×). Sperm moving forward were counted, whereas sperm moving in circles or backward or else showing pendulating movement were omitted (Herman *et al*., 1994).

Morphology of sperm head was assessed in dried semen smears by differential interference contrast (DIC) optics (BX 51, OLYMPUS, Tokyo Japan) with higher magnification (1000×) following the method of Freneau *et al.* (2009). Sperm acrosome, mid piece and tail morphology were observed by means of samples diluted in Buffered formal saline following technique described by Barth and Oko (1989), Jha *et al*., (2013). The Buffered formal saline sample was made by dissolving 6.2 gm disodium hydrogen phosphate, 2.5 gm potassium dihydrogen phosphate, 5.4 gm sodium chloride and 175 ml concentrated formaldehyde in 1000 ml of distilled water (Jha *et al*., 2013). Sperm viability was counted by using Eosin-Nigrosin staining (Evans and Maxwell, 1987). The Nigrosin–eosin was prepared by dissolving 10 gm Nigrosin, 1.7 gm eosin and 2.9 gm sodium citrate in 100 ml distilled water (Roostaei-Ali Mehr *et al*., 2013).

After examining of sperm concentration, motility and morphology, semen was extended with TRIS-citrate-egg yolk diluent (Sugulle *et al*., 2006). Briefly, the basic extender containing TRIS (297.6 mmol/L), citric acid (105.3 mmol/L), fructose (82.6 mmol/L), penicillin G sodium (1000 IU/ml) and streptomycin sulphate (1 mg/ml) was taken in glass-distilled water. Egg yolk was mixed with the buffer (20%; v/v). The whole extender was fractioned into two equal parts. Next, 12.8% glycerol was put in one part of the extender. Another part of the diluent was spent for initial dilution of semen. The two parts of diluent were then mixt together in four steps during a 3 to 4 hrs freezing technique as follows at +18ºC, +12ºC, + 8ºC and + 4ºC. The equilibrated semen was loaded into 0.25 ml plastic straws (0.25 ml straw, Minitube,

Verona, WI, 53593-1821 United States), sealed by an automatic filling-sealing machine (Minitube, Verona, WI, 53593-1821 United States) and frozen into a programmable biological freezer for cooling from 4°C to -140°C. Each semen sample was primarily cooled at the rate of -5°C/ min from 4° to -10°C. Once at -140°C, semen straws were instantly sunk into liquid nitrogen at -196°C for storage.

Computer assisted sperm analysis (CASA)

Total motility (TM%), progressive motility (PM %), VSL (µm/s), VCL (µm/s), VAP (µm/s), ALH (µm), BCF (Hz) and the ratios STR (VSL/VAP), LIN (VSL/VCL), and WOB (wobble, VAP/VCL) were evaluated objectively using CASA (IVOS II, IMV Technologies, 61302 L'Aigle, Cedex, France) using a phase-contrast microscope. After semen dilution, the equilibrated pre freezing bull semen was analysed. An aliquot (5 µL) of semen was laid on a microscope slide warmed at 38°C and covered with a coverslip (18 × 18 mm). For analysis of the kinematic patterns sperm images in eight fields were digitized. The mean values were counted for each of the following parameters focusing on approximately 1000 spermatozoa: total motility (%), progressive motility (%), VSL (µm/s), VCL (µm/s), VAP (µm/s), ALH (µm), BCF (Hz) and the ratios STR (VSL/VAP), LIN (VSL/VCL), and WOB (wobble, VAP/VCL). In case of frozen semen, before analysis semen was thawed at 37^{o}C temperatures for 10-15 seconds and kept in Eppendorf tube.

Artificial insemination and pregnancy diagnosis

A total of 500 Holstein-Friesian crossbred cyclic cows aged 3 to 4 years, weighing 300 - 350 kg, parity 2 - 3 with BCS 3.0 - 3.5 (1-5 scale) were selected. The oestrous signs were observed and the cows were inseminated artificially by trained AI technicians: information regarding semen deposition in the genital tracts was noted from AI log book of technicians. Trans-rectal palpation of reproductive tract was done for confirmation of pregnancy.

Statistical analysis

The data were presented in Microsoft excel sheet and expressed as the means and standard errors (mean ± se) and percentages (%). Statistical differences in the parameters among bulls were obtained using one-way analysis of variance with a Post Hoc least square mean test using SPSS 20.0 (Snedecor and Cochran, 1994) statistical package and $P<0.05$ was considered significant. The comparative analysis between equilibrated fresh semen and frozen-thawed semen were done by paired sample *t*-test. The univariable analysis of factors associated with pregnancy in cows was done by Chi square test. The relationships between sperm quality and pregnancy rate were estimated using the Pearson's correlation analysis.

Results and Discussion

The data on fresh semen are presented in Table 1. The volume of semen (6.7 ± 0.2 ml - 8.9 ± 0.5 ml) varied significantly between bulls and the semen volume of Bull 2 was significantly lower than the others ($P< 0.05$).

Table 1: Biophysical characteristics of fresh semen of five bulls

Parameters	Bull 1	Bull 2	Bull 3	Bull 4	Bull 5	P-value
Vol (ml)	8.9 ± 0.5^{a}	6.7 ± 0.2^{b}	8.5 ± 0.6^{a}	8.0 ± 0.2^{a}	7.9 ± 0.3^{a}	0.004
Density (1-5 scale)	3.0 ± 0.2^{ab}	3.5 ± 0.2c	2.6 ± 0.2^{b}	2.7 ± 0.2^{b}	3.4 ± 0.3^{ac}	0.003
MA (1-5 scale)	3.6 ± 0.2^{a}	3.3 ± 0.2^{a}	3.5 ± 0.2^{a}	3.3 ± 0.2^{a}	3.5 ± 0.2^{a}	0.371
TC(million /eja)	12268 ± 2^{a}	11616 ± 13^{b}	7381 ± 52^{e}	9777 ± 17^{d}	10925 ± 15^{c}	<0.001
CON (milli/ml)	1409.0 ± 73.1^{b}	1751.0 ± 2.2^{a}	904.2 ± 56.4^{d}	1231.7± 37.4^{c}	1401.2 ± 60.0^{b}	<0.001
TM (%)	89.5 ± 1.1^{ab}	92.1 ± 0.6^{a}	83.0 ± 1.0^{c}	87.0 ± 1.2^{b}	77.0 ± 1.1^{d}	<0.001
PM (%)	85.4 ± 0.6^{a}	87.4 ± 0.6^{a}	75.6 ± 0.7^{c}	80.5 ± 1.2^{b}	67.0 ± 1.2^{d}	<0.001
SV (%)	85.4 ± 0.7^{a}	82.4 ± 0.5^{bc}	82.7 ± 0.5^{b}	81.0 ± 0.7^{c}	73.0 ± 0.7^{d}	<0.001
SA (%)	5.7 ± 0.3^{c}	7.8 ± 0.4^{b}	8.0 ± 0.4^{ab}	8.1 ± 0.7^{ab}	9.2 ± 0.5^{a}	<0.001

Mean ± SE in same rows with different superscripts showed significant (P≤0.05) mean differences; Vol = Semen volume, MA = Mass activity, TC = Total count, CON = Concentration, TM = Total motility, PM = Progressive motility, SV = Semen viability, SA = Semen abnormality, ANOVA AND POST HOC LEAST SQUAUE MEAN TEST

The result agrees with the observation of Hossain *et al*. (2012); Santoso *et al.* (2021) and Mandal *et al*. (2012) but differs from the findings of Shaha *et al*. (2008); Baharun *et al.* (2017) and Islam *et al*. (2020). The volume of fresh semen may be affected by age, body weight and season. Bulls with greater semen volume have higher fertility rate (Hossain *et al.*, 2012). The highest density of semen was in Bull 2 (3.5 ± 0.2) and lowest in Bull 3 (2.6 ± 0.2): the difference was significant (P<0.05) and was similar to the results of Sugulle *et al*. (2006); Santoso *et al.* (2021) but varied from Islam *et al.* (2020). The semen density varied due to age and degree of libido and frequency of semen collection (Ahmad *et al.,* 2003; Kumar *et al.,* 2015). The highest concentration was also in Bull 2 (1409.0 ± 73.1 million/ml), and the lowest in Bull 3 (904.2 ± 56.4 million/ml): the difference was significant (P<0.001). The mass activity did not differ significantly; the result supports the findings of Sugulle *et al*. (2006) and Islam *et al*. (2020) but varies from the findings of Rabidas *et al*. (2012); Santoso *et al.* (2021). The mass activity difference might be due to deviations in degree of sexual excitement, age of bulls and breed characteristics (Ahmad *et al*., 2003). However, the total count of sperm per ejaculate was higher in Bull 1 (12268 ± 2 million/ejaculation) and lower in Bull 3 (7381 ± 52 million/ejaculation) and differed significantly (P<0.001) between all bulls. The result is higher than the observation of Sugulle *et al.* (2006); Santoso *et al.* (2021). The number of sperm per ejaculate and sperm concentrations differ between bulls owing to age and body weight, season and method of semen collection (Rabidas *et al*., 2012, Siddiqui *et al*., 2007).

Table 2: Total sperm count, sperm motility, velocity and kinetics of frozen-thawed semen of five bulls evaluated using CASA (Mean ± SE)

Parameters	Bull 1	Bull 2	Bull 3	Bull 4	Bull 5	P-value
TC million /straw	26.6 ± 1.2^{a}	23.1 ± 1.1^{a}	23.0 ± 1.6^{a}	22.5 ± 4.4^{a}	23.0 ± 1.2^{a}	0.698
TM (%)	59.0 ± 4.7^{a}	52.2 ± 2.3^{a}	50.1 ± 3.8^{a}	50.4 ± 2.6^{a}	52.3 ± 2.2^{a}	0.331
PM (%)	39.0 ± 1.6^{a}	32.0 ± 0.6^{b}	30.0 ± 1.2^{b}	31.0 ± 0.7^{b}	32.0 ± 1.2^{b}	<0.001
VSL (µm/s)	71.3 ± 0.7^{a}	57.2 ± 2.2^{b}	55.3 ± 3.7^{b}	47.0 ± 2.9^{c}	48.0 ± 1.3^{c}	<0.001
VCL (µm/s)	181.3 ± 10.9^{a}	152.0 ± 2.6^{b}	143.4 ± 6.9^{b}	142.0 ± 3.3^{b}	118.0 ± 2.8^{c}	<0.001
VAP (µm/s)	91.0 ± 2.9^{a}	77.0 ± 2.7^{b}	75.0 ± 3.4b^{c}	71.4 ± 1.2^{bc}	68.4 ± 2.6^{c}	<0.001
ALH (µm)	11.0 ± 0.3^{a}	10.0 ± 0.4^{ab}	9.0 ± 0.6^{b}	9.4 ± 0.3^{b}	10.0 ± 0.4^{ab}	0.041
BCF (Hz)	29.0 ± 1.2^{a}	27.4 ± 1.9^{a}	28.2 ± 1.6^{a}	28.0 ± 0.1^{a}	28.2 ± 1.6^{a}	0.973
STR (%)	79.0 ± 2.2^{a}	75.2 ± 4.1^{a}	76.0 ± 9.2^{a}	65.4 ± 3.2^{a}	71.0 ± 4.3^{a}	0.427
LIN (%)	40.0 ± 2.6^{a}	38.0 ± 1.2^{a}	39.4 ± 4.6^{a}	33.2 ± 2.5^{a}	41.0 ± 1.8^{a}	0.366
WOB (%)	51.0 ± 3.8^{abc}	50.6 ± 2.2^{a}	52.2 ± 1.2^{abc}	50.5 ± 1.8^{ab}	58.1 ± 2.4^{c}	0.210

Rows with different superscripts showed significant (P≤0.05) mean differences; TC = Total count, TM = Total motility, PM = Progressive motility, VSL = Straight linear velocity, VCL = Curvilinear velocity, VAP = Average path velocity, ALH = Average lateral head displacement, BCF = Beat cross frequency, STR = Straightness, LIN = Linearity, WOB = Wobble, ANOVA AND POST HOC LEAST SQUAUE MEAN TEST

The total motility and progressive motility of bulls ranged from 77.0 ± 1.1% – 92.1 ± 0.6% and 67.0 ± 1.2% – 87.4 ± 0.6%, respectively, and differed significantly between the bulls, supporting the findings of Indriastuti *et al*. (2020) and Santoso *et al.* (2021) but higher from the findings of Islam *et al*. (2020) and Said *et al.* (2014). The motility of sperm among bulls varies due to age, scrotal circumference, ionic composition of seminal plasma (Rabidas *et al*., 2012; Sundararaman *et al*., 2012; Kumar *et al.*, 2015). The sperm viability of the bulls was good (73.0 ± 0.6% to 85.4 ± 0.7%) and the differences between bulls were significant. The result was similar to the observation of Rabidas *et al*. (2012) who reported 65.7 ± 4.0 to 85.0 ± 1.0% viability but lower (80.6 ± 10.7- 89.5 ± 5.3%) than the findings of Sugulle *et al.* (2006). The sperm abnormalities (5.7 ± 0.3% to 9.2 ± 0.5%) were in acceptable range, below 10%, and the result was higher than the observation of Indriastuti *et al*. (2020) and Said *et al.* (2014) but lower than the findings of Sugulle *et al*. (2006). The variation might be due to age, genotype, condition of the genital tract etc. (Sugulle *et al*., 2006; Rabidas *et al*., 2012; Kumar *et al.*, 2015). The variation might be due to age, genotype, condition of the genital tract etc. (Sugulle *et al*., 2006; Rabidas *et al*., 2012; Kumar *et al.*, 2015).

The sperm count, motility, velocity and kinetic characteristics of frozen thawed semen of five bulls are presented in Table 2. No significant difference was found among the bulls in total sperm count, which was lower than the findings of Bhuiyan *et al.* (2019).

The total motility of sperm (50.1 ± 3.8 % to 59.0 ± 4.7%) did not differ significantly between bulls and was similar to the findings of Patel and Dhami (2016) and Kumar *et al*. (2015). This result differs from that of Hasan *et al*. (2020) who reported lower values, but Goshme *et al*. (2021) found higher total motility compares to present findings. The progressive motility was significantly higher in Bull 1 (39.0 ± 1.6%) compared to other bulls and the results are in agreement with the findings of Singh *et al*. (2013) and Patel and Dhami (2016) but differ from the findings of Islam *et al*. (2020) and Morrell *et al*. (2018). The sperm motility differences between the bulls might be due to age and bodyweight, genetics, temperature, degree of sperm maturation, energy stores, or ionic composition of seminal plasma (Sundararaman *et al*., 2012; Blasco, 1984). The velocity traits of VSL, VCL and VAP of sperm ranged from 48.0 ± 1.3 - 71.3 ± 0.7 µm/s,118.1 ± 2.8-181.3 ± 10.9 µm/s and 68.4 ± 2.5 to 91.0 ± 2.9 µm/s, respectively. Bull 1 showed significantly higher VSL (71.3 ± 0.7 µm/s), VCL (181.3 ± 10.9 µm/s) and VAP (91.0 ± 2.9 µm/s) compared to others. This observation supports the findings of Najjar *et al*. (2013) but differs from the findings of Hoflack *et al*. (2007); Morrell *et al*. (2018) and Islam *et al*. (2017) who found lower values in Holstein-Friesian and Brahman bull semen. This result differs from that of Amanda (2011) who reported higher sperm velocity in Holstein-Friesian bull semen. The high velocity of sperms indicated that the sperm were hyperactive, which implied high energy state of sperm to penetrate through cervical mucus and successfully fertilize the ovum (Atiken *et al*., 1985; Kasai *et al*., 2002; Islam *et al*., 2017).

ALH of sperms was higher in Bull 1 (11.0 ± 0.3 µm) than in other bulls (9.0 ± 0.6 µm - 10.0 ± 0.4 µm), but Hoflack *et al*. (2007); Islam *et al*. (2017); Morrell *et al*. (2018) and Sundararaman *et al*. (2012) found lower ALH of sperms. BCF (27.4 ± 1.9 Hz- 29.0 ± 1.2 Hz), LIN (33.2 ± 2.5% - 41.0 ± 1.8%) and STR (65.4 ± 3.2% - 79.0 ± 2.2%) showed no significant differences between the bulls. The BCF and STR results agree with the findings of Islam *et al*. (2017) in Brahman bulls, but are higher than reported by Hoflack *et al*. (2007) in Holstein- Friesian bulls. The WOB was similar to the findings of Islam *et al*. (2017). The LIN differs from the findings of Islam *et al*. (2017) and Patel and Dhami (2016) in Brahman and Buffalo bulls, who found higher LIN. Viability of frozen-thawed semen was lower in Bull 5 (73.0 ± 1.71%) compared to others (Table 3). The result agrees with the findings of Farooq *et al*. (2015). The percentage of abnormal sperm (7.4 ± 1.1% - 11.6 ± 0.1%) did not differ significantly between the bulls: it is lower than the findings of Singh *et al*. (2013) and Mahmoud *et al*. (2013).

Table 3: Sperm viability and sperm abnormality of frozen thawed semen (Mean ± SE)

Parameters	Bull 1	Bull 2	Bull 3	Bull 4	Bull 5	P-value
SV (%)	79.3 ± 1.3^{a}	79.0 ± 0.1^{a}	78.3 ± 1.3^{a}	78.0 ± 1.4^{a}	73.0 ± 1.2^{b}	0.020
SA (%)	7.4 ± 1.1^{b}	11.2 ± 1.0^{a}	11.2 ± 1.1^{a}	10.0 ± 1.0^{ab}	11.6 ± 0.1^{a}	0.054

SV = Sperm viability, SA = Sperm abnormality

Comparison between fresh and frozen-thawed semen quality showed significant ($P<0.05$) differences (Table 4) in total and progressive motilities, sperm velocity and

kinetics, sperm viability and sperm abnormality. In frozen-thawed semen, the total motility and progressive motility were reduced significantly from 77.0 ± 0.8% to 53.0 ± 1.5% and 65.3 ± 1.5% to 32.4 ± 0.8%, respectively. Similarly, the sperm velocity VSL, VCL, VAP and ALH declined significantly from 84.0 ± 3.2 µm/s - 56.0 ± 2.0 µm/s, 171.0 ± 6.0 µm/s - 147.3 ± 4.8 µm/s, 94.3 ± 2.7µm/s to 76.4 ± 1.9 µm/s and 17.0 ± 0.5 µm- 10.0 ± 0.2 µm, respectively. The other sperm kinetics LIN (49.3 ± 1.3% - 38.2 ± 1.3%), STR (89.0 ± 2.0 - 73.2 ± 2.3%) and WOB (56.0 ± 1.2% - 52.4 ± 1.2%) reduced significantly in frozen-thawed semen compared to pre-freezing semen. The BCF did not show any significant difference after freezing. The results agree with the findings of Kumar *et al*. (2015); Baharun *et al*. (2017); Mandal *et al.* (2013) and Said *et al.* (2014). This decline might be due to damage to sperm during freezing (Sundararaman *et al*., 2012; Kumar *et al*., 2015; Mandal *et al*., 2013; Borchardt *et al*., 2018).

Table 4: Motility, kinetics, viability and abnormality of diluted fresh and frozen-thawed semen evaluated under CASA (Mean±SE)

Parameters	Pre-freezing	Frozen-thawed
Total motility (TM), %	77.0 ± 0.8[a]	53.8 ± 1.5[b]
Progressive motility (PM), %	65.3 ± 1.5[a]	32.4 ± 0.8[b]
Straight linear velocity (VSL), µm/s	84.0 ± 3.2[a]	56.0 ± 2.1[b]
Curvilinear velocity (VCL), µm/s	171.0 ± 6.0[a]	147.3 ± 4.7[b]
Average path velocity (VAP), µm/s	94.3 ± 2.7[a]	76.4 ± 1.2[b]
Average lateral head displacement (ALH), µm	17.0 ± 0.5[a]	10.0 ± 0.2[b]
Beat cross frequency (BCF), Hz	29.3 ± 0.7[a]	28.1 ± 0.6[a]
Mean straightness (STR), %	89.0 ± 2.0[a]	73.2 ± 2.3[b]
Linearity (LIN), %	49.3 ± 1.3[a]	38.2 ± 1.3[b]
Wobble (WOB), %	56.0 ± 1.2[a]	52.4 ± 1.2[b]
Sperm viability (%)	81.0 ± 0.7[a]	77.3 ± 0.8[b]
Sperm abnormality (%)	7.7 ± 0.2[b]	10.3 ± 0.5[a]

Rows with different superscripts showed significant (P≤0.05) mean differences paired sample *t*-test

Pregnancy rates in 500 cows inseminated with frozen semen are presented in Table 5. The overall pregnancy rate was 55.6%, the highest in cows inseminated with bull 1 semen (62.0%) and the lowest in cows inseminated with bull 3 semen (48.0%). The result supports the report of Shamsuddin *et al*. (2001) and Haque *et al*. (2015), who found 54.3% - 57.9% pregnancy rate. The pregnancy rate is higher than the findings of Kamal *et al*. (2013); Mahmoud *et al*. (2013) but lower than those of Howlader *et al*. (2019) and Khatun *et al.* (2014).

Table 5: Pregnancy rate of cows artificially inseminated with five different bulls

Bulls no.	Number of inseminations	Number pregnant (%)	P-value	95% CI
1	100	62 (62.0)		52.3 - 71.9
2	100	58 (58.0)		48.2 - 67.8
3	100	48 (48.0)	0.362	38.0 - 58.0
4	100	54 (54.0)		44.1 - 63.9
5	100	56 (56.0)		46.1 - 65.9
Total	500	278 (55.6)		51.2 - 6.0

Chi square test

The correlation matrix of sperm motility, velocity, kinetics and morphology of frozen-thawed semen with pregnancy rate are presented in Table 6. Pregnancy rate had positive correlation with sperm motility and sperm VCL, VSL, VAP, ALH, LIN, and STR, but had negative correlation with WOBB and SA. The present study revealed that pregnancy rate had positive correlation with total motility and progressive motility, TC, VCL, VSL, VAP, ALH, BCF, LIN, STR and sperm viability but had negative correlation with WOB and sperm abnormality. The results are similar to the findings of Farooq *et al*. (2015) and Mahmoud *et al*. (2013).

Table 6: Correlation matrix of motility, kinetics, viability, and abnormality parameters of frozen-thawed semen with pregnancy rate

	TC	TM	PM	VSL	VCL	VAP	BCF	STR	LIN	WOB	SV	SA	PR
TC	1												
TM	0.965**	1											
PM	0.960**	0.997**	1										
VSL	0.930*	0.859	0.852	1									
VCL	0.844	0.750	0.775	0.911*	1								
VAP	0.951*	0.880*	0.887*	0.978**	0.962**	1							
BCF	0.731	0.656	0.633	0.508	0.393	0.549	1						
STR	0.694	0.612	0.572	0.870	0.641	0.749	0.320	1					
LIN	0.402	0.419	0.344	0.429	0.019	0.262	0.393	0.712	1				
WOB	-0.257	-0.131	-0.189	-0.423	-0.730	-0.517	0.156	-0.179	0.563	1			
SV	0.422	0.259	0.299	0.613	0.833	0.669	0.010	0.430	-0.320	-0.957*	1		
SA	-0.902*	-0.803	-0.821	-0.929*	-0.989**	-0.981**	-0.527	-0.654	-0.092	0.645	-0.770	1	
PR	0.683	0.836	0.855	0.577	0.540	0.619	0.230	0.298	0.162	-0.108	0.098	-0.537	1

**Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed); TC = Total count, TM = Total motility, PM = Progressive motility, VSL = Straight linear velocity, VCL = Curvilinear velocity, VAP = Average path velocity, BCF = Beat cross frequency, STR = Mean straightness, LIN = Linearity, WOB = Wobble, SV = Sperm viability, SA = Sperm abnormality, PR = Pregnancy rate

The factors affecting the pregnancy rate of inseminated cows are depicted in Table 7. The cows inseminated with good signs of oestrus, with clear mucus discharge, had 68.0% pregnancy rate, higher than the cows inseminated with poor signs without mucus. The result agrees with the findings of De Kruif (1978); Shamsuddin *et al*. (2001); Garcia *et al*. (2011) and Khatun *et al*. (2014). The insemination of cows with good intensity of oestrus and clear mucus discharge could increase the pregnancy rate. The cows showing oestrus signs in the morning and inseminated in the evening had 64.1% pregnancy rate, higher than the cows inseminated in the morning. Cows wrongly identified as in oestrus in the evening and inseminated in the next morning had lower pregnacy rate. The result supports the findings of Nebel *et al.* (1994); Saacke *et al*. (2000); Noakes *et al*. (2018c). The semen deposition in the body of uterus produced significantly higher pregnancy rate (62.0%) than others. The result agrees with the findings of Kurykin *et al*. (2016). The pregnancy rates were significantly higher in cows inseminated in dry season (65%) than in wet season. The result supports the report of Alam and Ghosh (1988); Paul *et al*. (2011) and Khatun *et al*. (2014). During dry season, the inseminated cows were less stressed with good weather.

Table 7: Univariable analysis of factors associated with pregnancy in cows through AI

Factors	No. of cow inseminated	No. of cow pregnant	Pregnancy rate (%)	Odds ratio (95% CI)	P-value
Intensity of oestrus					<0.001
Strong with mucus	312	207	68.0	3.3 (2.2 - 4.7)	
Weak without mucus	188	71	35.1	1	
Time of AI					<0.001
Evening	315	202	64.1	2.6 (1.8 - 3.7)	
Morning	185	76	41.1	1	
Semen deposition					<0.001
Body of uterus	376	233	62.0	3.1 (1.8 - 5.2)	
Cervix	52	20	38.4	1.2 (0.6 - 2.5)	
Horn of uterus	72	25	34.7	1	
Season of AI					<0.001
Dry season (November-April)	286	186	65.0	2.5 (1.7 - 3.6)	
Wet season (May-October)	214	92	43.0	1	

Chi square test

Conclusions

The fresh and frozen-thawed semen of breeding bulls supplied in North-east Bangladesh for AI programme were good quality. Heat detection and insemination timing need to be improved to increase the pregnancy rate.

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