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A COMPARISON OF FREEZING METHODS AND DILUENTS TYPES ON POST-THAW SPERM QUALITY OF RAM SPERM

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The effect of freezing methods and diluents types on post-thaw sperm quality of Bangladeshi ram semen was studied. Two freezing methods and three diluents was tested as pooling effects (freezing methods or diluents) on post-thaw sperm parameters; sperm motility (SM), viability (SV), plasma membrane integrity (SPMI) and acrosome integrity (SAI), respectively. From selected ten rams, eight ejaculates were used for each freezing group (freezing methods × diluents). Semen samples were diluted by using two-steps for hand-made tris-based diluents (20% egg yolk): D1 (7% glycerol) and D2 (5% glycerol), and one-step dilution for commercial diluents: D3 (Triladyl®) at 35°C. After 4h of equilibration of temperature at 5°C, diluted semen samples was aspirated into 0.25 mL straws, and sealed. Straws were frozen in liquid nitrogen (LN_2) vapour using two methods: F1 (manually in Styrofoam box, using three-steps method; +5°C to -80°C at -11.33°C/min, -80°C to -120°C at -26.66°C/min, and -120°C to -140°C at -13.33°C/min) and F2 (programmable bio-freezer, using two-steps method; +5°C to -100°C at -20°C/min and -100°C to -140°C at -10°C/min). Two semen straws from each batch were evaluated (37°C for 20 sec) for sperm parameters. In pool effects between freezing methods; SAI differed significantly ($P < 0.001$). The SM (56%) and SV (72%) were observed competitive. However, SPMI (67.58 ± 2.02%) and SAI (76.13 ± 1.42%) were higher in F1. Among diluents, SM ($P < 0.006$), SV ($P < 0.008$), SPMI ($P < 0.012$) and SAI ($P < 0.019$) differed significantly. The SM (61.25 ± 1.80%), SV (77.13 ± 1.47%), SPMI (68.31 ± 1.91%) and SAI (74.75 ± 1.64%) were highest in D3. In conclusion, the combination of manual freezing (three-steps) and hand-made tris-based diluents (20% egg yolk, 5% glycerol) is suitable and sustainable method for cryopreservation of ram semen.

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INTRODUCTION

Now a days Bangladeshi farmers are showing their increasing interest towards sheep farming. They rear sheep mainly for livelihood, income generation and food security. Bangladeshi sheep (*Wera*) are non-descript indigenous type, small sized (mature body weight 15-25 kg) and capable of bi-annual lambing with litter size commonly 1-2, rarely 3 (Bhuiyan, 2006). Due to this poor genetics of indigenous sheep, farmers are looking for selective breeding technology to increase and improve production. Selection of ram of superior genetics, cryopreservation of semen and use of artificial insemination (AI) are the method of choice for improving genetics and increasing productivity (Benson et al, 2012). The success of AI depends largely upon the quality of frozen semen. Types of diluents, dilution-cooling-freezing method and thawing protocol eventually affects the quality of frozen ram semen (Salamon and Maxwell, 2000; Watson, 2000; Forouzanfar et al, 2010; Nur et al, 2010). Usually, during freezing-thawing process of semen, the sperm undergoes several vital biological and functional changes due to cold shocks and cryo-injuries, especially affects membranes that distresses their functional abilities (Nur et al, 2010; Chelucci et al, 2015). To establish a simple and sustainable cryopreservation protocol for ram semen in Bangladesh, efforts are focused on different types of diluents, dilution and freezing methods (Mahmuda et al, 2015; Rekha et al, 2016; Rekha et al, 2018; Jha et al, 2019). Evidence from previous studies, a hand-made tris-fructose-citrate (20% egg yolk and 5-7% glycerol) and commercial diluents Triladyl®, two-steps dilution (fractional procedure) and three-steps manual freezing (in LN_2 vapourusing styrofoam) are seemed to be recommended protocol for freezing ram semen in Bangladesh, already extended for field fertility testing (Rekha et al. 2016; Rekha et al. 2018; Jha et al, 2019). However, more effort needs to standardize and select a sustainable frozen ram semen production technology, both in terms of dilutes and freezing methods. Therefore, this study aimed to compare the freezing methods and diluents types, and their interaction on post-thaw quality of ram sperm.

MATERIALS AND METHODS

All procedures were approved by the Department of Surgery and Obstetrics (DSO), Bangladesh Agricultural University's (BAU) Animal Experimental Ethics Committee (AEEC/DSO-BAU/02/2015). The study was carried out from July 2016 to June 2017 in the Laboratory of Theriogenology, DSO, BAU, Mymensingh-2202. All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Animals

Ten Bangladeshi rams (*Wera*) aged 2.5-3 years old, body weight 26-32 kg, scrotal circumference 20-24 cm belonged to Sheep Research Farm, DSO, BAU were used as semen donors (Jha et al, 2018). Health management like; deworming (internal and external parasites), vaccinated (Tetanus, Foot and Mouth Disease, *Peste des Petits Ruminants*) and, husbandry management like; feeding, grazing, watering and housing remained as routinely done in Sheep Research Farm (Jha et al, 2018).

Experimental design

A factorial design (2×3) was used to observe the effects of freezing methods and diluents types, as pooling effects (freezing method or diluents) on post-thaw sperm parameters. Eight ejaculates were used for each treatment combination. Two semen straws from each experimental batch were tested for sperm quality.

Semen collection and evaluation

Semen was collected routinely twice a week. Artificial vagina, especially designed for sheep and goat (Minitube, Germany) was used for semen collection (Jha et al, 2018). Semen was evaluated at 35°C for semen volume, sperm motility (SM), concentration (SC; 10^6/mL), viability (SV), plasma membrane integrity (SPMI), and acrosome integrity (SAI) (Jha et al, 2019). Briefly, SC was calculated by fixing the semen sample (sperm cells) in buffered formol saline (1:200) and counting onto haemocytometer (Neubauer counting chamber) at 400×. The SM was estimated by placing 5 µL semen and cover slip at 100×). The SV was estimated using nigrosine-eosin staining technique at 400×.Sperm cells counted as alive that ignored stain

and dead that stain eosin. The SPMI was estimated using hypo-osmotic swelling test (HOST) technique at 400×. Sperm with swollen and coiled tails were recorded as intact plasma membrane integrity. Acrosomal integrity was evaluated by fixing the sperm cells in buffered formol saline (1:10) and observed the presence or absence of a normal apical ridge of the sperm head at 1000×. At least 200 spermatozoa were examined from each smear.

Diluents

Three diluents, two hand-made (Tris-based) and one commercial (Triladyl®, Minitube, Germany) were used for semen dilution. The hand-made tris-based diluents were prepared in two fraction procedure: Fraction-A (without glycerol) and Fraction-B (with glycerol) according to Jha et al. (2019). Briefly, two hand-made tris-based diluents: D1 (7% glycerol) and D2 (5% glycerol) consists of tris 3.63g, fructose 0.5g, citric acid 1.99g, yolk 20 mL, deionized water, penicillin G (sodium salt) 100,000 IU and dihydrostreptomycin sulphate 100 mg to prepare 100 mL final volume. Similarly, the commercial diluent; (Triladyl®; D3) was prepared by adding 1 volume Triladyl®, 3 volumes of deionized water and 1 volume of egg yolk. The diluents were prepared on the day of semen collection at room temperature and stored at 5°C.

Semen dilution and cooling

Only ejaculates with volume ≥ 0.5 mL, SM ≥ 80%, SV ≥ 90%, SC ≥ 2500×10^6 spermatozoa/mL, SPMI ≥ 85% and SAI ≥ 90% were considered for processing. Semen samples were diluted using two-steps for D1 and D2, and one-step for D3 to obtain a final sperm concentration 800×10^6 spermatozoa/mL (Jha et al, 2019). In two-steps dilution, calculated volume of diluents fraction-A was added to the semen at 35°C. The diluted semen sample (semen + Fraction-A) was allowed to cool down to 5°C for 120 min (-0.25°C/min). The second dilution was carried out with remaining calculated volume of diluents Fraction-B (previously cooled to 5°C). The diluted semen sample (semen + Fraction-A + Fraction-B) was further maintained at 5°C for 120 min for temperature equilibration. In one-step dilution, calculated volume of diluents was added to the semen sample as a whole fraction at 35°C and maintained at 5°C for 240 min for temperature equilibration. Finally, the diluted semen sample was aspirated into 0.25 mL French mini straws and sealed with polyvinyl alcohol.

Semen freezing

The semen straws were frozen in liquid nitrogen (LN_2) vapour using two methods: F1 [manually in Styrofoam box, using three-steps method (Jha et al, 2019); where the semen straws were placed 9 cm above the surface of LN_2 for 7.5 min (+5°C to -80°C at 11.33°C/min), 7 cm for 1.5 min (-80°C to -120°C at 26.66°C/min), and 5 cm for 1.5 min (-120°C to -140°C at 13.33°C/min)] and F2 [programmable bio-freezer (Freeze Control CL-3300 System; Cryologic Pty. Ltd., Australia) using two-steps method (García-Álvarez et al., 2010); where the semen straws were left for 5.25 min (+5°C to -100°C at -20°C/min) and for 4 min (-100°C to -140°C at -10°C/min)]. Finally the straws were plunged into the LN_2 (-196°C).

Post-thaw semen evaluation

Two semen straws from each batch was evaluated (37°C for 20 sec) for sperm parameters.

Statistical analysis

All data obtained from study were analyzed using SPSS (20.0 Version) statistical software. Analysis of variance (ANOVA) was carried to observe the pooling effects (freezing method or diluents) on sperm parameters. When ANOVA revealed a significant effect, the values were compared by Duncan's multiple range test (DMRT). To compare the pooling effects between the freezing methods on sperm parameters, independent samples *t* test was applied. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

The values of post-thaw sperm in fixed effects (freezing methods or diluents) are presented in Table 1 and 2. When observing the pooling effects between the freezing methods (F1 and F2) on post-thaw sperm parameters; the SM, SV and SPMI differed insignificantly, whereas, SAI differed significantly ($P < 0.001$). The SM (56%) and SV (72%) were comparable in both the freezing methods. Whereas, the SPMI (67.58 ± 2.02% vs 62.71 ± 1.52) and SAI (76.13 ± 1.42% vs 69.17 ± 1.01) observed higher in freezing methods F1 than in F2. Similarly, when observing the pooling affects among the diluents types (D1, D2 and D3) on post-thaw sperm parameters, the SM ($P < 0.006$), SV ($P < 0.008$), SPMI ($P < 0.012$) and SAI ($P < 0.019$) differed significantly. However, the sperm parameters in regards to SM, SV, SPMI and SAI between diluents D2 and D3 differed insignificantly. The SM (61.25 ± 1.80%), SV (77.13 ± 1.47%), SPMI (68.31 ± 1.91%) and SAI (74.75 ± 1.64%) scored highest in diluents D3.

Table 1. Percentage (%) of post-thaw sperm parameters (motility, viability, plasma membrane integrity and acrosome integrity) in different freezing methods (mean ± S.E.M)

Freezing methods	Motility	Viability	Plasma membrane integrity	Acrosome integrity
F1	56.25±1.98	72.38±2.11	67.58±2.02	76.13±1.42
F2	56.25±1.91	72.71±1.81	62.71±1.52	69.17±1.01
P-value	1.001	0.905	0.060	0.001

Table 2. Percentage (%) of post-thaw sperm parameters (motility, viability, plasma membrane integrity and acrosome integrity) in different diluents (mean ± S.E.M)

Diluents	Motility	Viability	Plasma membrane integrity	Acrosome integrity
D1	$50.94±2.47^{b}$	$67.13±2.83^{b}$	$59.88±2.35^{b}$	$68.75±1.19^{b}$
D2	$56.56±2.13^{ab}$	$73.38±2.05^{a}$	$67.25±2.06^{a}$	$74.44±1.94^{a}$
D3	$61.25±1.80^{a}$	$77.13±1.47^{a}$	$68.31±1.81^{a}$	$74.75±1.64^{a}$
P-value	0.006	0.008	0.012	0.019

Different letters (a, b) indicate differences among diluents $P<0.05$.

DISCUSSION

This study compared the association of two types of freezing method and three types of diluents on post thaw sperm quality in Bangladeshi ram semen. Many studies have been reported using various diluents, dilution protocol, freezing methods and freezing rates to improve the quality of cryopreserved sperm (Alcay et al, 2015a,b; Mahmuda et al, 2015; Ustuner et al, 2015; Alcay et al, 2016; Rekha et al, 2016; Rekha et al, 2018; Jha et al, 2019). In fact, during entire semen cryopreservation process, sperm undergoes several vital biological and functional changes due to cold shocks and cryo-injuries that affects membranes that distresses their functional abilities (Nur et al, 2010; Chelucci et al, 2015).

In the present study, it clearly stated that the freezing method F1 (+5°C to -80°C at -11.33°C/min, -80°C to -120°C at -26.66°C/min, and -120°C to -140°C at -13.33°C/min) observed superior compared to that of programmable bio-freezer freezing method F2. Similarly, hand-made diluents D2 (Tris-fructose-citrate, 20% egg yolk v/v and 5% glycerol) observed competitive performance compared to that of D3 (commercial diluents, Triladyl®). In this context, the combination of manual freezing method and hand-made Tris-based diluents could be a simple and sustainable protocol for freezing ram semen in Bangladesh.

The best preserved SM 50-61%, SV 67-77%, SPMI 60-68% and SAI 68-70% in this present study, all within the range reported by Anel et al. (2003), D'Alessandro et al. (2001), Chatterjee et al. (2001)and Valente et al. (2010), and this observation are comparable to freezing method F1 and F2, and diluents D2 and D3. However, there is variation in regards to breed, diluents formulation, dilution steps and freezing methods. The diluents used in those studies were tris-citrate based, but differed in egg yolk and glycerol concentration. The concentrations of egg yolk and glycerol used in the diluents were 20% and 4% (Anel et al, 2003), 20% and 5% (D'Alessandro et al, 2001) and 15% and 5.3% (Valente et al, 2010). Comparing the addition of glycerol as cryoprotectant and dilution method, Anel et al. (2003) and D'Alessandro et al. (2001) added glycerol at equilibrated temperature within 4-5°C in two-steps dilution and used both programmable bio-freezer and manual freezer (Styrofoam box technique), whereas Valente et al. (2010) added glycerol in single fraction as well as in second fraction at equilibrated temperature 5°C and used manual freezer (Styrofoam box technique).

In the present study, the post-thaw sperm quality revealed best with diluents D3 and D2, could be due to optimum concentration of egg yolk 20% and lower concentration of glycerol 5% (Holt, 2000; Salamon and Maxwell, 1995). Most semen extenders contain egg yolk as sources of liproprotein to prevent cold shock during semen processing at 4-5°C (Amirat et al, 2004). However, various experts are in against the use of egg yolk (Amirat et al, 2004). It is reported that products of animal origin could increase the risk of microbial contamination during AI (Aires et al, 2003), produces endotoxin and eventually reduce the potential fertilizing capacity of spermatozoa (Forouzanfar et al, 2010). In this regard, alternative to egg yolk, researchers are recommending soybean lecithin (or phosphatidylcholine) which is a phospholipid - a plant origin, plays an important role in the regulation of an animal cells' bio-membrane (Voet and Voet, 1995; Aires et al, 2003). As cryoproctactant, glycerol is one of the most commonly used for freezing ram semen (Salamon and Maxwell, 2000). It protects sperm cells from crystallization by entering into the cell by osmotic effects mechanism (Morris et al, 1972; Salamon and Maxwell, 1995). The amount of glycerol added to the freezing medium is limited by its toxicity (Emamverdi et al, 2013). It has been suggested that the optimum concentration of glycerol added to the freezing extender is between 2% and 10% (Alvarez-Rodriguez et al, 2011; De Paz et al, 2012). For optimum cryoprotection and to lessen the toxicity, rather than adding glycerol at a temperature close to 0°C, it is recommended using two-steps gradual addition at 2-5°C (Colas, 1975). Glycerol levels above 6% were detrimental to post-thaw survival of spermatozoa (Graham et al, 1978).

In the present study, both freezing methods; manual (F1) and programmable bio-freezer (F2) preserved better post-thaw sperm quality. This might be due to slow, steady and controlled drop of temperature from +5°C to -15°C in around 30-32 sec. This may be explained by less cytotoxic damage due to slow and consistent drop in freezing temperature (Salamon and Maxwell, 1995; Holt, 2000).

CONCLUSION

In conclusion, the combination of three-steps manual freezing and hand-made tris-based diluent (5% glycerol) is a suitable and sustainable protocol for freezing ram semen. However, further trial is suggested with soybean lecithin instead of using egg yolk to standardize the freezing protocol.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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