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Effects of duration of preservation and glycerol percentages on quality of frozen ram semen

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ABSTRACT

The aims of the present study were to determine the effectiveness of the undertaken freezing technique of indigenous ram semen (with different percentages of glycerol and different thawing times) by observing the post thaw motility at 24, 48 and 72 hours of preservation. Nine ejaculates were collected from each 6 mature rams using artificial vagina method. The ejaculates were frozen using Tris-citric acid egg yolk media having different percentages of glycerol (3, 5, and 7) in two step dilution method. The post thaw motility was observed in different temperature (37^0, 40^0 and 45^0C) and time (12, 12 and 10 seconds). The motility percentages after freezing for 24, 48 and 72 hours of duration varied from 8-50%, 8-50% and 4-45%. The significantly highest ($P<0.01$) motility (35.3 ± 7.8%) was observed after 24 hours of preservation compared with 48 and 72 hours (30.6 ± 8.0%, 28.8 ± 7.9%). The normal morphology percentages after freezing of ram semen for 24, 48 and 72 hours of duration varied from 63-89%, 60-85%, and 58-80%. The significantly highest ($P<0.05$) normal morphology (79.9 ± 4.0%) was observed after 24 hours of preservation compared with 48 and 72 hours (75.8 ± 4.4%, 73.3 ± 4.6%). The significantly highest ($P<0.01$) sperm motility (39.1 ± 5.8%) was observed with 5% glycerol, at 40^0C for 12 seconds thawing temperature and time compared with 37^0C for 12 seconds and 45^0C for 10 seconds, respectively. The study revealed a preliminary data which deserve further study to optimize the measures for sustainable freezing of indigenous ram semen.

Key words: preservation, glycerol, frozen semen, ram.

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INTRODUCTION

The population of sheep in Bangladesh is about 3.12 million (Bangladesh economic review, 2013). Of them, mostly are indigenous non-descript type and are sparsely distributed throughout the country with a relatively higher concentration in the coastal region of Noakhali and Cox's Bazar. The river basin of greater Rajshahi and Tangail districts are also concentrated area of sheep, where farmers maintain larger commercial (meat) flocks. They are of small sized breed known for its prolificacy, adaptation to the saline marshy land and lamb twice in a year, easy to rear and resistant to parasitic infestation (Rahman, 1989). However, population of sheep is decreasing day by day only due to lack of attention to this sector. Sheep contribute significantly to the subsistence, economic and social livelihoods of a large human population in low-input, small holder production systems in Bangladesh.

However, there is scarcity of breeding ram in Bangladesh to increase and improve the production. These need the cryopreservation of semen. Five to eight percent glycerol yielded highest sperm motility (54%) in frozen-thawed ram semen (First et al., 1961; Ali et al., 1994). Glycerol concentration of 7-7.5% was detrimental to the spermatozoa leading to death of ram spermatozoa (Watson and Martin, 1975; Graham et al., 1978). On research, in two-step dilution,

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equilibration at 5°C for 1.5 to 2 hours or over 2 hours yielded the best thawing motility (Evans and Maxwell, 1987; Shao-Guezhi et al., 1996). In order to maintain the benefit of a high rate of warming, ram semen frozen in straws has been thawed by most investigators at 38–42^{0}C (Salamon, Maxwell, 2000).

The success of AI in sheep depends on maintenance of viability, motility and fertility of spermatozoa during storage. Despite the improvements, post-thaw viability and fertility of the cryopreserved sperm are still reduced because of accumulated cellular injuries that arise throughout the cryopreservation process (Medeiros et al., 2002). International experience has been shown that cervical insemination of sheep with frozen-thawed semen usually yields unacceptably low pregnancy rates (10 to 30%) (Donovan et al., 1999). The pregnancy rate at day 70 for ewes inseminated by laparoscopy (48%) was higher than for ewes inseminated by trans-cervical intrauterine (32%) or cervical (9%) methods (Windsor et al., 1994). Technical aspects of laparoscopic AI influenced fertility-the pregnancy rate was 70% for ewes bred using frozen semen compared with 58 % when fresh semen was used (McCappin and Murray, 2011). However, there is no published literature on cryopreservation of ram semen so far in Bangladesh. Therefore the present study was designed to cryopreserve the indigenous ram semen with the following objectives:

- To observe the effectiveness of the undertaken frozen technique on the post thaw motility of frozen semen at 24, 48 and 72 hours of preservation.
- To determine the suitable glycerol percentages for obtaining acceptable post thaw motility of frozen semen.
- To determine the suitable thawing temperature and for obtaining the highest post thaw motility.

MATERIALS AND METHODS

Selection of rams and their management

The experiment was conducted on six indigenous rams at the Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh, during the period January-June, 2013. The rams were selected from parent flock of the research project funded by BAS-USDA in the same Department. They were allowed 6 to 7 hours natural grazing under supervision of a caretaker. Each ram was fed approximately 0.5 to 1.0 kg concentrate (Maize grit, wheat bran, wheat polish with salt) daily. Age, body weight, and scrotal circumference of the ram were 24 to 30 months, 17 to 21 kg and 20 to 25 cm, respectively. The rams were dewormed routinely with subcutaneous injection of Ivermectin (Ivermec Super ®, Advance Ltd. Bangladesh) at the dose of 1 ml/50 kg.

Preparation of media

Stock solutions for tris-fructose-citrate diluent were prepared by dissolving tris (3.63 mg), fructose (1.99 mg) and citrate (0.5 mg) in up to 84, 80, 76 ml distilled water. Antibiotic like penicillin (1 lac IU) and streptomycin (100 mg) were added to the media. The stock solutions were sterilized by filtration (0.22pm) and preserved at 4- 7°C for maximum 2 weeks.

At the day of collection, fresh well churned egg yolk 1 ml and glycerol either 0.3 or 0.5 or 0.7 ml were added with the stock solution to make 10 ml of complete medium that was part B diluent and part A contain egg yolk without glycerol. In part A diluent, distilled water 0.3 or 0.5 or 0.7 ml was added instead of glycerol.

Semen Collection

The rams were trained to ejaculate in artificial vagina (AV). After collection, semen was kept at 37°C in water- bath until the media and reagents were added to it. All these procedures were done at 37°C. The individual ejaculate was evaluated for volume, colour, density, mass activity, concentration, motility and morphology.

Semen evaluation

The volume of the ejaculate was measured by reading the value on graduated tube. Colour was observed by naked eye in the collecting tube immediately after collection. Density was 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.

Mass activity

To evaluate the mass activity, a drop (.5µl) of semen was placed on a pre-warmed slide (37^0C) 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.

Concentration

The concentration of spermatozoa (billion/ml) was determined by using hemocytometer (Herman and Madden, 1963). 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 the edge of pipette and spermatozoa were allowed to settle for 5-6 minutes before placing the haemocytometer on the stage of the microscope. The spermatozoa were counted in 5 large squares, each containing 16 small squares. The large squares to be counted are four at corners and one in the middle of 25 large squares. The head of the spermatozoa in the large squares were counted and recorded. The concentration of spermatozoa per ml of semen was calculated by multiplying the total number of spermatozoa in 5 large squares by 2×10^7 (10 million).

Motility

A drop (0.5) of semen diluted at 1:4 ratio with tris was placed on a dean pre-warmed slide (+37°C) and covered with a cover slip. The motility was determined by eye- estimation of the proportion of spermatozoa moving progressively straight forward at higher magnification (400X).

Morphology

A drop (0.5) of semen was fixed with 2 ml normal saline solution. The morphology was observed at high magnification (1000X). Semen and normal saline was always mixed at the same temperature to prevent any temperature related damage to the spermatozoa. At least 100 spermatozoa were individually examined. The percentages of normal morphology were recorded.

Viability

A drop of sperm (0.1µl) was placed on a glass slide. About 0.5 µl eosin-nigrosin stain was added. The semen sample and stain were mixed with a clean stick, and a homogenous thin smear was prepared. The smear was observed at high magnification (400X). Live spermatozoa appear unstained and dead spermatozoa stained pink against a brownish purple background. Care was taken to ensure that the semen and stain are at the same temperature as otherwise artifacts may be produced. At least 100 spermatozoa were examined from each smear.

Method of freezing

Two-step dilution method was used to freeze the semen in this experiment. Before semen collection, the diluents (Part A and Part B) were prepared and placed in the water bath at 37°C. After collection, individual ejaculate was diluted with calculated amount of diluent part A. One drop of semen was placed in a warm slide and the sperm motility was recorded. After that diluted semen and diluent B part were transferred to refrigerator for two hours. After two hours, calculated amount of diluent B was poured into the previous diluted semen in three divided parts. Then the semen was filled into the straws using micropipette. After filling, the straws were sealed by sealer. For equilibration, the sealed straws were placed in the refrigerator at +4°C for further 2 hours. After two hours, the motility was recorded by using one drop of semen placed on the previously warmed slide under the microscope. Liquid nitrogen was poured in a special box where rack was placed in the box keeping a gap of 5-6 cm above the surface of the liquid nitrogen and kept for 30 minutes to create vapour and to stable bubbling of liquid nitrogen. The freezing was done in liquid vapour (temperature -80°C) in a special box for 5-6 minutes. After that straws were transferred into the cryocan at-196°C.

Evaluation of frozen semen

Thawing of semen

Semen was thawed by plunging the frozen straws in water bath at 38-42°C for 10-12 seconds (Salamon and Maxwell, 2000). The motility of the thawed semen was observed at 0, 24, 48, 72 hours and morphology was done at 24, 48, and 72 hours after freezing as described above.

Analysis of data

Data generated from this experiment were entered in Microsoft Excel worksheet, organized and processed for further analysis. Descriptive statistics were performed to calculate mean standard deviations and percentages. Repeated measures were performed to know the effects of different percentages of glycerol and preservation time on post thawed frozen semen.

RESULTS AND DISCUSSION

In this study semen was collected twice in a week. In ram, semen collection twice a week for 12 weeks did not show any significant difference with respect to volume, concentration and proportion of motile spermatozoa (lhukwmere and Oker, 1990).

In our study volume varied from 1.3- 1.5 ml. The mean volume of semen was 1.4 ± 0.1 ml. In other published study, the average volume of ram semen varied from 0.8- 1.1 ± 0.1 ml (Guerrero et al., 2009; Pervage et al., 2009; Kulaksiz et al., 2012). The volume of semen in the present study was about similar, a bit higher than that of those published works. This difference could be due to differences in breed, climate, nutrition and experimental protocols.

Evaluation of semen immediately after collection

The volume, density, mass activity, concentration, motility, viability, percentages of normal morphology of semen collected from ram is shown in Table 4. The volume, colour, density, mass activity, concentration, motility, viability, normal morphology varied from 1.2-1.6 ml, creamy white-creamy, 2-3, 3-4, 3×10^9 - 6.8×10^9, 78-95%, 85-96%, 80-90%, respectively. However, their mean values were 1.4 ± 0.1 ml, 2.6 ± 0.3, 4.1 ± 0.4, 5.1 ± 1.0×10^9/ml, 89.2 ± 4.3%, 91.0 ± 3.6%, 84.2 ± 3.3%, respectively.

The good mass activity of fresh semen is the key factor to have the good motility of preserved semen. In this study, the mean mass activity was 4.1 ± 0.5. This result was consistent with the result of other published work within the same breed (Pervage et al., 2009). Mass activity is the outcome of collective movement of the spermatozoa. This means both sperm concentration and motility contribute to the mass activity. It is likely that, ram ejaculate more concentrated semen during the breeding seasons. Guerrero et al. (2009) found wave activity (4.4 ± 0.2., scale 1-5), similar to present observation.

In the present study the average sperm concentration varied from 4.1-6.1 $x10^9$/ml. In other studies, the concentration was 2.4 x 10^9 - 4.3 $x10^9$ (Pervage et al., 2009), ≥ 2.5×10^9 spermatozoa/ml (Kulaksiz et al., 2012) and 3.5 ± 0.1 $x10^9$/ml (Guerrero et al., 2009). The average concentration of sperm in the indigenous rams was also higher than other published works. This could be due to the same factors as stated above. In fresh semen, the sperm motility after addition of diluent varied from 78 to 95%. The mean sperm molitity was 89.2 ± 4.3%. Similar results were published by Pervage et al. (2009); Kulaksiz et al. (2012). Guerrero et al. (2009) found individual motility 87.0 ± 2.4%.

Table 1
Characteristics of indigenous ram semen (Mean ± SD).

Volume (ml) (1-4)	Density	Mass activity (1-5)	Concentration ($\times10^9$)/ml	Motility (%)	Viability (%)	Normal Morphology (%)
1.4 ± 0.1	2.6 ± 0.3	4.1 ± 0.5	5.1 ± 1.0	89.2 ± 4.3	91.0 ± 3.6	84.2 ± 3.3

Table 2
Effects of duration of preservation and different glycerol percentages and on sperm motility (Mean ± SD).

Glycerol (%)	Sperm motility (%)			
	0 hour before freezing	24 hours after freezing	48 hours after freezing	72 hours after freezing
3	$72.1_{a} \pm 5.9_{\alpha}$	$23.2_{b} \pm 6.5_{\alpha}$	$18.5_{c} \pm 6.1_{\alpha}$	$16.1_{d} \pm 5.6_{\alpha}$
5	$76.4_{a} \pm 5.8_{\beta}$	$35.3_{b} \pm 7.8_{\beta}$	$30.6_{c} \pm 8.0_{\beta}$	$28.8_{d} \pm 7.7_{\beta}$
7	$63.3_{a} \pm 7.5_{\gamma}$	$15.6_{b} \pm 3.9_{\gamma}$	$12.7_{c} \pm 3.7_{\gamma}$	$10.2_{d} \pm 3.55_{\gamma}$

abcd= The mean values within the same rows differs significantly (at least $P<0.05$)
$\alpha\beta\gamma$ = The mean values within the same columns differs significantly (at least $P<0.05$)

Table 3
Effects of duration of preservation and different percentages of glycerol on normal morphology of frozen-thawed spermatozoa (Mean ± SD).

Glycerol (%)	Normal morphology of spermatozoa (%)		
	24 hrs after freezing	48 hrs after Freezing	72 hrs after reezing
3	$76.8_{a} \pm 4.5_{\alpha}$	$73.2_{b} \pm 4.7_{\alpha}$	$70.6_{c} \pm 4.5_{\alpha}$
5	$73.0_{a} \pm 4.7_{\beta}$	$75.8_{b} \pm 4.4_{\beta}$	$73.3_{c} \pm 4.6_{\beta}$
7	$73.0_{a} \pm 4.7\ \gamma$	$68.7_{b} \pm 4.1\ \gamma$	$65.6_{c} \pm 3.8\ \gamma$

abc The mean values within the same rows differ significantly (at least $P<0.05$).
$\alpha\beta\gamma$ The mean values within the same columns differ significantly (at least $P<0.05$).

Motility of semen following freezing for different durations and having different percentages of glycerol. The motility percentages after freezing of semen for 24, 48 and 72 hours of duration varied from 8-50%, 8-50%, 4-45%. The mean sperm motility following freezing after different durations of preservation is shown in Table 2. The sperm motility was significantly decreasing with the increasing time of preservation with all percentages of glycerol. The significantly highest ($P<0.01$) motility (35.3 ±7.8%) was observed after 24 hours of preservation compared with 48 and 72 hours (30.6 ± 8.0%, 28.8 ± 7.9%). For cryoprotective effect, the motility percentages after freezing of semen with 3, 5 and 7% glycerol varied from 10-40%, 25-50%, 8-25%. The mean sperm motility percentages following freezing with different percentages of glycerol is also been shown in Table 2. The significantly highest ($P<0.01$) sperm motility percentages was observed with 5% glycerol (35.3 ± 7.8%), compared with 3% and 7% (23.2 ± 6.5% and 15.6 ± 3.9%).

Morphology of frozen-thawed spermatozoa

The percentages of preserved spermatozoa having normal morphology varied from 63-89%, 60-85%, 58-80% for 24, 48 and 72 hours of duration of preservation (Table 3). The normal sperm morphology was significantly decreasing with the increasing time of preservation in all percentages of glycerol. The significantly highest ($P<0.05$) normal morphology (79.9 ± 4.0%) was observed after 24 hours of preservation compared with 48 and 72 hours (75.8 ± 4.4, 73.3 ± 4.6). The mean normal sperm morphology percentages with different percentages of glycerol have also been shown in Table 3. For cryoprotective effect, the normal morphology percentages following freezing with 3%, 5% and 7% glycerol varied from 68-85%, 68-89%, 63-85%. The significantly highest ($P<0.05$) normal sperm morphology percentages was observed with 5% glycerol (79.9 ± 4.0%) compared with 3 and 7% (76.9 ± 4.5% and 73.0 ± 4.7%).

Motility of spermatozoa preserved for different temperature and time of frozen semen

The effect of different thawing temperature and time on motility of spermatozoa was observed following freezing for 24 hours of duration. The glycerol percentage used for this observation was 5%. The motility percentages after freezing of semen for 24 hours of duration varied from 15-50%. The mean motility with different thawing temperature and time of duration is shown in Table 4. The significantly highest (P<0.01) sperm motility (39.1 ± 5.8) was observed when frozen semen was thawed at 40^0C for 12 second of duration compared with 37°C for 12 seconds and 45^0C for 10 seconds, respectively.

Table 4. Effect of thawing with different temperature and time on motility of frozen sperm (Mean ± SD).

Parameter	Thawing duration		
	37 °C for12sec	40 °C for12sec	45°C for10sec
Motility (%)	26.8 ± 4.4a	39.1 ± 5.8b	20.1 ± 4.3c

The mean values within columns with different superscript letter differ significantly, at least P<0.05.

The proportion of morphologically abnormal spermatozoa correlates negatively with fertility (Söderquist, 1991; Shamsuddin et al., 1993). Nevertheless when both motility and normality of spermatozoa are used to grade the quality semen, the number of functionally normal spermatozoa appeared to be important determinant for fertility (Saacke et al., 1994). This means that the measures of semen parameters need to be more quantitive than qualitative to be able to predict fertility (Söderquist, 1991). The motility of spermatozoa and the percent of spermatozoa possessing normal structure are often correlated. For example, large numbers of spermatozoa with abnormal tails were associated with reduced sperm motility (Söderquist, 1991). It is well documented that the fertilizing capacity of spermatozoa depends on the innate fertility of male as well as deposition of optimum number of morphologically spermatozoa into the uterus in time (Saacke et al., 1994). In the present study, the percent of morphologically normal spermatozoa percentages was 84.2 ± 3.3%. In fresh semen, Guerrero et al. (2009) found abnormal sperm 11.8 ± 0.7% where in our study abnormal sperm morphology was 15.9 ± 3.6%. In fresh ram semen, Guerrero et al. (2009) found live sperm 90.2 ± 3.8%, which was similar to the present study (91.0 ± 3.6%).

In a research in two-step dilution, equilibration at 5°C for 1.5 to 2 hours or over 2 hours yielded the best thawing motility (Evans and Maxwell, 1987 and Shao- Guezhi et al., 1996). In our study, two-step dilution and two hours equilibrium at 4°C for 2 hours after addition glycerol was maintained.

In our study, for standardizing the thawing temperature, three different thawing temperature and time duration were selected at 37°C for 12 seconds, 40°C for 12 seconds and 45°C for 10 seconds. The significantly highest (P<0.01) sperm motility (39.1 ± 5.8%) was observed when frozen semen was thawed at 40°C for 12 seconds of duration compared with 37°C for 12 second and 45°C for 10 seconds, respectively. Thawing temperature between 37°C and 75°C have been examined for both wet and dry thawing, and generally improvements in post-thaw motility rates were observed with increasing temperature. In order to maintain the benefit of a high rate of warming, ram semen frozen in straws has been thawed by most investigators at 38-42^0C (Salamon and Maxwell, 2000). Cabrera et al. (2011) conducted a research in ram where thawing was done at 38^0C for 15 seconds and progressive individual motility was 56.8-62%. The difference of the effect of thawing time on motility between present study and Cabrera et al. (2011) could be due to experimental differences due to purity of chemicals or skill of work.

Glycerol is the most commonly used protective substance in diluents for freezing ram semen. For semen frozen by the slow "conventional" method, and using mainly hypertonic diluents, most investigators found that the optimal glycerol concentration was within the range of 6-8%; spermatozoa frozen rapidly by the pellet method survived best with 3-4% glycerol in the diluent. Graham et al. (1978) reported that glycerol levels above 6% were detrimental to post-thawing survival of spermatozoa. Ali et al. (1994) observed best post thaw motility of ram spermatozoa in tris-citric acid-fructose diluent with 10% egg yolk and

5% glycerol. The sperm motility varied depending on the percentages of glycerol (3, 5, and 7%) added in this study. In the present work, the motility percentages varied from 10-40%, 25-50%, 8-25% following addition of 3, 5 and 7% of glycerol. The significantly highest ($P<0.01$) sperm motility percentages was observed with 5% glycerol (35.3 ± 7.8%), compared with 3 and 7% glycerol (23.2 ± 6.5% and 15.6 ± 3.9%). Five to eight percent glycerol yielded highest sperm motility (54%) in frozen-thawed ram semen (First et al., 1961; Ali et al., 1994). In the present work the motility percentages after freezing of semen for 24, 48 and 72 hours of duration varied from 8-50, 8-50 and 4-45%. The sperm motility was significantly decreasing with the increasing time of preservation with all percentages of glycerol. The significantly highest ($P<0.01$) motility (35.3 ± 7.8%) was observed with 5% glycerol after 24hours of preservation compared with 48 and 72 hours (30.6 ± 8.0, 28.8 ± 7.9). In other research, with 6% glycerol the progressive motility was 45.7 ± 2.3% after 24 hours of preservation (Nalley and Arifiantini, 2008). In goat with 7% glycerol the post thaw motility was 51% following 24 hours of preservation (Biswas, 2001).

In the present study the abnormal percentages of ram semen with 7% glycerol varied from 20.1 ± 4.0, 24.2 ± 4.8 and 26.7 ± 4.6% after freezing for 24, 48 and 72 hours of duration. The significantly highest ($P<0.05$) normal morphology (79.9 ± 4.0%) was observed after 24 hours of preservation compared with 48 and 72 hours (75.8 ± 4.4%, 73.3 ± 4.6%). In a study, with 7% glycerol abnormality of ram spermatozoa was 10.2 ± .3, 26.5 ± .4 and, 37.3 ± .3% in 0 hour, 24 hours and 48 hours, respectively (Kulaksiz et al., 2012). This little discrepancy for abnormality of spermatozoa following 24 hours of preservation could be due to differences of several factors during freezing, thawing and observation between the works. This similarity and dis-similarity of post thaw motility percentages of spermatozoa with 3-8% glycerol following different times of preservation within the same work or between the works in the ram indicates that still there is much work need to perform for obtaining the full success of ram semen preservation for long time.

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