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## Post Mortem Viability of Epididymal Sperm from Philippine Native Water Buffalo (*Bubalus bubalis*)

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Post-mortem spermatozoa recovery is an important method to obtain germplasm reserves from genetically valuable animals or endangered species. The objective of the study was to determine the viability of water buffalo epididymal spermatozoa from excised testicles stored at two temperature conditions either at 5 to 8°C or at 18 to 22°C which is maintained for 10 to 12 hours and 15 to 17 hours. Six pairs of water buffalo paired testicles were collected after slaughter and transported to the laboratory. The testicles were placed in a styropore box previously calibrated with the target storage temperature. Epididymal sperm from one testicle was recovered after 10 to 12 hours while the other testicle at 15 to 17 hours post-mortem. Epididymal spermatozoa recovery was by slicing and swim up method in Tris-citric acid-egg yolk extender with fructose and raffinose. Sperm viability was assessed by determining the percentage sperm motility using conventional method. Total motility and progressive motility were determined using an automated sperm analyzer CASA IVOS II (Hamilton Thorne). Percentage live sperm was determined after eosin nigrosin staining of smeared sperm slide preparations. Morphological observation for the presence of abnormal spermatozoa and cytoplasmic droplets was similarly performed. Based on our findings, two way ANOVA revealed that the main effect of storage temperature and its interaction with storage time did not affect the viability of epididymal spermatozoa. However, a significant decline on the quality of spermatozoa was notable in the prolonged post mortem storage duration ( $p \leq 0.05$ ). On the other hand, acceptable percentage of abnormal spermatozoa of less than 20% was obtained and was not influenced by storage temperature and storage time ( $p \geq 0.05$ ). Therefore, viable water buffalo epididymal sperm can be recovered after 10-12 hours of animal death from testicles maintained at storage temperature of either 18-22°C and 5-8°C.

**Keywords:** water buffalo, post mortem epididymal sperm, motility, liveability

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## **Introduction**

Native water buffaloes in the Philippines have a long history of use to farming villages. They are very important draught animals in rice fields and can be a source of milk and meat. Their resilience, adaptability to local environmental conditions and perceived innate traits related to disease and heat tolerance make them likely candidates for genetic diversity conservation. However, these animals usually end up in local slaughterhouses to supply the high demand for meat.

Previous research on the utilization of post-mortem buffalo ovaries for the production of embryos by the IVF technology with resultant live births have been demonstrated by several authors (Ocampo et al.,1996 and Duran et al.,2004). Therefore, slaughterhouse derived post mortem testicles can be potential sources of gametes for sperm preservation of these animals. However, this is not always possible especially when the slaughterhouse is too distant to the laboratory or when wild species die unexpectedly.

In this study, we therefore determined the effect of two holding temperature conditions (5-8°C and 18-22°C ) and storage time of 10-12 and 15-17 hours post mortem (hpm) on the quality of sperm recovered from the cauda epididymis of water buffaloes.

## **Materials and Methods**

### ***Testes collection and transport***

Paired testicles with intact scrotal sac were collected from matured native carabaos immediately after the animal was slaughtered.

Collected samples were grouped into two, namely Group 1 testicles, to be or stored at chilled temperature of 18 to 22° C and Group 2 stored at 5 to 8° C. To maintain the holding temperature during transport, the testes were placed on a styropore box previously calibrated with the target storage temperature. The temperature inside the ice chest was monitored using a thermometer .

Epididymal sperm was recovered from one testicle after 10 to 12 hours of storage and the other one 15 to 17 hours later .

***Preparation of Tris-Egg Yolk, Glycerol (TEY) with Fructose and Raffinose extender***

A buffer solution composed of 250 mM of Tris hydroxymethyl amino methane, 80 mM of citric acid monohydrate, 69 mM of fructose, 54 mM of raffinose with gentamycin (25µg/mL) was prepared a day before testicle collection. The Tris Egg yolk (TEY) extender was prepared by adding egg yolk (20% v/v) to the buffer solution on the day of collection.

***Epididymal sperm collection and dilution***

The cauda epididymis was sliced into half in a petri dish and was flushed with the TEY solution. Then the flushing medium was pipetted out and transferred in a conical test tube to be centrifuged at 10,000 rpm for 5 minutes to form a pellet. The volume of the pellet was determined and recorded. Shortly thereafter, the sperm pellet was diluted with TEY extender in 1:1 proportion using a calibrated micropipette.

***Sperm Motility by Conventional method***

The motility of the semen was evaluated using an inverted microscope (Nikon Eclipse Tx10i) following procedures of the wet mount technique. A micro droplet (10 to 20µl) of initially diluted epididymal sperm was placed in a cleaned pre-warmed (38° C) microscope slide and covered with a clean cover slip. Subjective method of evaluation for sperm motility in at least five microscopic fields was performed and was recorded using the imaging software (NIS elements). The percentage motility was based on the movement of sperm from the scoring system adapted from the protocol of Mamuad et al.,(2005) in Table 1.

**Table 1.** Scoring system for the motility of sperm cells (Mamuad et al.,2005).

<b>MOTILITY (%)</b>	<b>GRADE</b>	<b>CHARACTERISTICS</b>
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.
Less than 40	Poor Motility	Less than 40% of the sperm is in motion. The motion is not progressive but mostly weak and oscillatory.
0	Zero Motility	No motility is discernable.

***Assessment of sperm motility using Computer Assisted Sperm Analyzer (CASA)***

Sperm motility was evaluated using a CASA system (HTM-IVOS-Ultimate; Hamilton Thorne Biosciences, Beverly, MA, USA). The total motility (CASA MOT) and progressive motility (CASA PMOT) were recorded for every sample analyzed. Prior to analysis, all samples were diluted to  $25 \times 10^6$  sperms/ml in phosphate buffered saline or PBS. Diluted sperm were kept at  $38^\circ \text{C}$  prior to analysis. Five microliters ( $5 \mu\text{l}$ ) sample was drawn from PBS-extended ( $25 \times 10^6$  sperm cells/ml) pooled epididymal sperm. The sample were

mounted on a pre-warmed (38° C) Leja© (SC20.01.FA; Leja®, Nieuw-Vennep, The Netherlands) disposable chamber and allowed to settle for 1 min on the MiniTherm© stage warmer. Seven fields were selected for analysis of total motility (MOT %) and progressive (PMOT%) motilities.

### ***Sperm concentration***

The sperm concentration was determined using a hemocytometer. A sample of epididymal sperm was sucked up to the 0.5 mark of the RBC dilution pipette. Then, the diluting fluid which consisted of 3% NaCl with tinge of eosin stain was sucked up to the 101 mark avoiding air bubble formation. The diluted sample was shaken in a figure of eight motion to mix the sperm with the diluting fluid. Shortly thereafter, about 4 to 5 drops of the diluted sperm was discarded. Then the hemocytometer was loaded with 10 µl of the sperm suspension in each of the upper and lower grid chambers. The spermatozoa were allowed to settle for 5 to 6 minutes before placing the hemocytometer on the stage of the microscope. The number of spermatozoa in five large squares was counted with the head of the spermatozoa considered as the reference in counting. As some spermatozoa transcended the lines at the edge of the squares, only spermatozoa on the top right lines were included in the count. The spermatozoa in any five of the 25 squares were counted and multiplied by the dilution factor and by 10,000 to yield a sperm count of  $n \times 10^7$  sperm cells/ml.

### ***Evaluation of Live Sperms***

Percentage of live and dead spermatozoa was determined after eosin nigrosin staining of semen samples. Live sperm appear white while dead sperm took the color of eosin and appeared pink or purple. A total of five hundred spermatozoa were observed on different microscopic fields. The percentage of live sperms was calculated using the formula used by Mamuad et al.,(2004):

$$\% \text{ Live Sperm} = \frac{\text{Number of live sperm counted}}{\text{Total number of sperm observed}} \times 100$$

### ***Sperm Morphology***

Morphological assessment of the eosin nigrosin stained sperm samples was performed by examining the shape of the head, neck or midpiece and the tail. At least three hundred spermatozoa were observed on different microscopic fields and the percentage of normal and abnormal sperms were calculated using the formula used by Mamuadet al., (2004):

$$\% \text{ Abnormal Sperm} = \frac{\text{Number of abnormal sperm counted}}{\text{Total number of sperm observed}} \times 100$$

### ***Statistical Analysis***

The collected data were subjected to different statistical models. Factorial analysis was laid out to determine the effect of several factors (storage temperature and storage time) on epididymal sperm quality. T-test was used to compare the main effects of storage temperature and post-mortem time on epididymal sperm quality with statistical level of significance set at 5% ( $p < 0.05$ ).

### **Results**

There was a decline in the volume of sperm recovered after a prolonged storage of the testicles in both temperature conditions. The average volume of sperm obtained was 0.75mL at 10-12hours post mortem (hpm) and 0.58 ml at 15-17 hpm for testicles stored at 5-8 degrees Celsius. Testicles stored at a temperature of 18-22 degrees Celsius registered an average sperm volume of 0.5 ml at 10-12 hpm and 0.4ml at 15-17 hpm.

In terms of sperm concentration, the average sperm concentration was found to be greater ( $339.3 \times 10^7$ ) for testicles stored within 10-12 hours at 18-22 degrees Celsius followed by testicles stored at a temperature of 5-8 degrees Celsius with  $326.6 \times 10^7$  sperm per mL. There was a decline in the sperm concentration in either temperature conditions after 15-17 hours post mortem as depicted in Table 2.

**Table 2.** Effect of storage temperature and storage time on the volume and sperm concentration of recovered epididymal sperm

PARAMETERS	STORAGE TEMPERATURE			
	5 – 8°C		18 – 22°C	
	STORAGE TIME			
	10-12hours post-mortem	15-17hours post-mortem	10-12hours post-mortem	15-17hours post-mortem
Volume (ml)	0.75	0.58	0.5	0.4
Sperm Concentration (n x10 <sup>7</sup> sperm cells/ml)	326.6	303.3	339.3	222.2

**Table 3.** Percentage sperm motility by conventional method and total sperm motility (MOT) and progressive motility (PMOT) assessment by computer assisted sperm analyzer (CASA)

PARAMETERS	STORAGE TEMPERATURE			
	5 – 8°C		18 – 22°C	
	STORAGE TIME			
	10-12hours post-mortem	15-17hours post-mortem	10-12hours post-mortem	15-17hours post-mortem
Percentage Motility (Conventional Method)	63.3 ± 2.1 <sup>a</sup>	46.7 ± 3.3 <sup>b</sup>	60 ± 0.0 <sup>a</sup>	46.7 ± 2.1 <sup>b</sup>
CASA Total Motility	64.8 ± 4.9 <sup>a</sup>	38 ± 6.8 <sup>b</sup>	75 ± 1.5 <sup>a</sup>	33 ± 3.2 <sup>b</sup>
CASA Progressive Motility	25 ± 6 <sup>a</sup>	16 ± 5.04 <sup>c</sup>	39.3 ± 4.0 <sup>b</sup>	11.5 ± 3 <sup>c</sup>

Mean + s.e.m. values having the same letter superscripts on each row are not significantly different from each other at 5% level of significance

The table 3 depicts the sperm motility percentages assessed by conventional method and the CASA total motility (MOT) and Progressive motility scores (PMOT). Conventional method of sperm motility evaluation revealed that at 10-12 hours post mortem, epididymal sperm displayed good motility (60-65%) from testicles stored in both temperature storage conditions. However, the percentage motility after 15-17 hpm was reduced and did not differ ( $p \geq 0.05$ ).

In terms of the more objective approach of CASA sperm analysis, the total motility of after 10-12 h from testicles stored at 18-22°C was greater (75 ± 1.5) compared with those recovered from samples maintained at 5-8 °C (64.8

$\pm 4.9$ ) but did not statistically differ ( $p \geq 0.05$ ). However, as the duration of post mortem time was prolonged, the total motility declined considerably at 15-17hpm with a percentage total motility of  $33 \pm 3.2$  at 18-22 °C and  $38 \pm 6.8$  at 5-8°C.

Factorial analysis confirmed that storage temperature has no significant effect on percent motility (conventional method) and total motility (CASA) ( $p \geq 0.05$ ) while post mortem time displayed a significant effect. ( $p \leq 0.05$ ). Percentage motility and total motility was not influenced by the interaction between the storage temperature and post mortem time ( $p \geq 0.05$ ).

Progressive motility (CASA PMOT) scores after 10 to 12 hours post mortem time were significantly ( $p \leq 0.05$ ) higher from the testes stored at 18 to 22° C (39.3%) compared with those stored at 5 to 8° C (25%). Factorial analysis confirmed that storage temperature and post mortem time has a significant effect on progressive motility. However, there was a remarkable reduction in progressive over time (15-17 hpm) in both storage temperature conditions that did not statistically differ ( $p \geq 0.05$ ).

The percentage live and abnormal sperm after eosin-nigrosin staining is presented on table 4. There is no significant difference ( $p \geq 0.05$ ) between the percentage live sperm of epididymal spermatozoa recovered from testes stored at 5 to 8° C and 18 to 22° C after 10 to 12 hours post mortem time (62.9% vs. 57.5%). Similarly, there was no significant difference found on the percentage live sperm (58.2% Vs. 54.5%) between 5 to 8° C and 18 to 22° C after 15 to 17 hours post mortem time ( $p \geq 0.05$ ). Factorial analysis confirmed that percentage live sperm was not affected by either storage temperature or post mortem time ( $p \geq 0.05$ ). Lower percentage sperm abnormalities obtained in both storage temperature conditions and post mortem period was not influenced by storage temperature and storage time ( $p \geq 0.05$ ).

**Table 4.** Mean percent live sperm and total abnormal spermatozoa of recovered epididymal sperm from cauda epididymis

PARAMETERS	STORAGE TEMPERATURE			
	5 - 8° C		18 - 22° C	
	STORAGE TIME			
	10-12hours post-mortem	15-17hours post-mortem	10-12hours post-mortem	15-17hours post-mortem
Live Sperm (%)	$62.9 \pm 2.6^a$	$58.2 \pm 1.4^a$	$57.5 \pm 1.5^a$	$54.5 \pm 1.5^a$
Total Abnormal Spermatozoa (%)	$4.4 \pm 1.87^a$	$10.7 \pm 1.87^a$	$8.16 \pm 1.87^a$	$6.3 \pm 1.87^a$

Means + s.e.m. having the same letter superscripts on each row are not significantly different from each other at 5% level of significance.



## Discussion

There was a variation in the volume of sperm pellet obtained on the study which is a reflection of the individuality of the slaughtered male buffalo animals. The volume of recovered sperm pellet from cauda epididymis is relatively low compared with the volume of ejaculated semen because theoretically, ejaculated semen contains seminal plasma (Garner and Hafez, 2000). The sperm concentration obtained was not associated with storage temperature or post mortem time but was rather influenced by the size of testes and epididymis of the donor animal. Nonetheless, the volume of sperm recovered was acceptably sufficient considering the amount of time that had lapsed after the animal was slaughtered and the transport duration from the slaughterhouse to the laboratory.

Several authors reported that motile epididymal sperm from buffalo can be obtained with the same quality as ejaculated sperm (Yulnawati et al., 2009, Barati et al., 2009, Lambrechts et al., 2009, Herold et al., 2004). Barati et al., (2009) reported that epididymal sperm with motility of 76.5% can be obtained on buffalo testes stored at  $\pm 4^{\circ}$  C for 24 hours. On rams, epididymal sperm with motility of 69.1% can be obtained on testicles stored at refrigerator for 24 hours (Shakeri et al., 2008).

In the present study, epididymal sperm recovered from testes of buffaloes stored at 5 to  $8^{\circ}$  C displayed good motility, CASA total motility and CASA progressive motility on the first 10 to 12 hours of sperm recovery. However, a significant decrease in percentage motility was obtained 15 to 17 hours later. Yulnawati et al., (2009) reported that  $> 40\%$  sperm motility of epididymal sperm from the Indonesian spotted water buffalo is still acceptable for the artificial insemination (AI) application.

Storage of buffalo testes at 5 to  $8^{\circ}$ C can decrease sperm metabolism and maintain viability which then allows for transport to the laboratory so that adequate manipulation can occur. According to Fernández-Santos et al., as cited by Martins et al., (2009), epididymis has suitable conditions that provide the optimal environment for gamete storage in physiological conditions. Although the cauda epididymis offers good conditions to keep sperm viability for an extended period, when long periods are needed until sperm recovery, several studies agreed that storage at refrigerated conditions ( $\pm 4^{\circ}$  C) is necessary to minimize damage of sperm integrity (Yu and Leibo, 2002; Martínez-Pastor et al., 2005; Fernández-Santos et al., in press).

One beneficial effect of refrigeration on epididymal sperm quality especially on motility is the decreased metabolic rate of sperm cells (Salamon and Maxwell, 1983 as cited by Shakeri et al., 2008). On the other hand, little

information has been recorded on the storage of buffalo testes at 18 to 22° C with good sperm motility, total motility and progressive motility after 10 to 12 hours post mortem time sperm recovery and a markedly decrease after 15 to 17 hours but the values are still acceptable. Bertol et al., (2013) obtained a sperm motility of 66.25% on epididymal spermatozoa recovered after 12 hours on testes of bulls stored at 18 to 20° C. Kaabi et al., (2003) reported a good epididymal sperm motility of sheep and Muradas et al., (2006) on stallions on epididymis stored at 18 to 20° C.

Our findings revealed that epididymal sperm displayed higher progressive motility values within 10-12 hours post mortem when testes were held in 18-22 degrees Celsius compared with those held in 5-8 degrees. This method of storing was made by putting cubes of ice in water where the sample is submerged and can be of practical use if an animal with unique genetic trait unexpectedly die.

Decreased sperm motility across post mortem time in the present study may be correlated with the changes associated to body death and decomposition (Martinez-Pastor et al.,2005). One of the changes happens in the epididymal lumen. Prolonged post mortem storage of epididymis resulted in a severe decrease in the pH of epididymal lumen in wild ruminant which affects the sperm motility (Martinez-Pastor et al.,2005).

Data in this study on the percentage live sperms (53-65%) is in accordance with other findings that only sperm motility is affected by storage temperature or post mortem time. Bertol et al., (2013) confirmed that the most affected sperm parameters by storage time is the percent motility of bull epididymal spermatozoa recovered from the epididymis stored at 18 to 22° C. Martins et al., (2009) stated likewise that the only variable that is affected by storage time is the total motility and not the live sperm population of bull epididymal spermatozoa recovered from the epididymis stored at refrigerated condition.

According to Ax et al., (2000) as cited by Yulnawati et al., (2009), normal fertile semen samples should contain not more than 20% abnormal spermatozoa. Our findings revealed that the epididymal sperm samples registered acceptable number of abnormal spermatozoa below 15% which is reflective of the quality of the sperm from the buffalo animals.

In conclusion, epididymal spermatozoa recovered from testes stored at 5 to 8° C and 18 to 22° C remain viable after 10 to 12 hours post mortem. The results demonstrate the possibility of obtaining good quality epididymal sperm of native carabao after several hours post mortem. If this valuable animal die and the epididymal sperm cannot be processed right away, it may still possible

to obtain viable sperm many hours later to be used in assisted reproductive techniques for the conservation of native carabao.

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