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Viability of carabao (*Bubalus bubalis carabanensis*) epididymal sperm from post mortem testes in semen extender at refrigerated temperature

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ABSTRACT

Post mortem epididymal sperm (ES) is an excellent source of germplasm for conservation of animal genetic resources. In this study, the viability of post mortem ES from carabao after collection at ambient temperature (AT) and after 24 hr and 48 hr of storage at refrigeration temperature (RT) were evaluated. ES were collected through slicing of epididymides from 7 carabaos. The mean ES volume was 0.4 ml with sperm concentration of 2.5×10^9 cells/ml. The mean percentage livability of fresh ES, after 24 hr and 48 hr of storage at RT were 81.93%, 65.93% and 43.7%, respectively. The mean percentage abnormalities of fresh ES, after 24 hr and 48 hr of storage at RT were 44.15%, 38.5% and 43.47%, respectively. The mean percentage motility of fresh ES, after 24 hr and 48 hr of storage at RT were 60.0%, 50.0% and 17.24%, respectively, after analysis through conventional means. Through CASA, the mean percentage motility was observed to be lower significantly at 26.12%, 37.94% and 18.32% for fresh ES, after 24 hr and 48 hr of storage at RT, respectively. The mean progressive motility of fresh ES, after 24 hr and 48 hr storage at RT were 10.02%, 16.47% and 10.87%, respectively. The results suggest that ES potential for use in fertilization studies remained viable when used immediately or after storage at RT for the first 24 hr.

Key words: Epididymal sperm,Post mortem,Viability,Storage

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INTRODUCTION

In the Philippines, carabao (*Bubalus bubalis carabanensis*; 2n=48) constitute 99.6% of the 2.9 million water buffaloes in the country. It belongs to a swamp type water buffalo commonly found in the East and SouthEast Asian countries and used primarily as draft animal and secondly for meat and milk purposes. Farmers preference to carabao is attributed to their docile temperament and endurance for work, the reason why it is considered as farmers inseparable partner in their farming activities. Therefore, preservation of this valuable genetic stock is important in maintaining genetic variability in domestic farm animal breeds. At present, the Philippine Carabao Center operates both an *in situ* and *ex situ* conservation of water buffalo genetic resources. Current approaches is in improving the efficiency of reproductive programs including artificial insemination [19], semen freezing [18], embryo transfer technologies [14, 15, 23, 33], in vitro embryo production [22, 25, 26] and related technologies [2, 13, 25, 28] to help ensure maintenance of genetic diversity. When using female gametes, oocytes are collected either *in vivo*, through TUFA [1, 3, 5, 10] or *in vitro,* by aspiration of antral follicles from the ovaries of

slaughtered buffalo cows [7, 8, 24, 27]. When using male gametes, the semen collection is usually done through the use of an artificial vagina, digital manipulation or electro ejaculation. Oftentimes, an extensive training of males is required to get high quality semen. Another approach is the use of ES as an alternative source of genetic material for preservation. Collection of ES from slaughtered male is simple, rapid and cheap. In case of dead or dying male of high genetic importance, of economic interest, from wildlife and/or endangered species, ES is an excellent source of germplasm for conservation of animal genetic resources. In this study, we used carabao as a model for ES recovery and preservation to demonstrate its suitability for genetic preservation and possible use in Tamaraw (*Bubalus mindorensis*), an important close relative of carabao that is endemic only in the Philippines and is in the brink of extinction.

MATERIALS AND METHODS

Medium preparation

The chemicals used were of reagent grade and were purchased from Sigma-Aldrich (USA) except for Tris-base (Promeg Corp., Madison, WI, USA). Tris-citric acid-lactose-raffinose buffer (TLB medium) was prepared a day before collection of cauda epididymides. A one liter preparation composed of 15.7 g Tris-hydroxymethyl amino methane, 8.8 g citric acid monohydrate, 14.1 g lactose, 25.4 g raffinose and gentamycin solution (50 µg/ml) using an ultra-pure water (Milli-Q, Integral 5). Before use, the medium was sterilized by filtration using a 0.2 µm syringe filter. For the extender, TLB medium was supplemented with 20% egg yolk and 7% glycerol (v/v).

Preparation of epididymides and sperm recovery

Cauda epididymides were aseptically excised from the testicles of carabaos (n=7) sent for slaughter in local abattoir, washed aseptically and incised longitudinally using a sterile scalpel blade and placed in a 50 ml conical tube with 25 ml TLB medium and transported to the laboratory at AT (22-24°C) within 4-5 hr. This allows the ES to swim-up. Immediately upon arrival at the laboratory, the upper 2/3 of the medium were collected, transferred to a 15 ml conical tube, centrifuged at 4,000 rpm for 5 min to form a pellet and the volume assessed using a graduated sterile tube. Thereafter, the sperm pellet were diluted with the extender (1:1, v/v) and stored in RT for up to 48 hr. The acidity of the ES was examined using Brom Thymol Blue pH paper.

Sperm motility

Sperm motility evaluation was done through conventional means using an inverted microscope (Nikon Eclipse Tx10i) at 40-100x magnification and by using a computer assisted sperm analyzer (CASA; HTM-IVOS-Ultimate, Hamilton Thorne BioSciences, Beverly MA, USA). Briefly, a sample of semen was diluted with TLB medium and about 10-20 µl was pipetted into a clean pre-warmed (37°C) microscope slide. A coverslip was carefully lowered into the sample, avoiding formation of air bubbles before examination. Visual motility was recorded using the imaging software (NIS elements) for at least ten widely-spaced fields to provide an estimate of percentage motility using the scoring system (Table 1). Through CASA, each sample were diluted (25×10^6 cells/ml) in TLB medium and kept at 38°C water bath pending analysis. Then pre-warmed (38°C) chamber slide (SC20.01FA; Leja®, Nieuw-Vennep, The Netherlands) was loaded with 10 µl sample, allowed to settle for 1 min on MiniTherm© stage warmer before analysis. At least 5 fields per sample were selected and observed for motility or progressive motility and expressed in percentage.

Sperm concentration

The sperm concentration was determined using a haemocytometer. Briefly, the sperm sample was diluted at 1:200 (5 µl sperm sample + 995 µl saline solution) in an RBC pipette and the sperm counted in the central large area of the Neumbauer haemocytometer which consists of 25 squares and each square consists of 16 smaller squares. The dimensions of the large central area of the Neumbauer counting chamber are 1 mm (width) x 1mm (height) x 0.1 mm (depth) for a volume of 0.1 cubic millimeter (mm^3) or 0.1 µl. Since the sperm concentration is expressed in number per cubic centimeter (cm^3), the sperm count must be multiplied by a factor of 10,000. The sperm (n) was counted in five (5) squares. The sperm concentration was computed using the following formula:

Sperm concentration per ml = n x dilution factor x 50,000

= n x 200 x 50,000

= n x 10,000,000

= n x 10^7

Table 1: Scoring system for the motility of sperm cells [19].

Motility (%)	Grade	Characteristics
91-100	Excellent Motility	90% or more of the spermatozoa is very rigorous in motion. Swirls caused by the movement of the sperm are extremely rapid and constantly going forward progressively.
76-90	Very Good Motility	Approximately 75-90% of the spermatozoa is in vigorous rapid motion. Waves and eddies form rapidly but not as rapid as in excellent motility.
60-75	Good Motility	About 60-75% of the spermatozoa is in motion. Motion is vigorous but waves and eddies formed move slowly across the field of vision
40-59	Fair Motility	From 40-55% of the sperm is in motion. The movements are largely vigorous or eddies are formed.
< 40	Poor Motility	Less than 40% of the sperm is in motion. The motion is not progressive but mostly weak and oscillary.
0	Zero Motility	No motility is discernible.

Morphological assessment

The ES viability (percentage live and dead sperm) and morphology (percentage with normal shape) were evaluated using a 1:2 dilution of semen sample and eosin-nigrosin stain. Briefly, 5 µl ES sample was dropped in a clean glass slide and added with 10 µl eosin-nigrosin stain before mixing gently using the tip of the pipette to minimized secondary abnormalities. After mixing, both edge of another glass slides was dipped into the mixture and smeared throughout another glass slide, forming a feather like smear (thin smear) and air-dried for 15-30 min. Nikon imaging software was used in examining the percentage viability and morphology of ES.

The nigrosin stain created a dark background for the stained samples under the microscope whereas, the eosin stain penetrated the head of dead sperm due to the degradation of their cell membrane resulting to either pink or dark violet coloration. Live sperm appeared colorless or translucent. Percent live and dead sperm were determined from 10 separate fields under a magnification of 40x. The following equation was used in the percentage estimation:

$$\% \, Live = \frac{\begin{matrix} total\ number\ of\ live \\ sperm\ \ in\ the\ field \end{matrix}}{\begin{matrix} total\ number\ of\ counted \\ sperm\ \ in\ the\ field \end{matrix}} x100$$

$$\% \, Dead = \frac{\begin{matrix} total\ number\ of\ dead \\ sperm\ \ in\ the\ field \end{matrix}}{\begin{matrix} total\ number\ of\ counted \\ sperm\ \ in\ the\ field \end{matrix}} x100$$

The ES morphology was evaluated using CASA to avoid subjectivity. The percentage abnormal sperm was based on sperm head abnormalities including those with small, tapered, pyriform, round or amorphous head and on sperm with tail abnormalities including those with coiled tail, bent tail, the presence of proximal and distal cytoplasmic droplets. The percentage normal and abnormal sperm was determined by using the following equation:

$$\% \, Normal = \frac{\begin{matrix} total\ number\ of\ normal \\ sperm\ \ in\ the\ field \end{matrix}}{\begin{matrix} total\ number\ of\ counted \\ sperm\ \ in\ the\ field \end{matrix}} x100$$

$$\% \, Abnormal = \frac{\begin{matrix} total\ number\ of\ abnormal \\ sperm\ \ in\ the\ field \end{matrix}}{\begin{matrix} total\ number\ of\ counted \\ sperm\ \ in\ the\ field \end{matrix}} x100$$

Statistical analysis

One-way ANOVA was used to compare the mean percentage motility and livability of fresh ES and after storage to RT for 24 hr and 48 hr. A difference of $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

ES viability basically refers to the percentage "alive" sperm which are healthy and no defects of sort that might render it incapable of fertilizing the eggs. In order to get a more accurate picture of assessing ES fertility, the percentage sperm viability is evaluated alongside the percentage of motile sperm and its concentration. In this study, the mean volume of ES recovered was 0.4 ml with a mean sperm concentration of 2.5×10^9 cells/ml. In other ruminants, ES concentration of $4.8\pm98.1\times10^8$ - $3.6\pm102.0\times10^9$ in ovine [16, 29], 10.7×10^9 in bubaline [35], 1.2-9.6×10^9 in bovine [4, 30] and 0.5-1.2×10^9 cells/ml [31] to 17.5×10^9 cells/ml [9] reserved in goat have been reported. Such differences could be attributed to a number of factors that directly/indirectly influences the ES quality and quantity including the breed, age, size

and weight of the testes/epididymides, method of ES isolation/recovery and the collection time. For example, other researchers preferred the flushing method than the slicing method because it produced less contamination and higher quality semen [20], besides being an acceptable method in the field when collecting ES [11]. There are different methods in recovering epididymal sperm and each has its advantages and disadvantages. Therefore, determining which method to use is just another concern when preparing ES for a certain purpose.

The mean percentage live sperm of fresh ES, after 24 hr and 48 hr at RT were 81.93%, 65.93% and 43.7%, respectively. The mean percentage of fresh ES, after 24 hr and 48 hr at RT with coiled and bent tail were 3.93%, 5.21%, 17.57% and 14.07%, 13.79%, 15.68%, respectively. Similarly, the mean percentage of fresh ES, after 24 hr and 48 hr at RT with proximal and distal droplet were 25.36%, 18.86%, 19,02% and 0.79%, 0.64%, 1.2%, respectively (Table 2). In goat, the viability of ES, both fresh (94.1±2.53) and cold-stored (79.6±2.75) for up to 72 hr[31] was higher to our observation. In other ruminants, ram ES collected at various times and stored at 4°C had ≥70% [17] viability index while spotted buffalo ES had 85.02±2.4% [34, 36].

The estimated mean percentage motility of fresh ES through conventional approach was 60.0%. After storage in the RT for 24 hr and 48 hr, the mean percentage motility observed were 50.0% and 17.2%, respectively. Through CASA, the mean percentage motility of fresh ES, after 24 hr and 48 hr of storage at RT were 26.1%, 37.9% and 18.3%, respectively. The mean progressive motility of fresh ES, after 24 hr and 48 hr of storage at RT were 10.0%, 16.5% and 10.9%, respectively (Fig. 1).In spotted buffaloes found in Indonesia, the motility percentage of fresh ES was reported at 65.0% [35].

Table 2: Mean quality of fresh and RT stored ES.

Parameters	Fresh	24 hr	48 hr
Volume (ml)	0.4		
Concentration (ml)	2.5×10^9		
pH	6.8	6.8	6.4
Live (%)	81.93	65.93	43.7
Abnormalities (%)	44.15	38.5	43.47
Coiled tail	3.93	5.21	17.57
Bent tail	14.07	13.79	15.68
Proximal droplet	25.36	18.86	19.02
Distal droplet	0.79	0.64	1.2

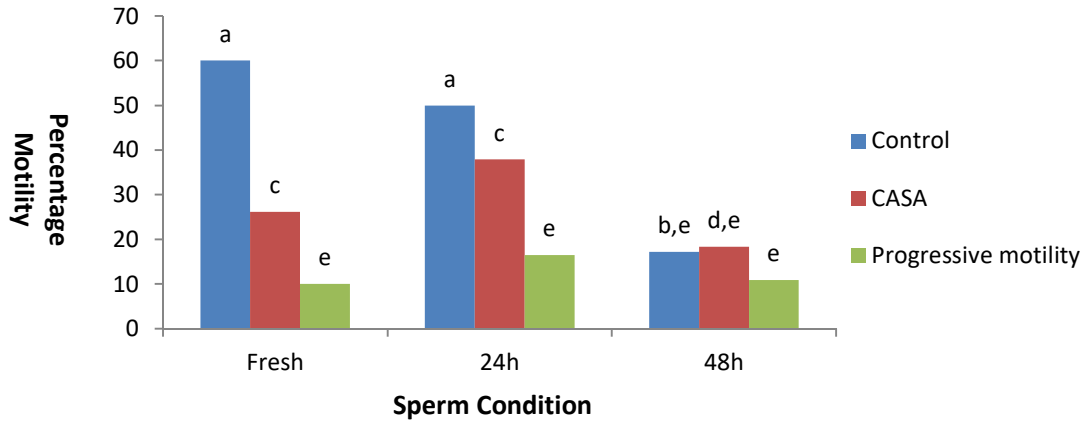

Fig 1. Sperm motility of ES throughout the experimentation from 7 replicates. Within each endpoint, bars with different letters (a,b,c, d,e) are significantly different (P<0.05).

In another study, the progressive motility percentage of buffaloes with different spotted types were reported to be between 65.0±0.00 to 73.0±2.5 [34, 36]. These values taken subjectively using phase contrast microscope in 10 fields of view were significantly higher than our observation using CASA at 10.0%. The wide discrepancies in the results could be attributed to a number of factors, including the expertise of the person doing the analysis, the processing method used in the recovery of ES and bull differences. In other ruminants, fresh ES motility were reported between 70-80% [21] in bull, ≥ 80% [17, 29, 32] in ram, 70-80% [12] in sika deer and ≥ 80% in goat [31] and springbok, impala, blesbok [6].

CONCLUSION

The results of the study showed that the quality of carabao ES declines when stored at RT for up to 48 hr. However, the ES potential for use in fertilization studies remained viable in the first 24 hr after animals death.

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Authors' contributions
LBS, MSS and ACB designed the experiments and performed the experimental works. MBO, LCO contributed ideas, gave critical feedback during data analysis and manuscript write-ups. All authors read and approved the final manuscript.

Conflict of interest
The authors declare that they have no competing interest.

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