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Short communication

Comparison of commercial Triladyl extender with a tris-fructose-egg-yolk extender on the quality of frozen semen and pregnancy rate after transcervical AI in Bangladeshi indigenous sheep (*Ovis aries*)

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ABSTRACT

The objective of this study was to compare the effects of commercial and locally manufactured tris-fructose-egg-yolk semen extenders on the quality of frozen semen and pregnancy rates (PR) after AI of Bangladeshi indigenous sheep. Semen was collected weekly by artificial vagina (AV) from 6 mature indigenous rams (*Ovis aries*). Each semen sample was diluted with commercial (Triladyl®) and locally manufactured (tris, fructose, egg-yolk, 7% glycerol) extenders, to a final concentration of 200, 400 and 600×10^6 sperm/mL. Semen samples were frozen in 0.25 mL mini-straws in liquid nitrogen (LN) vapor for 6 min and then stored in LN. Post-thawing motility, viability, functional integrity and normal morphology were evaluated on Days 1, 5 and 30 of storage. Post-thaw sperm quality parameters were better ($p < 0.001$) in Triladyl than in the locally manufactured extender; and quality parameters were also higher for a pre-freezing concentration of 400×10^6 sperm/mL ($p < 0.001$) than for other concentrations, regardless of extender. Storage time did not affect semen quality parameters ($p > 0.001$). Indigenous ewes ($n = 46$) were inseminated transcervically using semen from both extenders at a sperm concentration of 400×10^6/mL. There was no difference in the pregnancy rates between sperm frozen in Triladyl compared with the locally manufactured extender ($p > 0.70$). In conclusion, whilst post-thaw sperm quality was better in semen frozen in Triladyl, this did not result in any significant benefit compared with the locally-prepared extender on PR after TCAI in ewes.

1. Introduction

Although the AI of sheep has been undertaken for many years, much research is still being undertaken to find ways of improving the pregnancy rates that can be achieved by cryopreserved semen (Motamedi-Mojdehi et al., 2013). Moreover, for many indigenous or locally-important breeds of sheep (e.g., the Bangladesh indigenous sheep), little is known about the optimal conditions for the dilution, cooling, freezing and thawing of their semen, nor how these affect pregnancy rates. The ability of cryopreserved semen indefinitely superior to fresh/chilled semen in terms of its use for underpinning selective breeding programmes (Salamon and Maxwell, 2000; Tekin et al., 2006) via selection for, and dissemination of, superior male characteristics. Thus, it is an important target of the sheep industry in Bangladesh for the cryopreserved semen of indigenous sheep to give equally good conception rates through TCAI as those that can be achieved with chilled/fresh semen. Indeed, achieving acceptable conception rates is very important for the uptake of sheep AI services, as the relatively conservative farming community in Bangladesh will reject AI services unless conception rates meet its expectations.

The quality of frozen semen one of the most important factors that influences the conception rate after TCAI. Much of the

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research on the cryopreservation of ram semen has been focused upon the use of various extenders, dilution rates and freezing protocols (Salamon and Maxwell, 2000; Tekin et al., 2006). For example, tris citric acid, Triladyl®, Skim milk and INRA 96® are extenders that have been widely used for semen freezing (Kulaksiz et al., 2012); although the results from this and other extenders have been remarkably variable (Byrne et al., 2000; Joshi et al., 2005). Furthermore, prefreezing sperm concentration is another important factor that affects sperm quality after freezing (Alvarez et al., 2012).

Therefore, the present study was designed to compare Triladyl with a tris/fructose/egg-yolk extender to determine the effect of extender and initial sperm concentration on the quality (i.e., post-thaw semen quality and the pregnancy rate after TCAI) of frozen-thawed semen from Bangladesh indigenous sheep.

2. Materials and methods

2.1. Study area and period

The study was conducted at the Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh-2202 (N 24.73 and E 90.44) during the period from 21 January to 30 June 2013. The area receives on average 174 mm of rainfall. Mean annual minimum and maximum temperatures experienced at the site are 16.5 and 29.1 °C, respectively.

2.2. Animals and their management

Six indigenous rams (3–3.5 years old, 17–26 kg body weight, scrotal circumference of 20–24 cm) were used for collection of semen. Anthelmintic and vaccination against rabies and tetanus were performed routinely. The animals were maintained on natural grazing supplemented with concentrates (300 g/head/day) that consisted of wheat bran (50%), crushed maize (25%), soy bean meal (20%), fish meal (1%), dicalcium phosphate (DCP) powder (2%), vitamin–mineral premix (0.5%), and salt (1.5%) with water always available.

2.3. Preparation of extender

Two different extenders; commercial (Triladyl® Minitube, Germany) and a locally-manufactured extender were used. The locally-manufactured extender (tris, fructose, egg-yolk: TFE) was prepared according to Salamon and Maxwell (2000) (tris-base: tris 3.4 g, fructose 0.5 g, citric acid 2.0 g, penicillin 100,000 IU, streptomycin 100 mg, deionized water to 100 mL). The stock was stored at 4 to 5 °C and, on the day of semen collection, the final extender was prepared by adding egg-yolk (10%), after which the extender was split into two fractions: Fraction 1 (no glycerol) and Fraction 2 (7% v/v glycerol). Similarly, on the day of collection, the final Triladyl® was prepared by adding 1 volume of Triladyl (contains glycerol, tris, citric acid, fructose, tylosin, gentamicin, lincomycin and spectinomycin according to the manufacturers' specifications) to 3 volumes of demonized water and half volume of egg-yolk. After mixing the egg-yolk the mixture was filtered by filter paper (Whatman™, 125 mm Ø × 100 circles, GE Healthcare UK Limited, Amersham Place, China). Both extenders contained 10% egg-yolk and 7% glycerol. Triladyl and TFE fraction 1 extenders were kept on water bath at 32–35 °C before semen collection whereas fraction 2 kept at 4 °C.

2.4. Semen collection, freezing and evaluation

Rams semen was collected once per wk per ram using AV. A total of 57 ejaculates were collected from 6 rams during the study. Each ejaculate was examined macroscopically and microscopically. The volume of semen was measured and color was estimated. Mass motility was estimated by assessment of wave motion of fresh undiluted semen at 10× magnification (0–5 scale) (Domínguez et al., 2008). The concentration of spermatozoa was calculated by haemocytometer count (1:200 dilutions). Thereafter, each semen sample was divided into six equal tubes for dilution either into TFE extender or Triladyl, l with final concentrations of 200, 400 and 600×10^6 sperm/mL, for each extender. For Triladyl, the semen was diluted slowly in a single step at room temperature, loaded into 0.25 mL mini straws, sealed and then held at 4 °C for 4 h. For TFE extender, the semen was extended with two fractions. Fraction 1 was mixed with semen at room temperature and cooled at 4 °C for 2 h, then Fraction 2 was added in three aliquots. Thereafter, the extended semen was loaded into 0.25 mL ministraws, sealed and held for next 2 h at 4 °C. Semen was cooled in a refrigerator. After 4 h of equilibration, all the straws were placed horizontally on a wire Plate 4 cm above the nitrogen level for 6 min and subsequently stored in liquid nitrogen until thawing. After 1, 5 and 30 days of storage in liquid nitrogen, samples were thawed in water at 39 °C for 14 s and evaluated for motility, viability, functional integrity (by hypo osmotic swelling HOS test) and morphology.

Sperm motility was assessed (400× magnification) directly on a microscope slide. A semen sample (5 μL) placed on a grease-free microscope slide, pre-warmed at 37 °C and covered by a cover slip. For each sample, different microscopic fields were examined. The mean of the three successive evaluations was recorded as the final motility score (Ax et al., 2000).

Eosin-nigrosin staining was used to determine the viability of spermatozoa. A small drop of semen and one drop of eosin–nigrosin stain were placed on a clean slide and mixed with a clean stick; a thin smear was then made, dried in air and examined at 400× maginification. At least 200 spermatozoa were examined from each smear to calculate the percentages of live spermatozoa. Sperm that were white (unstained) were classified as live and those that showed any pink or red coloration were classified as dead, with the sole exception for sperm with a slight pink or red appearance restricted to the neck region, which, according to the criteria of Mortimer (1994) were classified as live.

The HOS solution was prepared by mixing 9 g fructose and 4.9 g sodium citrate into 1 L deionized water. For the test, 20 μL of extended semen was mixed with 200 μL of HOS solution and incubated at 37 °C for 60 min. After incubation, 5 μL of HOS/semen mix was taken into pre-warmed slide and examined under microscope 400×. Sperm with curled tails were considered to be HOS test positive. At least 200 spermatozoa were observed in different microscopic fields to calculate the proportion of reacted sperm (Revell and Mrode, 1994).

Morphology was assessed after staining with Spermac (Minitube, Box 152, Wellington 7654, South Africa) under a differential interference contrast (DIC) (Olympus, Japan) microscope at 1000×. At least 500 spermatozoa were examined from each smear to calculate the percentage of normal spermatozoa. Abnormalities were classified into those of the acrosome, head, midpiece and tail (Schafer and Holzman, 2000).

2.5. Oestrus synchronization

Oestrus was synchronized by administering cloprostenol (Ovuprost sterile injection, Bomac, Laboratories Ltd., New Zealand; 0.4 mL) twice, 9 days apart, to each ewe. Vasectomized (teaser) rams and direct observation were used to detect oestrus.