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Assessment of Post Mortem Epididymal Sperm from Non-Descript Bucklings *(Capra Hircus)*

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ABSTRACT

Recent progress in gamete preservation have utilized epididymal sperm for animal genetic resource cryobanking of traditional livestock breeds and endangered animals not trained for semen collection. Therefore, appropriate analysis to ensure its quality is paramount. In this study, post mortem epididymal sperm of bucklings (n=8) were evaluated to determine its possible use in related assisted reproductive techniques. The epididymides were sliced longitudinally and the sperm collected through swim-up method. Significant variations on semen quality and quantity among individual buckling were observed. The mean volume recovered was0.65 ml with 6.3 pH. The sperm motility ranged from 50-75% with a mean concentration of 1.92×109 cells/ml. The percentage viability was ≥80% with percentage normality range of 48.95-82.2%. The common head and tail abnormalities were the small, pyriform type and cytoplasmic droplets in the proximal region. The results showed that the epididymides of buckling contain a significant population of normal sperm that could be used for fertilization studies both in vivo or in vitro.

Keywords: Bucklings, Post-Mortem, Epididymal Sperm, Semen Analysis

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INTRODUCTION

In the Philippines, the immediate concerns for the conservation of animal genetic resources (AnGR) are food security, economic development and preservation of breeds (both indigenous, introduced and endangered). During the preparations of strategies and action plans for AnGR, several learning institutions (eg., colleges/universities) and/or agencies (eg., government, non-government organizations) were tapped in spearheading the program. The Philippine Carabao Center is one and it operates on the utilization of some genetic resources through cryobanking of semen, oocytes and embryos. The samples being considered were subjected to examination and evaluation protocols to ensure their safety and usefulness. Efforts are primarily directed on improving their quality and survival post thawing/warming to produce live birth. So far, instances of the animals getting sick, having unexpected serious injuries resulting to death and/or retired due to old age are the only reasons that served as the constraint. In these cases, the only alternative approach in sourcing their genetic material for storage and use is to utilize the epididymides and/or ovaries for epididymal sperm (ES) and oocyte collection, respectively. In particular, the ES obtained post mortem have been reported alive and remained viable for fertilization [1], though is influenced by the recovery method of choice [2, 3]. So far, most of the studies conducted on ES of mammals were taken from matured males considering their sexual maturity and the belief that it has a greater proportion of matured spermatozoa [1, 2, 4-9]. Often, the testicles of younger animals are disregarded despite of cauda epididymis containing immature, maturing and matured spermatozoa. By using the testicles of non-descript buckling as a model, this study assessed the semen characteristics of epididymal sperm to determine its potential for use in fertilization studies.

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.

Preparation of epididymides

Cauda epididymides were aseptically excised from the testicles of non-descript goats (n=8; 8 mo-1 yr old) sent for slaughter in local abbatoir, washed aseptically using TLB medium and transported to the laboratory at ambient temperature. Subsequently, the epididymides were longitudinally sliced and submerged in 50 ml conical tubes (1 piece/tube) with 20 ml TLB medium maintained at 37°C water bath for 15 min, allowing the epididymal sperm (ES) to swim-up. This was followed by recovering the upper 2/3 of the medium (about 15 ml) before transferring to a 15 ml conical tube, centrifuged at 15, 000 rpm for 5 min to form a pellet. The supernatant was then recovered and the volume of the pellet measured against the graduation lines of the conical tube. The acidity of the ES was examined using Bromo Thymol Blue pH paper.

Motility evaluation

Microscopic evaluation of ES was undertaken using the inverted microscope (Nikon Eclipse Tx10i) at 40-100x magnification. 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) [10].

Table 1: Scoring system for the motility of sperm cells

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 and rapidly but not so 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 discernable.

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 1mm (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 × dilution factor × 50,000

= n × 200 × 50,000

= n × 10,000,000

= $n \times 10^7$

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:

$$\%\ Live = \frac{total\ number\ of\ live\ sperm\ in\ the\ field}{total\ number\ of\ counted\ sperm\ in\ the\ field} \times 100$$

$$\%\ Dead = \frac{total\ number\ of\ dead\ sperm\ in\ the\ field}{total\ number\ of\ counted\ sperm\ in\ the\ field} \times 100$$

The ES morphology was evaluated using a computer assisted sperm analyser (CASA; HTMIVOS- Ultimate, Hamilton Thorne BioSciences, Beverly, MA, USA) 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:

$$\%\ Normal = \frac{total\ number\ of\ normal\ sperm\ in\ the\ field}{total\ number\ of\ counted\ sperm\ in\ the\ field} \times 100$$

$$\%\ Abnormal = \frac{total\ number\ of\ abnormal\ sperm\ in\ the\ field}{total\ number\ of\ counted\ sperm\ in\ the\ field} \times 100$$

Statistical analysis

Data from individual buckling on sperm parameters considered were expressed in percentage and analyzed by one-way ANOVA. Differences of $P<0.05$ were considered significant.

RESULTS AND DISCUSSION

Table 2 shows the values of some parameters evaluated in the ES of non-descript bucklings (8 pairs) processed in this study. The quantity and quality of isolated ES using the swim-up method showed significant individual variation. Buckling No. 1 and 2 had the lowest sperm volume recovered at 0.5 ml with the rest having between 0.7-0.8 ml (mean of 0.65 ml). This value was significantly lower than the collected volume of ES in 50 Alpine bucks at 1.5-2.8 ml [7] but was similar with that of Red Sokoto goats which are indigenous in northern Nigeria [11] at 0.83 ml and Cashmere bucks at 0.7 ml [12]. In spotted buffalo found in Indonesia, the ES volume reported was 0.45 ml [13].

Table 2: Some ES parameters from non-descript bucklings.

Buckling(No.)	Volume (ml)	pH	Sperm concentration($\times 10^7$)	Sperm Motility (%)
1	0.5	6.4	52^a	50^a
2	0.8	6.6	46^a	75^c
3	0.7	6.4	373^c	70b,c
4	0.5	6.4	462^c	60a,b
5	0.8	6.2	175^b	50^a
6	0.7	6.2	69^a	50^a
7	0.7	6.2	196^b	50^a
8	0.7	6.2	163^b	50^a
Mean	0.65	6.3	192	56.8

$^{a, b, c}$Values differ significantly ($P>0.05$).

In this study, the mean pH of ES obtained was 6.3. The normal semen pH was reported to be between 7.2-7.8 in mammals [14] which is most favorable for sperm cell motility [15, 16]. The reasons for obtaining an acidic pH from bucklings used was unknown. Nonetheless, a pH of 6.4 was observed in South African indigenous goats when using an electro-ejaculator [17] which could have stimulated the buck to release acidic urine, thus contaminating the semen [18, 19]. The percentage ES motility of buckling No. 2, 3 and 4 were higher (60-75%) than the other bucklings (50%). This differences could be attributed to the time lapsed of ES collection from slaughter to recovery in the laboratory. ES collected at less than 1 hr post mortem had a higher percentage motility than those collected at 5 hr post mortem. Apparently, keeping the testis and epididymides inside the scrotum should be enough to protect the ES from desiccation, but since the cauda epididymides used in this study were immediately excised and placed in the transport medium at ambient temperature, the ES motility has been compromised. This suggest that temperature effect is related to changes in ES metabolic activity. In other studies, better ES percent motility were obtained when epididymides were maintained at refrigeration temperature of 4-5°C [20-23] during transport and storage to the laboratory of up to 72 hr before collection. Determination of sperm concentration is not really a component of semen quality evaluation, rather it is used as a tool in monitoring the health and reproductive status of a particular buck for optimizing its genetic potential. In this study, significant variations on the ES

concentration of individual bucklings used were observed. For instance, buckling No. 4 with sperm volume of 0.5 ml has the highest sperm concentration of 462×107 cells/ml whereas, buckling No. 2 with 0.8 ml sperm volume has the lowest sperm concentration of 46×107cells/ml. Overall, the mean ES concentration obtained from 8 buckling was 192×107 cells/ml. Others have reported a 0.5-1.2×109 cells/ml [7] to 17.5×109 cells/ml [24] reserved in goat epididymides. In some ruminants, ES concentration of 4.8±98.1×108 - 3.6±102.0×109 in ovine [21, 25], 10.7×109 in bubaline [26] and 1.2-9.6×109 in bovine [3, 4] 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 [27], besides being an acceptable method in the field when collecting ES [28].

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 percentage viability of ES from 8 buckling showed no difference (73.8-85.2%) though the percentage of morphologically normal ES from buckling No. 4 (48.9%) was significantly lower compared to others (63.3-82.2%), despite having the highest number of sperm/ml concentration and acceptable percentage motility of 60 % (Table 3). In contrast, buckling No. 5 and 8 with 50% ES motility had the highest percentage normality at ≥80%. The percentage normality could be an effective tool in the evaluation of viability and provide information on the nature of a given ES in relation to its quality than relying just on the percentage motility. Therefore, the least ES samples if we are to make a recommendation for use either for in vitro or in vivo fertilization studies are those from bucklings No. 1, 3, 4 and 6 because of higher percentage abnormalities. Among the common head abnormalities observed were the small and pyriform type while the tail abnormalities were the coiled (1-5%) and bent tail (2-13%) type. ES samples from all buckling with cytoplasmic droplets in the proximal region was ≥60%. The presence of cytoplasmic droplets was reported to be not deleterious to sperm motility [29] although may be predictive of some forms of male infertility [30, 31]. In another study, the viability of ES, both fresh (94.1±2.53) and cold-stored (79.6±2.75) for up to 72 hr from matured bucks [23] was comparable to our observation. In other ruminants, ram ES collected at various times and stored at 4°C had ≥70% [32] viability index while spotted buffalo ES had 85.02±2.4% [8, 9].

Table 3: Percent viability and normality of ES from non-descript bucklings

Buckling (No.)	Live (%)	Dead (%)	Normal (%)	Abnormal (%)
1	82.78	17.22	63.30^{b}	36.69
2	83.11	16.89	68.14^{b,c}	31.85
3	73.77	26.23	64.67^{b}	35.32
4	80.08	19.92	48.95^{a}	51.04
5	85.18	14.82	82.20^{c}	17.79
6	81.77	18.23	59.90^{b}	40.09
7	83.29	16.71	70.10^{b,c}	29.89
8	79.24	20.76	80.58^{c}	19.41

$^{a, b, c}$ Values differ significantly (P<0.05).

CONCLUSION

The epididymides from bucklings consist of a significant population of normal sperm that could be used for fertilization studies both *in vivo* or *in vitro*.

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