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Quality of chilled indigenous ram semen using skim milk-based extenders

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Abstract

This study aimed to evaluate the effects of extenders using skim milk from different species on sperm quality during storage at 5°C over time. Eight ejaculates were preserved in each of five home-made semen extenders: cow skimmed milk (CSM), goat skimmed milk (GSM), sheep skimmed milk (SSM), buffalo skimmed milk (BSM) and commercial dried skim milk (CDSM). Sperm motility, plasma membrane integrity, and normal morphology (%) were assessed at 0, 24, 48, 72, 96, and 120 hours. At 48, 72, 96, and 120 hours, sperm motility in the GSM extender was higher than in the BSM, SSM, and CDSM extenders. Plasma membrane integrity (%) and normal sperm morphology (%) in the GSM and CSM extenders were significantly higher than in the BSM, SSM, and CDSM extenders at 24, 48, 72, 96 and 120 hours. Overall, motility, membrane integrity, and normal morphology were superior in the GSM extender. Further research is needed to validate sperm quality in relation to lambing rates. (*Bang. vet.* 2025. Vol. 42, No. 1 – 2, 11 – 16)

Introduction

Artificial insemination using frozen ram semen has not been widely adopted, largely due to the extensive nature of sheep breeding and the low fertility rates associated with intra-cervical insemination of frozen semen (Paulenz *et al*., 2000). Freezing reduces sperm motility, compromises morphological integrity, increases embryonic loss, and results in low fertility rates. These detrimental effects are less pronounced in diluted and chilled semen compared with frozen–thawed semen (Fiser and Fairful, 1984; Aisen *et al*., 2000). The principal advantage of liquid semen is improved fertility rates (Anzar *et al*., 2003), even when using fewer spermatozoa.

Bangladeshi sheep are renowned for their adaptability, disease resistance, and prolificacy. They generally have lower mortality rates than goats and are less susceptible to extreme weather conditions. To enhance and diversify livestock

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production in Bangladesh, sheep farming could be an optimal choice (Ahmmed *et al*., 2016).

Milk-based semen extenders are widely used and reduce cold shock during semen preservation. Skimmed or whole milk can serve as buffering media in which semen is directly diluted and stored at 4°C (Kakar and Ganguli, 1978). Milk-based extenders have been shown to be as effective as egg-yolk-based extenders in protecting sperm (Chen *et al*., 1993). The key protective component of milk is casein micelles, which safeguard ram sperm during storage at 4 – 5°C. Although a few studies have examined skim-milk-based extenders for chilled ram semen, no comprehensive investigation has been undertaken to identify the most effective formulation. Therefore, this study was designed to evaluate the efficacy of different skim-milk-based extenders for preserving ram semen at 5°C for up to 120 hours.

Materials and Methods

Fresh milk from disease-free cows, buffaloes, goats, and sheep were warmed at 37^{o}C and milk fat was removed by centrifugation at 6000 rpm for 15 minutes and filtered through cotton wool. Skim milk was incubated at 92^{o}C for 10 minutes and cooled at room temperature for further processing to inactivate the spermicidal factor (Gordon, 1999) and preserved at 4 - 5^{o}C. Fat percentage was determined by Babcock fat test.

Preparation of Skim Milk Based Extenders*:* Five extenders were prepared: Cow skim milk (CSM), Buffalo skim milk (BSM), Goat skim milk (GSM), Sheep skim milk (SSM) and Commercial dried skim milk (CDSM) extender. Finally, 100 ml of milk-based extenders contained 90 ml skim milk, 10 ml egg yolk, 0.9 gm glucose and 100 mg streptomycin. For CDSM, 10 ml extenders contained 90 ml distilled water, 10 gm dried skim milk, 10 ml egg yolk, 0.9 gm glucose and 100 mg streptomycin.

Semen Collection and Processing*:* Four healthy rams of 2.5 to 3 years old grazing 6 to 8 hours daily were selected as semen donors. Once a week semen was collected between 6 - 7 AM using artificial vagina. Only ejaculates with motility >75%, sperm concentration of >2.8 × 10^{9} spermatozoa/ml and semen volume of >0.5 ml were included. After collection, five semen samples were diluted with CSM, BSM, GSM, SSM and CDSM extenders at a concentration of 400X10^{6} spermatozoa/ml. Motility, plasma membrane integrity and normal morphology (%) were examined immediately after dilution of semen. For refrigeration, some straws from all extenders were kept at 5°C.

Evaluation of semen*:* A drop of 5 µl from each of three straws semen diluted with TRIS at 1 : 4 ratios was placed on a clean warm glass slide (37°C) and covered with a cover slip. The motility was determined by estimation of the percentage of spermatozoa moving progressively straight forward at medium magnification (40X).

Hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane. This was performed by incubating 20 µl semen with 200 µl of hypo-osmotic solution containing 9 gm fructose and 4.9 gm sodium citrate in 1000 ml distilled water at 37°C for 60 minutes. After incubation, 5 µl of the mixture was spread with a cover slip on a warm slide and a total of 200 sperms were evaluated in different microscopic fields at 40X objective. The percentage of sperm with swollen and curled tails (HOST+ve) was recorded.

A drop (10 µl) of formal saline-fixed semen was placed on a clean glass slide with a cover slip and the edges were soaked with tissue paper to remove excess fluid. The slide was held for five minutes to allow spermatozoa to settle down and examined under microscope at high magnification (100X). At least 200 spermatozoa were evaluated to determine the abnormalities in head, mid piece and tails.

Statistical analysis*:* The results were expressed as mean ± standard deviation. Means were analyzed by one-way analysis of variance, followed by the Tukey test to determine significant differences in all the parameters among all diluents using the SPSS computer program (Version 20.0; SPSS). Differences with values of $P<0.05$ were considered to be statistically significant.

Results and Discussion

Sperm motility, plasma membrane integrity (HOST-positive), and the proportion of morphologically normal spermatozoa (%) were measured after 0, 24, 48, 72, 96 and 120 hours of storage. The effects of the diluents and preservation time on these parameters are summarised in Tables 1, 2 and 3.

Sperm motility decreased significantly ($P<0.05$) with increasing preservation time. From 48 to 120 hours, motility in the GSM extender was significantly higher than in BSM, SSM, and CDSM extenders. The motility of chilled semen in GSM at 0 hours (84.5 ± 1.00%) was comparable to that reported by Kulaksiz *et al*. (2012), who recorded 84.0 ± 0.1% at the same time point. Similarly, Azizunnesa *et al*. (2014) reported motility of 84.0 ± 0.4% and 76.1 ± 0.5% at 24 and 48 hours, respectively, which were in close agreement with the results for GSM and CSM in this study. At 72 hours, motility remained at approximately 70%, slightly higher than the 66.5 ± 4.2% reported by Foote *et al*. (2002) for skim-milk-extended semen. In the present study, GSM maintained 72.0 ± 0.8% motility up to 96 hours, exceeding the values reported by Foote *et al*. (2002).

Plasma membrane integrity (%) in GSM and CSM extenders was significantly higher than in BSM, SSM, and CDSM at all times from 24 to 120 hours. Azizunnesa *et al*. (2014) recorded plasma membrane integrity values of 82.0 ± 0.4% and 73.8 ± 0.6% at 24 and 48 hours, respectively, which were comparable to the results obtained with GSM and CSM in the present study. The proportion of HOST-positive spermatozoa was higher than the 60 ± 1.4% reported by Akhter *et al*. (2015) for skim-milk-extended semen.

Normal sperm morphology (%) was significantly higher in GSM and CSM than in BSM, SSM and CDSM at all times from 24 to 120 hours. Kulaksiz *et al*. (2012) reported 82.3% normal spermatozoa in skim-milk-extended chilled semen at 24 hours, similar to the morphology recorded in GSM (83.0 ± 0.8%) at the same time point.

Table 1. Sperm motility (%) of ram spermatozoa in different extenders stored at 5°C

Extender	Fresh semen	0 h	24 h	48 h	72 h	96 h	120 h
CSM	86.3 ± 1.3^{a}	84.0 ± 1.4^{a}	82.3 ± 2.1$^{a\beta}$	79.8 ± 1.7^{a}	75.0 ± 2.5^{a}	71.8 ± 2.4^{a}	69.3 ± 1.5^{a}
BSM	85.5 ± 1.7^{a}	80.3 ± 2.6$^{a\beta}$	78.0 ± 3.2$^{\beta\gamma}$	73.8 ± 1.0$^{\beta\gamma}$	70.3 ± 0.5$^{\gamma}$	59.8 ± 1.3$^{\beta}$	49.5 ± 6.1$^{\gamma}$
GSM	87.3 ± 1.0^{a}	84.5 ± 1.0^{a}	82.5 ± 0.6$^{a\beta}$	80.3 ± 0.5^{a}	76.5 ± 1.3^{a}	72.0 ± 0.8^{a}	70.3 ± 0.5^{a}
SSM	86.5 ± 1.3^{a}	83.3 ± 1.3$^{a\beta}$	76.5 ± 1.7$^{\gamma}$	72.3 ± 1.9$^{\gamma}$	68.3 ± 2.5$^{\gamma}$	61.3 ± 2.5$^{\beta}$	52.5 ± 2.9$^{\beta\gamma}$
CDSM	86.0 ± 0.8^{a}	84.8 ± 0.5^{a}	80.3 ± 1.3$^{a\beta\gamma}$	75.5 ± 1.0$^{\beta}$	71.8 ± 2.4$^{\gamma}$	61.8 ± 2.5$^{\beta}$	56.8 ± 2.4$^{\beta}$

^{abcdef}-The mean values with different superscript within the same row differs significantly (P<0.05).
$^{\alpha\beta\gamma}$-The mean values with different superscript within the same column differs significantly (P<0.05).

Table 2. HOST-positive (%) of ram spermatozoa in different extenders stored at 5°C

Extender	Fresh semen	0 h	24 h	48 h	72 h	96 h	120 h
CSM	85.8 ± 0.5^{a}	84.5 ± 0.6^{a}	82.8 ± 0.5$^{a\beta}$	80.8 ± 1.0$^{a\beta}$	78.0 ± 1.4^{a}	73.5 ± 1.7^{a}	70.3 ± 0.5^{a}
BSM	84.5 ± 0.6^{a}	82.3 ± 1.3^{a}	81.0 ± 1.4$^{\beta\gamma}$	76.3 ± 2.5$^{\beta\gamma}$	72.5 ± 1.7$^{\beta}$	63.8 ± 2.5$^{\beta}$	54.0 ± 4.6$^{\gamma}$
GSM	86.8 ± 1.3^{a}	85.3 ± 1.0^{a}	84.3 ± 1.0^{a}	81.8 ± 1.3^{a}	78.0 ± 2.2^{a}	74.8 ± 0.5^{a}	72.0 ± 1.4^{a}
SSM	85.0 ± 2.2^{a}	82.8 ± 1.9^{a}	78.5 ± 1.5$^{\gamma}$	75.0 ± 1.6$^{\gamma}$	71.5 ± 2.4$^{\beta}$	64.3 ± 1.7$^{\beta}$	56.5 ± 2.4$^{\beta\gamma}$
CDSM	85.5 ± 0.6^{a}	82.8 ± 2.2^{a}	78.3 ± 2.4$^{\gamma}$	75.8 ± 3.9$^{\beta\gamma}$	72.5 ± 3.3$^{\beta}$	64.8 ± 1.0$^{\beta}$	60.5 ± 3.3$^{\beta}$

^{abcdef}-The mean values with different superscript within the same row differ significantly (P<0.05).
$^{\alpha\beta\gamma}$-The mean values with different superscript within the same column differ significantly (P<0.05).

Table 3. Normal morphology (%) of ram spermatozoa in different extenders stored at 5°C

Extender	Fresh semen	0 h	24 h	48 h	72 h	96 h	120 h
CSM	84.8 ± 1.3^{a}	83.0 ± 2.2^{a}	81.5 ± 1.7$^{a\beta}$	79.3 ± 1.5$^{a\beta}$	75.8 ± 1.5$^{a\beta}$	70.3 ± 1.3$^{a\beta}$	66.3 ± 1.5$^{a\beta}$
BSM	84.3 ± 1.7^{a}	81.5 ± 1.3^{a}	79.0 ± 1.2$^{\beta}$	75.0 ± 1.4$^{\gamma}$	69.5 ± 1.7$^{\gamma}$	64.5 ± 3.1$^{\gamma}$	56.8 ± 2.2$^{\gamma}$
GSM	85.8 ± 1.0^{a}	84.5 ± 1.0^{a}	83.0 ± 0.8^{a}	81.3 ± 1.5^{a}	79.3 ± 1.3^{a}	73.0 ± 2.2^{a}	70.3 ± 1.3^{a}
SSM	84.8 ± 1.0^{a}	82.0 ± 1.4^{a}	79.8 ± 1.3$^{\beta}$	76.8 ± 2.1$^{\beta\gamma}$	71.3 ± 3.0$^{\beta\gamma}$	66.0 ± 2.7$^{\beta\gamma}$	60.3 ± 4.5$^{\beta\gamma}$
CDSM	85.5 ± 1.3^{a}	82.3 ± 1.7^{a}	80.0 ± 1.6$^{\beta}$	77.0 ± 2.2$^{\beta\gamma}$	72.0 ± 2.5$^{\beta\gamma}$	66.3 ± 2.5$^{\beta\gamma}$	62.0 ± 4.0$^{\beta\gamma}$

^{abcdef}-The mean values with different superscript within the same row differ significantly (P<0.05).
$^{\alpha\beta\gamma}$-The mean values with different superscript within the same column differs significantly (P<0.05).

Since skimmed milk is as effective as whole milk in protecting sperm during storage at 4°C or cryopreservation (Rasteh and Divandi, 2015), lipids do not appear to be the main component responsible for the protective effect. Caseins, the major milk proteins and recognised cryoprotectants (Foote *et al*., 2002), may protect sperm function by preventing the binding of bull seminal plasma (BSP) proteins to sperm. This prevents

BSP protein-mediated stimulation of lipid loss from the membrane during preservation. BSP proteins, secreted by the seminal vesicles, bind to sperm at ejaculation and alter the sperm membrane by removing cholesterol and phospholipids, potentially impairing preservation capacity. Similar to bovine semen, ram seminal plasma contains specific proteins (RSP proteins) associated with fertility and semen quality (Bergeron *et al*., 2007). Interactions between sperm membranes and RSP proteins may occur during freezing and thawing, with casein potentially preventing such interactions and membrane damage. The composition of casein varies among goat, cow, buffalo, and sheep milk (Yue *et al*., 2009).

In the present study, GSM proved superior to all other extenders in preserving semen quality, with CSM ranking second. The differences in preservation efficacy may be attributed to variations in casein composition, as goat's milk and cow's milk share a similar fatty acid profile and saturated/unsaturated fatty acid ratio (Ham *et al*., 2010; Castillo *et al*., 2006). Fatty acids in goat, buffalo, sheep, and cow skim-milk extenders may have adversely affected semen freezability, but the unique casein structure in goat's milk may have counteracted these detrimental effects (Chilliard *et al*., 2006). Moreover, goat's milk contains higher levels of copper, manganese, zinc, iron, calcium, and magnesium-though lower potassium and sodium-compared with cow's milk (Yue *et al*., 2009). Differences in mineral content between goat and cow milk may have influenced semen preservation.

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