Article

Conception rate following intra-cervical artificial insemination using frozen semen at field level in indigenous sheep of Bangladesh

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Abstract: This study was undertaken to study the AI conception rate using frozen semen at field level. Five farms in Mymensingh, Bangladesh were selected for AI Trial in field ewes. Four rams were selected for semen collection, evaluation, and frozen semen production and further to study conception rate followed by intra-cervical AI in both natural and synchronized ewes. Conception rate were confirmed by non-returned rate and ultrasound scanning at 30-40 days of post insemination. The volume, colour, mass activity, sperm motility, viability, concentration, HOST +ve (%) and normal spermatozoa percentages were 0.8±0.3 ml, 3.9±0.3, 4.4±0.6, 81.3±5.0%, 90.0±4.0%, 3519.0±545.6x10^6/ml, 87.4±3.3% and 85.6±1.8%, respectively. The sperm concentration of ram R#6 was significantly higher (P<0.05) (4120.5±93.5x10^6/ml) compared with other rams. The mean motility and viability of pre-dilution, 120 minutes of addition of Part-A, 240 minutes of addition of Part-B and post-thaw were (83.8±4.8%, 81.3±2.5%, 80.0±4.1% and 41.3±9.5%) and (93.3±1.0%, 90.0±1.4%, 88.8±1.0% and 58.3±8.7%), respectively. There were no significant difference (P>0.05) between pre-dilution and post-dilution sperm motility and viability percentage however, post-thaw sperm motility and viability significantly (P<0.05) decreased compare with the motility and viability of pre-dilution and post-dilution values. Motility and viability percentages of frozen semen did not decrease significantly (P> 0.05) with the increase of preservation time. The mean motility and viability at 24 hrs, day 7, day 15 and day 30 were 41.3±9.5%, 41.5±8.5%, 41.8±9.9% and 40.5±10.2%; and 59.0±10.1%, 58.5±7.7%, 59.0±8.8% and 57.8±8.3%, respectively. The conception rates in natural and synchronized estrous were 26.7% and 25%, respectively. There was no significant difference in conception rates between the natural and synchronized oestrous in field level. However, the present non-return rate and conception rate indicate the suitability of produced frozen semen application in the field level.

Keywords: artificial insemination; conception rate; frozen semen; sheep; HOST; Intra-cervical AI

1. Introduction

Sheep are important in Bangladesh, providing meat and wool. During the last twelve years sheep population increased 2.5 times, with annual growth rate of 5% (Sarker *et al*., 2015). There are 3.3 million sheep (DLS, 2016), of which 32% are reared in three ecological zones. Barind, Jamuna basin and Coastal areas. Most of the sheep are indigenous, with few crossbreds (Bhuiyan, 2006) and are capable of bi-annual lambing and multiple births. The fecundity gene (Booroola) in Australian Merino may be derived from this Bengal line (Piper and Bindon, 1996). Native sheep (*Ovis aries*) might have originated from the wild Urial (*Ovis orientalis vignei*) of Asia.With the introduction of artificial insemination (AI), sheep producers in many countries are using the technologies for controlled reproduction; including synchronization of estrus, fixed-time AI, early pregnancy diagnosis and synchronization of lambing (Halbert *et al*., 1990; Eppleston and Maxwell, 1993; Donavan *et al*.,

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2001). AI is practicing with the best quality rams to exploit rapid dissemination of desirable genetic merit. It is the basic assisted reproductive technique (ARTs) to inseminate and make large numbers of ewes pregnant from single ejaculation. However, insemination outcome is affected by many factors (intrinsic and extrinsic) related to female (handling, seasonality, genital morphology, etc.), male (seasonality, sperm quality, sperm conservation, etc.), farm (environmental conditions, sanitary status, handling, etc.) and the technique itself (route of application, spermatozoa/dose, technician, etc.) (Shackell *et al*., 1990; Donovan *et al*., 2004; Paulenz *et al*., 2005). Artificial insemination (AI) with frozen thawed semen has been proved as the most potent method for rapid genetic improvement in domestic animals. Each stage of the semen cryopreservation includes the entire process like semen collection, dilution, and equilibration and freezing. However, this technique permits limitation of the spermatozoa to lose the ability to fertilize normally (Watson, 1995). The conception rates varies (10 to 80%) with the type of semen (fresh, freshly diluted, chilled and frozen) and AI (vaginal, cervical, intra-cervical, transcervical and intra-uterine) used. Conception rates with fresh semen are good (65 to 75%) while with frozen semen (10 to 30%) (Olesen, 1993; Gordon, 1997). In Bangladesh, some works have been performed on reproductive performances, estrus synchronization protocol, protocols for semen preservation, AI with chilled and frozen semen in DSO, BAU research station (Azizunnesa *et al*., 2014, 2016). However, about the use of AI is limited to research level with some initiation at farm level. The use and fruitful results of AI have drawn attention among farmers. Sheep could contribute a significant amount in livelihood promotion and in meat subsector of Bangladesh. Therefore, the present research was performed to study the AI conception rate using frozen semen at field level.

2. Materials and Methods
2.1. Study area
The cryopreservation of semen was conducted at the Reproduction Laboratory of the Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202. Sheep farmer's information was taken from the district livestock officer and then five farms were visited before implementation of AI in farmer's ewes. AI trial with frozen ram semen was conducted at three farms at Trishal in Mymensingh, from January to December 2016.

2.2. Farmers training workshop for AI trial at field level
A day long farmers training workshop in Mymensingh was arranged for informing them the importance of AI to improve sheep breeds. Also existing sheep information from the farmers was taken.

2.3. Animal selection for semen freezing and AI trial
Four apparently healthy indigenous adult rams (2 to 2.5 years old, 20 to 22 kg) were selected from the project entitled "Speed up of genetic improvement of indigenous sheep through Multiple Ovulation and Embryo Transfer (MOET) technique". Ewes (n = 23) were included from selected farms which were selected for AI trial. The age, parity and body weight of ewes were 1.5-3 years, 1-3 and 14-20 kg, respectively. The general health of the selected animals for this experiment was clinically examined. Both ballottement of abdomen and ultrasonography were applied to detect non pregnant ewes. The animals were reared under the field conditions on different farms. They were kept under natural field conditions, having access to good quality grasses and maintained in good health.

2.4. Semen freezing for AI
2.4.1. Extender preparation
Locally-manufactured extender (tris, fructose, egg-yolk: TFE) was prepared according to Azizunnesa *et al.* (2016); (tris-base: tris 3.4 g, fructose 0.5 g, citric acid 2.0 g, penicillin 100,000 IU, strepto-mycin 100 mg, 7% glycerol deionized water 100 mL and egg-yolk 10%).

2.4.2. Semen collection
Semen was collected twice in a week during the period of research (January to December 2016). Collection was always performed in the morning between 7- 9 AM using properly maintained artificial vagina (AV). After collection, semen was kept at 37°C in water-bath until the media were added with it.

2.4.3. Evaluation of semen
The volume of semen was measured with a calibrated collection vial. Colour was observed visually. Sperm concentration was measured by haemocytometer method (Figure 1) (Herman and Madden, 1963). Density was

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measured observing the viscosity of sperm against the slant position of the tube. Density was scored in 4 scales; 1= watery, 2=milky, 3=creamy, 4=creamy to grainy. Sperm concentration was measured by haemocytometer method (Herman and Madden, 1963). After diluting the semen with tris (1:4) motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward at higher magnification (400x) and expressed as percentage. To evaluate the mass activity, a drop (5μl) of semen was placed on a pre-warmed slide (37°C) without any cover slip and examined under microscope equipped with phase-contrast optics (100x). The mass activity was scored into 5 scales: 1= no motion, 2=free spermatozoa moving without forming any waves, 3= small, slow moving waves, 4= vigorous movement with moderately rapid waves and eddies and 5= dense, very rapidly moving waves and distinct eddies. Viability test was performed after mixing of semen with eosin-nigrosin stain; live spermatozoa appeared unstained and dead spermatozoa stained pink against a brownish purple background (Figure 2).The sperm morphology were evaluated by using William's staining (especially for sperm head) and buffered formol saline for acrosome, mid-piece and tail (Figure 3). Plasma membrane integrity test by hypo-osmotic swelling test (HOST) according to Ramu and Jeyendran (2013). The percentage of spermatozoa with swollen and curled tails was recorded as HOST+ve cells (Figure 4). The ram semen was preserved according to the procedure of Azizunnesa *et al.* (2016). Two-step dilution method was used to freeze the semen in this experiment. The frozen semen containing straws were thawed at 37°C for 10-15 seconds. Then post-thawed semen was placed on a clean glass slide and covered with (18x18mm) cover slide. The motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward at higher magnification (400X) and expressed as percentage. Also eosin-nigrosin stain was used for determining live and dead sperm percentage in frozen semen. Motility and viability percentage of frozen semen are measured at day 0, day 7, day 15 and day 30.
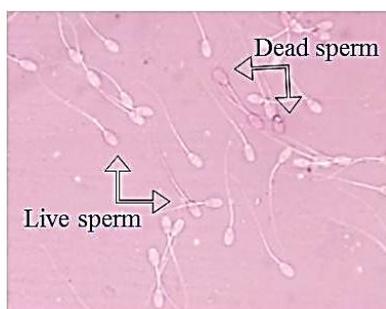

Figure 1. Sperm concentration by Haemocytometer.

Figure 2. Viability test by Eosin-nigrosin stain.

2.5. Estrous synchronization of farmer's ewes

Estrus was induced in the ewes by intramuscular administration of two injections of PGF2α (Ovuprost®Bayer, Newzealand) @ of 150 μg (0.6 ml) per ewe. The first injection was given ignoring the stage of the estrous cycle. After 9 days the same doses were injected to all ewes of regardless of estrus.

2.6. Estrus detection at field level

The onset of estrus was detected by the use of normal breeding special apron wearing ram.
The apron wearing rams were introduced in the flock after injecting PGF2α intramuscularly and for a total period of 96 hours (Figure 5).
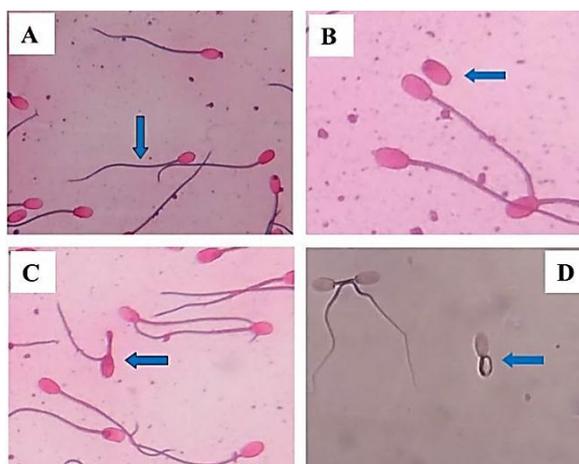
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Figure 3. Sperm morphology evaluated by using William's staining method.
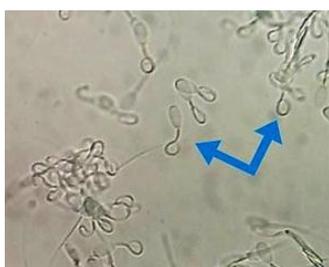**A: Normal spermatozoa, B: Detached head C: Bent tail, D: Coiled tail**

Figure 4. HOST (+ve) spermatozoa.

Figure 5. Ram wearing special apron and heat detection by apron wearing ram.

Figure 6. Pregnancy diagnosis by ultrasonography.

2.7. Process of Intra-cervical AI (ICAI) at field level

All natural and synchronized ewes were inseminated Intra-cervically within 14-20 hrs of 1st observed heat (Windsor *et al.,* 1994). A standard, commercially available (eccentric insemination pipette for Sheep/Goat; Minitube, Slovakia) insemination pipette with a sharp pointed bent tip, designed for use in ewes was used for trans-cervical insemination. After thawing 0.25ml mini straws at 37°C warm water for 10-15 seconds were dried with the help of tissue paper. Then the straw containing 200 x 106 spermatozoa was loaded into the AI pipette. The estrous ewes were kept in standing condition just only hind quarter up and restrained by the animal owner. Then the vulva of the ewe was cleaned with a dry paper towel and the vaginal speculum was lubricated with

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non-spermicidal water soluble lubricant (KLY ®gel). The vaginal speculum was introduced into the vagina and forwarded to locate the cervix guided specialized light source for vaginal speculum. The inseminating pipette was then inserted into the cervix and pushed forward as much as it easily passes through the rings of the cervix. Then the semen was expelled deep into the cervix by pressing the plunger. After deposition of semen the pipette was kept in position for a period of 5 minutes before withdrawn from the cervix. Similar procedure was applied for insemination in all ewes.

2.8. Pregnancy diagnosis
All inseminated ewes were monitored for non-return to estrus by the aid of an apron wearing ram at the 17 days following insemination. The ewes which were in non-return to estrous were allowed to ultrasonographic scan for confirmation of pregnancy using trans-abdominal ultrasonography within 30-40 days of post insemination (Figure 6).

2.9. Assumption of Non-return rate and Conception rate
Non-return (NR) rate (%) = (Number of ewes non-return to oestrus x100)/Number of ewes inseminated
Conception rate (%) = (Number of ewes conceived x 100)/Number of ewes inseminated

2.10. Statistical Analysis
All values relating to semen evaluation parameters were expressed as Mean ± SD. The statistical analyses were done using SPSS IMB 20.0 version software program. One way analysis of variance (ANOVA) was done to find out significant differences in reproductive parameters. One-way ANOVA: Post Hoc multiple comparisons (Duncan test) were done to find out significant differences at 5% level of significance (P< 0.05) in semen parameters. Conception rate was expressed as percentage.

3. Results
3.1. Evaluation of fresh ram semen used for freezing
The mean values of semen parameters regarding volume, color, mass activity, sperm motility, viability, concentration, HOST+ percentage and morphology percentage are shown in Table 1. The volume, colour, mass activity, sperm motility, viability, concentration, HOST+ve(%) and normal spermatozoa percentage varied from 0.4-1.3 ml, 2-4 (creamy to creamy-grainy), 2-5, 75-92%, 80-95%, $2500\text{-}5000\times10^6$/ml, 75-90% and 75-95%, respectively. However, their mean values were 0.8 ± 0.3 ml, 3.9 ± 0.3, 4.4 ± 0.6, $81.3\pm5.0\%$, $90.0\pm4.0\%$, 3519.0 ± 545.6 $x10^6$/ml, $87.4\pm3.3\%$ and $85.6\pm1.8\%$, respectively. The sperm concentration of ram R#6 was significantly higher (4120.5 ± 93.5 x 10^6/ml) compared with ram R#1, R#4 and R#5 (3496.7 ± 125.0 x 10^6/ml, 2795.4 ± 114.4 x 10^6/ml and 3663.2 ± 491.8 x10^6/ml). The sperm motility of ram R#6 and R#5 were significantly higher ($83.8\pm4.8\%$ and $85.0\pm4.1\%$) compared with Ram R#1 and R#4 ($80.0\pm4.1\%$ and $76.3\pm2.5\%$). Also the sperm viability of ram R#6 was significantly higher ($93.3\pm1.0\%$) compared with ram R#1, R#4 and R#5 ($89.3\pm2.8\%$, $85.0\pm3.6\%$ and $92.5\pm1.7\%$). Similarly, the mass activity of ram R#6 was significantly higher (5.0 ± 0.0) compared with ram ID R#1, R#4 and R#5 (4.3 ± 0.5, 3.8 ± 0.5 and 4.8 ± 0.5, respectively). The plasma membrane integrity percent (HOST +ve %) of ram R#5 was significantly higher ($90.8\pm2.2\%$) compared with ram R#1, R#4 and R#6 ($87.0\pm2.9\%$, $84.8\pm1.5\%$ and $87.3\pm3.6\%$, respectively). There was no significant difference (P>0.05) in semen volume and morphology % (normal sperm %) among the rams (0.7 ± 0.2, 0.6 ± 0.2, 1.0 ± 0.3 and 0.9 ± 0.2 ml, respectively) and ($84.8\pm1.0\%$, $85.3\pm1.5\%$, $87.3\pm1.7\%$ and $85.3\pm2.2\%$, respectively).

Table 1. Evaluation of fresh ram semen.

Ram ID:	Volume (ml)	Color (1-4)	Mass activity (1-5)	Sperm motility (%)	Viability (%)	Concentration (x10⁶/ml)	HOST (+) ve (%)	Normal sperms (%)
R#1 (n=4)	0.7 ± 0.2^a	3.5 ± 0.6^b	4.3 ± 0.5^{bc}	80.0 ± 4.1^{ab}	89.3 ± 2.8^b	3496.7 ± 125.0^b	87.0 ± 2.9^{ab}	84.8 ± 1.0^a
R#4 (n=4)	0.6 ± 0.2^a	4.0 ± 0.0^a	3.8 ± 0.5^c	76.3 ± 2.5^b	85.0 ± 3.6^c	2795.4 ± 114.4^c	84.8 ± 1.5^b	85.3 ± 1.5^a
R#5 (n=4)	1.0 ± 0.3^a	4.0 ± 0.0^a	4.8 ± 0.5^{ab}	85.0 ± 4.1^a	92.5 ± 1.7^{ab}	3663.2 ± 491.8^b	90.8 ± 2.2^a	87.3 ± 1.7^a
R#6 (n=4)	0.9 ± 0.2^a	4.0 ± 0.0^a	5.0 ± 0.0^a	83.8 ± 4.8^a	93.3 ± 1.0^a	4120.5 ± 93.5^a	87.3 ± 3.6^{ab}	85.3 ± 2.2^a
Mean	0.8 ± 0.3	3.9 ± 0.3	4.4 ± 0.6	81.3 ± 5.0	90.0 ± 4.0	3519.0 ± 545.6	87.4 ± 3.3	85.6 ± 1.8

[a-c]The mean values with different superscript within the same column differ significantly, at least (P<0.05)

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3.2. Characteristics of ram semen before and after freezing used for AI

The mean values of ram semen before and after freezing have shown in Table 2.

The mean motility and viability of pre-dilution, 120 minutes of addition of Part A, 240 minutes of addition of Part-B and post thaw were (83.8±4.8%, 81.3±2.5%, 80.0±4.1% and 41.3±9.5%) and (93.3±1.0%, 90.0±1.4%, 88.8±1.0% and 58.3±8.7%), respectively. There were no significant difference (P>0.05) in pre-dilution and post-dilution sperm motility and viability percentage. But post-thaw sperm motility (41.3±9.5%) and viability (58.3±8.7%) varied significantly (P<0.05) compared with the motility and viability of pre-dilution and post-dilution.

Table 2. Characteristics of ram semen before and after freezing used for AI (Mean ±SD); (n=4).

Parameters	Pre-dilution value of ram semen (n=4)	Post-dilution value of ram semen (n=4)		Post-thawed value of ram semen (n=4)
	Fresh semen	120 min	240 min	
Sperm motility (%)	83.8±4.8 [a]	81.3±2.5[a]	80.0±4.1[a]	41.3±9.5[b]
Sperm viability (%)	93.3±1.0 [a]	90.0±1.4[a]	88.8±1.0[a]	58.3±8.7[b]

[a-b] The mean values with different superscript within the same row differ significantly, at least (P<0.05).

3.3. Success of semen freezing evaluated in different preservation time

The effects of preservation time on motility and viability of frozen semen have shown in Table 3. Difference in Motility and viability of frozen semen was statistically non-significant (p> 0.05) with the increase of preservation time. The mean motility and viability percentages at 24hrs, day 7, day 15 and day 30 were 41.3±9.5%, 41.5±8.5%, 41.8±9.9% and 40.5±10.2%, respectively and 59.0±10.1%, 58.5±7.7%, 59.0±8.8% and 57.8±8.3%, respectively.

Table 3. Day wise Post-thaw evaluation of frozen ram semen.

Parameters	24 hrs	Day 7	Day 15	Day 30
Sperm motility (%)	41.3±9.5	41.5±8.5	41.8±9.9	40.5±10.2
Sperm viability (%)	59.0±10.1	58.5±7.7	59.0±8.8	57.8±8.3

3.4. Success of semen freezing observed by non-return rate and conception rate in farmers ewes

Non-return rate (NRR) and conception rate (CR) using thawed frozen ram semen at field level have shown in Table 4. The overall NR rate was 30.4%. The non-return rate following intra-cervical AI using frozen semen in natural and synchronized estrous were 33.3% and 25.0%, whereas the conception rates were 26.7% and 25%, respectively. The pooled over conception rate was 26.1%. There was no significant difference between the natural and synchronized estrus on the conception rate using frozen semen.

Table 4. Non-return rate and conception rate at field level using thawed frozen ram semen.

No. of Ewes inseminated	Nature of estrus	Methods of AI	No. of ewes non return to estrus	No. of ewes conceive	Non return rate (%)	Conception rate (%)
15	Natural	ICAI	5	4	33.3	26.7
8	Synchronized	ICAI	2	2	25	25.0
Total= 23			7	6	30.4	26.1

4. Discussion

Four rams (4 ejaculations from each) were used to collect semen for freezing to do AI in field ewes. The volume, colour, mass activity, sperm motility, viability, concentration, HOST +ve (%) and normal spermatozoa (%) varied from 0.4-1.3 ml, 2-4 (creamy to creamy-grainy), 2-5, 75-92%, 80-95%, 2500-5000×10^6/ml, 7590% and 75-95%, respectively. Similar semen quality parameters were observed in previous study of the same laboratory (Mahmuda *et al.,* 2015 and Azizunnesa *et al.,* 2014). In the present study the sperm concentration of ram R#6 was significantly higher (4120.5±93.5x 10^6/ml) compared with ram R#1, R#4 and R#5. The mean motility of pre-dilution, 120 minutes of addition of part A, 240 minutes of addition of part B during freezing and following 24 hrs.of freezing were 83.8±4.8%, 81.3±2.5%, 80.0±4.1% and 41.3±9.5%, respectively and viability

of pre-dilution, 120 minutes of addition of part A, 240 minutes of addition of part B during freezing and following 24 hrs. of freezing were 93.3±1.0%, 90.0±1.4%, 88.8±1.0% and 58.3±8.7%, respectively. There were no significant difference (P>0.05) between pre-dilution and post-dilution sperm motility and viability percentage. The post-thaw sperm motility (41.3±9.5%) and viability (58.3±8.7%) were significantly (P<0.05) decreased compared with the motility and viability of fresh and post-diluted semen. This result is similar with the observation of Mahmuda *et al.* (2015). However, the rate of motility and viability of post thawed semen in the present study were lower than observed by Azizunnesa *et al.* (2014). It is not clear to explain why there is difference from Azizunnesa *et al.* (2014), using the same protocol for freezing. This could be due to climatic condition, weather and season of the study. The mean motility of present study supported result observed with the progress of preservation time after freezing (Mahmuda *et al.,* 2015). The success is the post thaw motility was not significantly lowered with the progress of preservation time, indicating the suitable protocol of freezing process in the present study. During freezing and thawing, the quality of spermatozoa may be affected by the composition of the cryo-extender (Leibo and Songsasen, 2002). Different extenders have different effects on freezing and thawing of semen.

Both natural oestrus and synchronized oestrous ewes were inseminated with the produced frozen thawed semen in the laboratory. The non-return to oestrus percentages in natural oestrous was 33.3%, while that of synchronized oestrous was 25%. Paulenz *et al.* (2007) reported that the average 25-days non-return rate was 61.8%, ranging between 40.0 to 82.4% in the different farms. These differences could be due to differences in breed, semen, and laboratory, skillness of AI technician and time of AI. The conception rate following insemination with frozen semen was 26.7% in natural oestrus ewes and 25% in synchronized ewes. There was no significant difference in conception rates between the natural and synchronized oestrus ewes on the conception rate using frozen semen. The number of synchronized ewes used was lower than natural ewes. The conception rate (26.7% and 25%) in the present study was similar, higher or lower than other researchers (Olesen, 1993; Azizunnesa *et al.,* 2014 and Kumar and Naqvi, 2014).

5. Conclusion

This is the preliminary application of frozen semen in the farmer's ewes. However, the present non-return rate and conception rate indicate the suitability of produced frozen semen application in the field level. Although the pregnancy rates were not in higher percentages, however, modification and refinement of present freezing technique and insemination technique may lead to higher post thaw motility and higher pregnancy rates. Work is going on in the same laboratory for obtaining the frozen semen with higher post thaw motility, leading to higher pregnancy rates that could be acceptable to the farmers, increasing and improving the sheep production in Bangladesh.

Conflict of interest

None to declare.

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