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In vitro viability and longevity of cooled Beetal buck spermatozoa extended in skimmed milk and Tris-citric acid based extenders

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ABSTRACT

Two experiments were carried out to improve the quality of cooled Beetal buck spermatozoa stored for 72 h at 4 °C. Effect of seminal plasma (i) on membrane and acrosome integrities, motility and motion characteristics of spermatozoa extended in skimmed milk and Tris-citric acid based extenders (ii) longevity of spermatozoa extended in skimmed milk and Tris-citric acid based extenders were investigated. Semen of 5 Beetal bucks was extended to form 4 treatment groups: i) with seminal plasma in skimmed milk (T1); ii) with seminal plasma in Tris-citric acid (T2); iii) without seminal plasma in skimmed milk (T3); iv) without seminal plasma in Tris-citric acid (T4). Plasma membrane integrity was higher ($p < 0.05$) in T3 as compared with T2 and T4 at 48 h. There was no effect ($p > 0.05$) of storage time in T3 on plasma membrane integrity. Acrosome integrity was higher ($p < 0.05$) in T1 and T3 compared to T2 and T4 at 24 and 72 h. There was no effect ($p > 0.05$) of storage time in T1 and T3 on acrosome integrity. Progressive motility (%), linearity (%) and beat cross frequency (Hz) were significantly higher in T1 and T3 compared to T2 and T4 from 0 to 72 h. While straightness (%) was significantly higher in T1 and T3 compared to T2 and T4 at 0, 24 and 72 h. Regarding, longevity (% decrease in motion characteristics) of rapid and progressive motile Beetal buck spermatozoa at 72 h storage time, percent decrease in average path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement and beat cross frequency was significantly lower in T3 compared to other three treatment groups. In conclusion, *in vitro* viability and longevity of cooled Beetal buck spermatozoa was better preserved in T1 and T3 (skimmed milk based extenders with or without seminal plasma) up to 72 h of storage at 4 °C.

1. Introduction

Artificial insemination (AI) has been effectively used in goats for genetic improvement, controlled breeding, introduction of new breeds and conservation of indigenous breeds (Leboeuf et al., 2008). Timed AI in does with freshly diluted-chilled semen was found to be efficient in saving time, cost and labour (Faigl et al., 2012). Storage of chilled buck semen for more than 24 h is desirable as it would have greater flexibility to inseminate heat synchronized flocks.

A number of extenders *viz-a-viz* sodium citrate, Tris-citric acid, Tris, whole milk, skimmed milk and reconstituted milk with or without egg yolk for chilled buck semen have been tested in laboratory and in field fertility trials (Leboeuf et al., 2004). However, there was variability in the viability and fertility of chilled spermatozoa stored for 5–72 h in those extenders. This inconsistency was mainly due to the deleterious effect of bulbourethral gland secretions that exist in buck semen (Roy, 1957; Cabrera et al., 2005). While, Leboeuf et al. (2004) reported that irrespective of the extender used, the fertility rate was decreased with the increase in storage time (4–76 h) of chilled semen.

In recent years, different studies have been tried to define the best indicators of caprine semen quality *in vitro*. Different correlations between the characteristics in semen have been established. However, most of the indicators of semen quality have contrasting values in prediction of *in vivo* fertility (Christensen et al., 2005). Therefore, a simple approach is to use the *in vitro* sperm viability and longevity (incubation at 37 °C) and then, an *in vivo* fertility trial could be carried out if the initial results are encouraging. In the present study, we hypothesized that potential toxicity of seminal plasma in buck can be circumvented by milk based extender. Specifically, the aims were to compare the effect of skimmed milk and Tris-citric acid based extenders with or without seminal plasma on plasma membrane and acrosome integreties, motility and motion characteristics, and longevity of cooled Beetal buck spermatozoa at 0, 24, 48 and 72 h of storage.

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2. Materials and methods

Adult bucks (n = 5) of Beetal breed were used in the present study. Bucks were housed in a well facilitated shed, provided with grazing area, at National Agricultural Research Centre, Islamabad, Pakistan (33.42°N). Animals were fed on cotton seed cake (400 g/day/animal), wheat bran (200 g/day/animal) along with grazing on natural grasses (3–5 h/day). Clean drinking water was provided *ad libitum*.

2.1. Experiment I

2.1.1. Semen collection and dilution

Skimmed milk and Tris-citric acid based extenders were prepared as reported earlier (Mehmood et al., 2011) for buck semen. Semen samples (n = 25) were collected from five bucks with caprine artificial vagina (IMV, France) at 45 °C during peak breeding season (September and October, when majority of the females are cyclic). Semen samples were immediately brought to the laboratory and placed in a water bath at 37 °C. Samples were evaluated for volume, visual motility at ×400 by phase contrast microscope (Olympus, Japan) and sperm concentration by a digital photometer at 546 nm (Minitube, Germany). Semen samples with $3-4\times10^9$ sperm/ml and >80% visual motility were equally distributed into 2 parts. One part was split again into two equal aliquots and immediately extended either in skimmed milk with seminal plasma (Treatment 1; T1) or Tris-citric acid with seminal plasma (T2). The other part was diluted with normal saline at the ratio of (1:1 v/v) and subjected to centrifugation at 315 × *g* for 3 min for the removal of seminal plasma. The washed spermatozoa were split again into two equal aliquots and immediately extended either in skimmed milk without seminal plasma (T3) or Tris-citric acid (T4) without seminal plasma. Dilution with either T1 or T2 or T3 or T4 extender was carried out in a single step at 37 °C, to the final sperm concentration of 30×10^6 motile sperm/ml. Extended semen samples were cooled to 4 °C over 2 h for evaluation of sperm membrane integrity with hypo-osmotic swelling test, acrosome integrity with Coomassie Blue staining and motion characteristics with computer assisted semen analyzer (CASA) at 0, 24, 48 and 72 h of storage at 4 °C.

2.1.2. Membrane integrity

To evaluate plasma membrane integrity, hypo-osmotic swelling test was used (Jeyendran et al., 1984). Briefly, 50 μl of semen sample was diluted with 450 μl of 100 mM hypotonic solution composed of 9 g fructose plus 4.9 g sodium citrate/l of distilled water (osmolarity; ~100 mOsm/kg). After 30 min of incubation at 37 °C, a drop was placed on pre-warmed glass slide and cover slipped. Two hundred sperm were evaluated by using bright-field microscopy (×400; Olympus BX40, Japan). Sperm tail curling (%) was considered as sperm with intact plasma membrane.

2.1.3. Acrosome integrity

Acrosome integrity was assessed by Coomassie Blue G-250 staining with modified method as described for goat sperm (Zhao et al., 2009). Briefly, 50 μl of semen was washed twice with saline by centrifugation (200 × *g* for 5 min) and sperm pellet was fixed in 1 ml of 10% paraformaldehyde in Dulbecco's phosphate-buffered saline at room temperature for 30 min. Sperm suspension at the bottom of test tube was separated and washed in 1 ml of Dulbecco's phosphate-buffered saline (200 × g for 5 min). After centrifugation, the pellet was mixed with 500–800 μl of Dulbecco's phosphate-buffered saline and spread on a slide for air-drying. Smear was stained for 5 min with 0.22% Coomassie blue stain (G-250, ICN in 50% ethanol, 10% glacial acetic acid and 40% distilled water) and 200 sperm were examined under bright-field microscopy (×400; Olympus BX40, Japan). Sperm with intact acrosome had darkly stained apical portion of head.

2.1.4. Computer assisted semen analysis (CASA)

Cell Motion Analyzer (CEROS, Hamilton Thorne Biosciences, Beverly, USA) was used to evaluate sperm motion characteristics. The CASA system was programmed to the following settings: frames acquired, 30; frame rate, 60 Hz; minimum contrast, 56; minimum cell size, 5 pixels; minimum static contrast, 30; straightness threshold, 80%; average path velocity cutoff, 20 μm/s; progressive minimum average path velocity, 80 μm/s; curvilinear velocity cutoff, 0 μm/s; cell size, 2 pixels; cell intensity, 50; static head size, 0.71–10.00 and static head intensity, 0.79–1.41. A preheated slide (37 °C) was loaded with 7 μl of semen sample and microscopic fields with best visual motility were selected to have at least count of 200 spermatozoa. The motility parameters were percentage (%) of motile and progressive motile spermatozoa. Motion characteristics included average path velocity (μm/s), straight line velocity (straight line velocity; μm/s), curvilinear velocity (μm/s), amplitude of lateral head displacement (μm) and beat cross frequency (Hz). Description of these parameters of CASA can be found in the previous publication by Mortimer, 2000.

2.2. Experiment II

In experiment II, semen samples (n = 25) of 5 bucks were used to form one of the four treatment groups (T1, T2, T3 and T4) as described in experiment I. Chilled sample of each treatment group was incubated for 3 h at 37 °C to study the longevity of spermatozoa (Muiño et al., 2007) by measuring motion characteristics with CASA at 0, 24, 48 and 72 h of storage time at 4 °C. CASA setting was same as described in experiment I. Longevity was estimated by calculating percent decrease in sub-population of spermatozoa that moved rapidly and progressively as:

$$\%\ \text{decrease} = (\%\ \text{sperm at 0 h incubation} - \%\ \text{sperm at 3 h incubation}) \times 100/\text{Value at 0 h incubation}$$

A "supervised" method was implied i.e., there was a priori grouped dataset to guide the grouping. The sub-population of spermatozoa that moved rapidly and progressively was differentiated by average path velocity, straight line velocity, curvilinear velocity ≥ 120 μm/s, amplitude of lateral head displacement ≤4 (μm) and beat cross frequency ≥10 Hz as defined by clustering methods, and discriminate analysis was done to identify motile sperm subpopulations within the ejaculates as described for Florida goats (Dorado et al., 2010).

2.3. Statistical analysis

In experiment I, 4 (treatment groups) × 4 (storage time) factorial experiment with completely randomized design (CRD) was used to study the effect of treatment and storage time. In experiment II, 4 (treatment groups) × 4 (storage time) factorial experiment with CRD was used to study the longevity of spermatozoa analyzed on the basis of percent decrease in dependent variables (average path velocity, straight line velocity, curvilinear velocity, amplitude of lateral head displacement and beat cross frequency) of rapid and progressive motile sperm. Experiments were replicated five times. Results were presented as mean ± SD. Data were analyzed by using the generalized linear models (analysis of variance) of Minitab 12.22, 1996. Differences were further tested by Tukey's test and significance was set at a $p < 0.05$ level (Minitab 12.22, 1996).