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# Quality of ram spermatozoa separated with modified swim up method

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## ABSTRACT

Semen was collected from four rams, using artificial vagina and viability%, motility% and plasma membrane integrity% were measured. Fresh ejaculates (n = 32) were separated by modified swim-up separation using modified human tubal fluid medium. Four fractions of supernatant were collected at 15-minute intervals. The mean volume, mass activity, concentration, motility%, viability%, normal morphology and membrane integrity% (HOST +ve) of fresh semen were 1.0 ± 0.14, 4.1 ± 0.1 × $10^9$ spermatozoa / ml, 85.0 ± 1.3, 89.4 ± 1.0, 85.5 ± 0.7, 84.7 ± 0.5 respectively. There was no significant (P>0.05) difference in fresh semen quality parameters between rams. The motility%, viability% and HOST +ve % of first, second, third and fourth fractions were 53.4 ± 0.5, 68.2 ± 0.3, 74.8 ± 0.3 and 65.5 ± 0.4; 55.5 ± 0.4, 66.2 ± 0.4, 74.5 ± 0.3 and 73.6 ± 0.3 and 66.7 ± 0.5, 66.8 ± 0.5, 65.2 ± 0.4 and 74.7 ± 0.5 respectively. The motility%, viability% and membrane integrity% of separated semen samples differed significantly (P<0.05) between four fractions. The mean motility% and viability% were significantly higher (P<0.05) in third fraction (74.8 ± 0.3%), whereas the mean HOST +ve% was significantly higher (P<0.05) in fourth fraction (74.7 ± 0.5). All quality parameters of separated spermatozoa were significantly (P<0.05) lower than that of fresh semen. The pregnancy rates were higher with fresh semen (71%) in comparison to that of separated sample (57%). (*Bangl. vet*. 2016. Vol. 33, No. 2, 62 – 70)

## Introduction

Sperm separation for mammals is based on centrifugation. Although there are marked species differences between spermatozoa in their sensitivity to centrifugation, detrimental effects of centrifugation to ram (Gavella, 1983) and human (Aitken and Clarkson 1988) spermatozoa have been reported. Furthermore, centrifugation does not select for sperm quality (Lopata *et al.,* 1976).

Swim-up is one of the most commonly used techniques for sperm preparation and is preferred if the semen sample has a normal number of good sperms. By this technique, the spermatozoa are selected on their motility. The ability of spermatozoa to swim up from the ejaculate into the overlying medium has been used extensively as a method for separating highly motile spermatozoa (Berger *et al.,* 1985). The same principle was also used to evaluate sperm motility (Levin *et al.,* 1981). However, the standard swim-up procedure (Suttiyotin *et al.,* 1993) involves centrifugation, which decreases motility of sperm cells (Tanphaichitr *et al.,* 1987). A modified swim-up

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technique, without centrifugation, was developed by Alvarez *et al*. (1993) for the separation of motile human spermatozoa using a modified human tubal fluid medium (mHTF; Quin *et al.,* 1985). This technique does not affect the motility of spermatozoa. However, the relationship between sperm motility and fertility remains controversial (Kjaestad *et al.,* 1993). The study aimed to evaluate quality of separated spermatozoa of indigenous rams and to compare quality of normal and separated spermatozoa.

## Materials and Methods

The research was conducted at the Research Animal Farm, Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, from July to December 2016.

### *Selection and management of rams*

Four healthy indigenous rams, from 1.5 to 3 years old, were selected as semen donors. Body weight and scrotal circumference of these ram varied from 15 to 20 kg and 18 to 25 cm, respectively. All rams were apparently healthy. They were housed in a covered shelter with an open-air run and allowed 6 to 8 hours natural grazing. Routine vaccination and deworming programs were conducted. Each ram was fed approximately 0.5 to 1.0 kg concentrate daily.

### *Experimental design*

### *Semen collection and evaluation*

Semen was collected by artificial vagina (AV) using a homosexual teaser twice a week. Before semen collection warm water (40°C to 45°C) and air were injected into the AV. The rubber part of the AV was lubricated by sperm-friendly lubricant. Semen in vials was kept in a water bath (37$^{o}$C) as early as possible. Eight ejaculates were collected from each ram.

Immediate after collection, volume of semen was recorded. Colour of the semen was scored by naked eye 1 – 5. Only samples scored 3 or more were accepted for processing. Mass motility was scored from 1 – 5 using phase-contrast microscopes. A small drop of fresh semen was placed on pre-warmed (37°C) grease-free slide and observed under microscope at 10× without cover slip, and mass activity (0 – 5) score was recorded. The concentration of spermatozoa (billion / mL) was determined by using haemocytometer. Semen samples were diluted with distilled water (1 : 400) to kill the spermatozoa. A drop of diluted semen was placed on the counting chamber from a pipette, and spermatozoa were allowed to settle for 5 – 6 minutes before placing the haemocytometer on the stage of the microscope. The concentration of spermatozoa per ml of semen was calculated by multiplying the total number of spermatozoa in 5 large squares by $2 \times 10^7$.

Percentage of progressively motile spermatozoa was measured by phase-contrast microscope: 80% or more was considered acceptable. For sperm motility, a small drop (5 µL) of diluted semen was placed on a clean pre-warmed glass slide and covered with a cover slip. Motility was determined by eye under 40× magnification and expressed as percentage. The morphology of spermatozoa is used as an important criterion in the evaluation of semen. Spermatozoa having no abnormalities with respect to acrosome, midpiece and tail were considered as normal spermatozoa. Air dried smear of semen was stained with Farely stain® (Mintube, Germany) as per as manufacturer guideline. After drying, the stained smears were examined under microscope (×100) using oil immersion.

***Cell viability assessment***

5 µL Propidium iodide (PI) of semen sample was diluted with 1950 µl of saline extender (2.9% Na-citrate) and 20 µL of 6-CFDA, 20 µL of PI and 20 µL of formaldehyde were added. Stained sample was then incubated at 37°C for 15 min in dark and evaluated using fluorescence microscope. Viable sperm% (CF + PI-, green) and dead% (CF-PI+, red) was recorded.

***Sperm plasma membrane functional integrity***

The functional integrity of spermatozoa was evaluated by using hypo-osmotic swelling test (HOST). Osmolar solution (100 m Osm/L) with sodium citrate and fructose was use. A 1.0mL of hypo-osmotic solution was mixed with 0.1ml of undiluted semen and incubated at 37°C for 1 hour. After incubation, 200 spermatozoa were examined under phase contrast microscope (400×) and percentage of spermatozoa positive to HOST (with bent tail) was recorded.

***Swim-up separation***

Semen (0.25 mL) was mixed with mHTF (1.0 mL). The supernatant was collected and evaluated 4 times. Test tube was placed in a dry bath for 60 min for incubation. Motile spermatozoa were collected after swimming up after 15, 30, 45 and 60 minutes. Sperm from all supernatant samples were evaluated**.** Sperm of second fraction of supernatant was used for artificial insemination.

***Oestrous synchronization***

Oestrus was induced in the ewes by intramuscular administration of two injections of PGF2∞ @ 100 µg (0.5 mL) per ewe. The first injection was given ignoring the stage of the oestrous cycle. After 9 days the doses were repeated to all ewes regardless of oestrus. Teaser ram was used to detect the onset of oestrus.

***Process of cervical AI***

Basic equipment consisted of a speculum with a built-in light source, and a pipette connected to a 1 mL syringe. The vulva of the ewe was wiped with cotton wool. The speculum with lubricant was carefully inserted into the vagina to a depth of 10 – 13

cm. The cervix opening was located. The plunger of the syringe was withdrawn to 0.2 mL to have some air behind the semen. The appropriate amount of semen was drawn into the pipette from the semen collection tube in a water bath at 30°C – 34°C. The inseminator attempted to introduce the pipette into the cervix without using force. Semen was deposited into the cervix. The speculum was withdrawn before the pipette to prevent backflow of the semen.

### *Pregnancy diagnosis*

Ewes were monitored for non-return to oestrus by the aid of a vasectomized teaser ram at 15 – 17 days following insemination. Pregnancy was confirmed using MUIV ultrasonography (Korea) with transducer frequency 5 MHz 45 – 55 days after insemination.

### *Statistical analysis*

All data were entered in Excel program. Values relating to semen evaluation were expressed as mean ± Standard Error (SE). Two-way analysis of variance (ANOVA) was done to find out significant differences in semen parameters between rams. Pearson' correlation test was performed for the quality parameters of separated spermatozoa. All the statistical analyses were done using SPSS 17.0. Differences were regarded as significant when P was less than 0.05 ($P<0.05$).

## Results and Discussion

### *Evaluation of fresh semen*

The highest volume of semen was collected in ram k-82 (1.2 ± 0.2 mL). There was no significant ($P>0.05$) difference in semen quality parameters between rams (Table 1).

### *Effects of swim-up method on quality of separated sperm*

Four supernatants were collected at 15-minute intervals from each fresh semen sample and fractions were named first, second, third and fourth fractions. Motility%, viability% and HOST +ve% were monitored for each fraction: results are shown in Table 2. Semen quality parameters of fresh semen were used as control group.

The mean motility% of fresh semen samples was 85.0 ± 0.1. The motility% of first, second, third and fourth fractions were 53.4 ± 0.5, 68.2 ± 0.3, 74.8 ± 0.3 and 65.5 ± 0.4, respectively. The motility% was significantly higher ($P<0.05$) in third fraction. The mean viability% of the fresh semen sample was 89.4 ± 1.0. The mean viability% of first, second, third and fourth fractions were 55.5 ± 0.4, 66.2 ± 0.4, 74.5 ± 0.3 and 73.6 ± 0.3 respectively. The viability% was significantly higher ($P<0.05$) in third fraction (74.5 ± 0.3).

The mean HOST +ve% of the fresh semen was 84.7 ± 0.5. The mean HOST +ve% of first, second, third and fourth fractions was 66.7 ± 0.5, 66.8 ± 0.5, 65.2 ± 0.4 and 74.7 ±

0.5, respectively. The HOST +ve% was significantly higher (P<0.05) in fourth fraction (74.7 ± 0.5).

Viability%, motility% and plasma membrane integrity% of separated spermatozoa were significantly (P<0.05) lower than in fresh semen.

Table 1: Characteristics of fresh ram semen (Mean ± SD)

| Ram ID | Colour | Volume (m L) | Mass motility (1-5) | Concen-tration ($\times 10^9$) / mL | Motility (%) | Viability (%) | Morphology (%) | HOST+ (%) |
|---|---|---|---|---|---|---|---|---|
| K -73 | Creamy white | 0.9 ± 0.2 | 4.1 ± 0.6 | 3.9 ± 0.3 | 84.4 ± 5.6 | 89.6 ± 4.3 | 84.9 ± 3.3 | 85.1 ± 0.9 |
| K- 74 | Milky white | 1.0 ± 0.2 | 4.0 ± 0.5 | 4.1 ± 0.5 | 86.3 ± 3.5 | 90.1 ± 3.2 | 85.0 ±3.2 | 84.1 ± 1.1 |
| K- 78 | Creamy white | 0.9 ± 0.2 | 3.9 ± 0.6 | 4.0 ± 0.2 | 85.9 ± 4.5 | 90.0 ± 4.3 | 85.8 ± 1.7 | 84.6 ± 1.2 |
| K-82 | Creamy white | 1.2 ± 0.2 | 3.8 ± 0.5 | 4.3 ± 0.4 | 83.4 ± 3.3 | 87.9 ± 2.5 | 86.4 ± 2.1 | 85.1 ± 1.3 |
| Mean | | 1.0 ± 0.1 | 4.0 ± 0.1 | 4.1 ± 0.2 | 85.0 ± 1.3 | 89.4 ± 1.0 | 85.5 ± 0.7 | 84.7 ± 0.5 |

Table 2: Values of motility%, viability% and HOST +ve% in the fresh and supernatants

| Parameters | Fresh | First fraction | Second fraction | Third fraction | Fourth fraction |
|---|---|---|---|---|---|
| Motility% | $85.0 \pm 1.3^a$ | $53.41 \pm 0.5^b$ | $68.21 \pm 0.3^c$ | $74.80 \pm 0.3^d$ | $65.47 \pm 0.4^c$ |
| Viability% | $89.4 \pm 1.0^a$ | $55.53 \pm 0.4^b$ | $66.18 \pm 0.4^c$ | $74.52 \pm 0.3^d$ | $73.63 \pm 0.3^d$ |
| HOST+% | $84.7 \pm 0.5^a$ | $66.73 \pm 0.5^b$ | $66.80 \pm 0.5^b$ | $65.21 \pm 0.4^b$ | $74.72 \pm 0.5^c$ |

Different superscripts indicate significant differences in the same row (P<0.05)

***Effects of swim up method on sperm quality in relation to individual ram***

**S**eparated spermatozoa were collected from four rams by swim-up procedure, and samples were evaluated according to fraction for each ram. The results are presented in Tables 3, 4 and 5.

***Motility%***

Table 3 showed a significant (P<0.05) difference in motility%, in different fractions in rams. The motility% of first, second, third and fourth fractions was highest (P<0.05) in rams K-82 (55.5 ± 0.5), K-74 (69.8 ± 0.6), K-78 (76.5 ± 0.6) and K-74 (65.5 ± 0.4), respectively. There was no significant (P>0.05) difference in semen quality of fourth fraction between the rams.

Table 3: Motility% of sperm in the supernatants of rams

| Fractions | Ram ID : K-73 | Ram ID : K-74 | Ram ID : K-78 | Ram ID : K-82 |
|---|---|---|---|---|
| First | $54.8 \pm 0.6^{ac\alpha}$ | $52.7 \pm 0.9^{ab\alpha}$ | $50.6 \pm 0.8^{c\alpha}$ | $55.5 \pm 0.5^{a\alpha}$ |
| Second | $68.3 \pm 0.6^{ab\beta}$ | $69.8 \pm 0.6^{a\beta}$ | $67.9 \pm 0.5^{b\beta}$ | $66.9 \pm 0.5^{b\beta}$ |
| Third | $74.5 \pm 0.5^{ab\gamma}$ | $73.3 \pm 0.4^{b\gamma}$ | $76.5 \pm 0.6^{c\gamma}$ | $74.9 \pm 0.5^{ac\gamma}$ |
| Fourth | $64.3 \pm 0.6^{\delta}$ | $65.5 \pm 0.4^{\delta}$ | $64.8 \pm 0.7^{\delta}$ | $64.4 \pm 0.6^{\beta}$ |

α, β, γ, δ indicate significant difference between fractions at P<0.05; a, b, c, d indicate significant difference between rams at P<0.05

***Viability%***

The viability% of first, second, third and fourth fractions was highest (P<0.05) in rams K-78 (57.8 ± 0.5), K-73 (67.4 ± 0.7), K-82 (75.8 ± 0.7) and in K-78 (74.2 ± 0.7), respectively. There was no significant difference in third and fourth fraction between the rams.

Table 4: Viability% of sperm in the supernatants of rams

| Fractions | Ram ID : K-73 | Ram ID : K-74 | Ram ID : K-78 | Ram ID : K-82 |
|---|---|---|---|---|
| First | $53.8 \pm 0.7^{a\alpha}$ | $54.9 \pm 0.5^{ac\alpha}$ | $57.8 \pm 0.5^{b\alpha}$ | $55.6 \pm 0.6^{a\alpha}$ |
| Second | $67.4 \pm 0.7^{a\beta}$ | $64.9 \pm 0.6^{b\beta}$ | $66.8 \pm 0.6^{ab\beta}$ | $67.4 \pm 0.7^{ab\beta}$ |
| Third | $74.1 \pm 0.4^{\gamma}$ | $74.3 \pm 0.3^{\gamma}$ | $73.9 \pm 0.4^{\gamma}$ | $75.8 \pm 0.7^{\gamma}$ |
| Fourth | $72.8 \pm 0.4^{\gamma}$ | $73.7 \pm 0.5^{\gamma}$ | $74.2 \pm 0.7^{\gamma}$ | $73.8 \pm 0.5^{\gamma}$ |

α, β, γ, δ indicate significant difference between fractions at P<0.05; a, b, c, d indicate significant difference between rams at P<0.05

***HOST +ve%***

There were significant differences in plasma membrane functional integrity% between fractions of all four rams. The HOST +ve% of first, second, third and fourth fractions was significantly higher (P<0.05) in rams K-78 (69.3 ± 0.8), ram K-74 (69.2 ± 0.7), K-73 (66.3 ± 0.9) and k-82 (78.2 ± 0.8), respectively. There was no significant difference in third fraction between the rams.

Table 5: Values of HOST +ve% of sperm in the supernatants

| Fractions | Ram ID : K-73 | Ram ID : K-74 | Ram ID : K-78 | Ram ID : K-82 |
|---|---|---|---|---|
| First | $65.3 \pm 0.9^{a\alpha}$ | $67.9 \pm 0.5^{ab\alpha}$ | $69.3 \pm 0.8^{b\alpha}$ | $64.5 \pm 0.7^{ac\alpha}$ |
| Second | $65.3 \pm 0.8^{a\alpha}$ | $69.2 \pm 0.7^{b\alpha}$ | $64.9 \pm 0.9^{a\beta}$ | $67.8 \pm 0.7^{bc\beta}$ |
| Third | $66.3 \pm 0.9^{\alpha}$ | $65.3 \pm 0.5^{\alpha}$ | $64.4 \pm 0.6^{\beta}$ | $64.4 \pm 0.6^{\alpha}$ |
| Fourth | $71.3 \pm 1.1^{a\beta}$ | $73.7 \pm 0.5^{ab\beta}$ | $75.7 \pm 0.7^{b\gamma}$ | $78.2 \pm 0.8^{c\gamma}$ |

α, β, γ, δ indicate significant difference within fraction at P<0.05; a, b, c, d indicate significant difference within rams at P<0.05

***Correlation among quality parameters of separated sperm***

Pearson's correlation analysis was performed to understand the relation among the three quality parameters of separated sperm (Table 6). Results showed a significant ($P<0.01$) positive correlation among viability%, motility% and membrane functional integrity%.

Table 6: Correlation among quality parameters

| | | HOST +ve% | Motility% |
|---|---|---|---|
| Viability% | r value | 0.3** | 0.8** |
| | p value | 0.0 | 0.0 |
| HOST +ve% | r value | - | -0.1 |
| | p value | - | 0.2 |

***Pregnancy rates***

The results of pregnancy rate in indigenous ewes are presented in Table 7. Ewes were inseminated by cervical artificial method (CAI).

Table 7: Pregnancy rates (%)

| No. of ewes inseminated | No. of ewes pregnant | Pregnancy rate (%) |
|---|---|---|
| Insemination with fresh semen (n = 7) | 5 | 71 |
| Insemination with separated semen (n = 7) | 4 | 57 |

Semen was collected once a week and showed no significant difference in volume, concentration, viability%, motility% and plasma membrane integrity%. Mean volume per ejaculate was 1.0 ± 0.142 mL. This result is consistent with the finding of Guerrero *et al*. (2009). Azizunnesa *et al*. (2014) has reported that the average volume of ram semen varied from 0.75 ± 0.1 to 1.4 ± 0.01 in Bangladesh. The good mass activity of fresh semen is a key factor to have good motility of preserved semen. The sperm concentration and motility contribute to the mass activity. In this study, the mean mass activity was 4.0 ± 0.13 (Scale 1 – 5) similar to the results of other published work within the same breed (Pervage *et al.,* 2009*).* In fresh semen, the sperm motility after addition of diluents varied from 75 – 92%. The mean sperm motility was 85.0 ± 1.34*%.* Similar results were observed by Pervage *et al*. (2009). Guerrero *et al*. (2009) found individual motility 87.0 ± 2.4%. In the present study, the mean concentration was 4.1 ± 0.17 x $10^9$ / mL, similar to the findings of Gundogun (2009). They found average sperm concentration 4.2 ± 0.2 x$10^9$ spermatozoa / mL with a range from 2.5 x $10^9$ / mL –5.0 x $10^9$ / mL. In the present study, the percentage of morphologically normal spermatozoa was 85.5 ± 0.71%, which is acceptable for insemination. In other studies morphologically normal spermatozoa percentage was 94.0% (Hernandez *et al*., 2012). Guerrero *et al*. (2009) found 1.8 ± 0.7% abnormal sperm where in our study 15.8 ± 0.7% sperms were abnormal.

The mean viability of fresh ram semen was 89.4 ± 1.02. Viability% of indigenous ram semen is 91.7 ± 3.6% (Hassan *et al*., 2009). Functional integrity of spermatozoa is an important parameter for semen assessment. The hypo-osmotic swelling test was developed to evaluate the functional integrity of sperm membrane. In our study, we found 84.7 ± 0.84% of HOST positive spermatozoa. However, Juyena (2011) reported 76% HOST positive spermatozoa in fresh semen in Padovana rams, lower than the present result.

In this study, the mean motility% of separated sperm in first, second, third and fourth fractions was 53.41 ± 0.48, 68.21 ± 0.32, 74.80 ± 0.32 and 65.47 ± 0.36, respectively. These results correspond well with the result of other published work. In the present study the highest motility% of sperm was in the third fraction. The mean percentage of spermatozoa reacting positively for hypo-osmotic environment with swelling (HOST+) was significantly lower ($P<0.05$) in swim-up (74.72 ± 0.52) than in control samples (84.7 ± 0.48). Pearson's correlation test showed a negative correlation between motility% and plasma membrane functional integrity%. This finding is in agreement with Valcárcel *et al.* (1994) who stated that spermatozoa having good motility showed membrane damage. Fresh semen contains spermatozoa, while motile, and exhibit membrane damage, thus being incapable of fertilizing an ovum (Valcilrcel *et al.,* 1994).

The study concludes that there was no significant ($P>0.05$) difference in fresh semen quality parameters between the four rams tested. The motility%, viability% and plasma membrane integrity varied significantly ($P<0.05$) between fractions and between rams. Pearson's correlation showed a strong positive ($P<0.001$) relation between viability% and motility%.

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