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Effect of acid phosphatase and alkaline phosphatase on epididymal spermatozoan motility during maturation process in black Bengal buck

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Abstract
The aim of this study was to evaluate spermatozoan motility and the physiological level of acid phosphatase (ACP), alkaline phosphatase (ALT) in the luminal fluid collected from the three different epididymal segments from slaughtered Black Bengal bucks and the relationship between ACP and ALP level to sperm motility. The percentage of motile sperm was highest in the cauda segment (81.1 ± 1.23) followed by corpus (21.9 ± 0.84) and totally absent in caput region with significant difference ($p<0.01$) in between regions. Moreover, the correlation of spermatozoan motility between corpus and cauda was related (r = 0.812) which was highly significant ($p<0.01$). Concentration (U/L) of both ACP and ALP was highest in caudal segment of epididymis (6.95 ± 0.77 vs 7.97 ± 0.82) compare to corpus epididymis (6.14±0.43 vs 6.64±0.39) and caput epididymis (6.03 ± 0.35 vs 6.72 ± 0.46) respectively. Spermatozoan motility with ACP (r = 0.432) and ALP (r = 0.538) was highly positively correlated ($p<0.01$). The value of ACP and ALP was varied significantly ($p<0.05$) between caput and corpus, between caput and cauda (r = 0.513) and non-significantly between corpus and cauda (r = 0.305). Present investigation confirms that ACP, ALP and sperm motility, exhibited a good correlation with all the parameters studied, might be used as a simple analytical tool to monitor as one of the indicator for sperm maturation process in comparison with other conventional evaluations which require further analysis.

Keywords: Acid phosphatase, alkaline phosphatase, epididymis, black Bengal buck

1. Introduction
The epididymis is a complex organ where spermatozoa acquire motility and ability to fertilize the egg. In mammals testicular sperm are not able to fertilize an oocyte and require additional post testicular changes during their transit through the epididymis to achieve fertilization competence (Bedford, 1975) [2]. Each region of the epididymis is organized into lobules separated by connective tissue septa that serve for maturation, transportation, concentration and storage of sperm (Orgebin-Crist, 1967; Robaire and Hermo, 2002) [26, 29] and also as a functional separation which allows for selective expression of genes and proteins within each individual lobule (Robaire *et al*., 2006; Turner *et al*., 2003) [30, 33, 34]. Proper luminal microenvironment of the epididymis is essential and plays pivotal role for successfully sperm maturation (Foley, 2001) [15]. During sperm maturation process, many epididymal secretory proteins (Cuasnicu *et al*., 2002; Cornwall, 2009) [10, 9] including hormone, enzymes, vitamins, ions, cations, trace elements are associated with the sperm surface and play important roles in male reproduction (Massanyi *et al*., 2004; Massanyi *et al*., 2005) [24, 25].
Good motility is an important factor associated with sperm quality, a property often regarded as being of the utmost importance for fertility. Motility is the most evident change in the epididymal sperm, with irregular and asymmetric flagella beating in the caput epididymis becoming symmetrical with propagation of waves on each side of the flagella, inducing forward motility of the spermatozoa when it reaches the cauda (Chevrier and Dacheux, 1992; Dacheux and Dacheux, 2014) [8, 11].
ACP are known to provide phosphate to tissue that show energy requirements especially during development, growth and maturation (Blum, 1970; Hurkudli *et al*., 1985) [4, 20]. ACP is localized in Golgi area of epithelial cells in the head segments of the duct. Special cells in the head, body and tail of the epididymal canal showed high levels of activity generally distributed in their cytoplasm. ACP containing lysosomes are similarly found in spermatogonia and late spermatids in rat testes, and a significant increase in ACP activity observed in the Golgi complex during spermatogenesis (Chemes, 1986) [7].

ACP activity is present in head membranes of spermatozoa from the testes, the caput and a less extent from the cauda epididymis, but absent in spermatozoa from the ductus deferences. ALP is dephosphorylated enzyme acting on many tissues and organ, including bone, liver, kidney, intestine, lung, and placenta (Hoffmann *et al*., 1989) [18]. ALP activity is not uniform in the epididymis, but is concentrated at high levels in the lumen of the epididymal tail and can be used as a clinical ejaculatory marker to differentiate azoospermia or oligospermia (Turner and Macdonell, 2003) [33].

Phosphatase enzymes such as ALP and ACP levels in seminal plasma are very important for sperm metabolism as well as sperm functions (Brooks, 1990) [5]. Estimation of these enzymes have been recommended as biomarkers for assessment of semen quality (Pesch *et al*., 2006) [28].

Therefore, the present study was conducted to evaluate the motility that exhibited by the spermatozoa and to estimate the physiological level of ACP and ALP in the luminal fluid collected from the three different epididymal segments from slaughtered bucks and whether the variation of ALP and ACP influence the spermatozoan motility.

2. Materials and Methods

The present study was conducted in the Department of Veterinary Gynaecology and Obstetrics, WBUAFS, Kolkata - 700037, India. Normal testes attached with intact epididymis were collected from adult and healthy Bucks (n=60) from local abattoir immediately after slaughter and placed in Styrofoam container at 5 ℃ in a plastic bag container 0.9% saline solution (NSS) and brought to the laboratory within 2 hours.

Tunica albuginea were removed from the testes and washed thoroughly with NSS. Ligatures were placed unilaterally at the proximal end of the vasdeferences, ampulla and cauda epididymis separately and distal to caput epididymis and vasefferentia. After ligations, epididymis along with vasdeferences were dissected out from each testis and washed thoroughly by NSS. Left epididymis of each animal was considered for biochemical estimation and the corresponding right epididymis was considered for evaluation of spermatozoan motility. The ligated left epididymis were placed immediately in chilled PBS into individual beaker and kept at 5 ℃ in a refrigerator.

Each ligated portion of the right epididymis was cut gently into three parts, *viz* caput, corpus and cauda and placed individually into three separates polystyrene Petri dishes containing 2 ml of 0.15 M Phosphate buffer saline (PBS, pH 7.4) at 37 ℃. The individual portions were minced carefully and luminal content from each segment was collected by giving gentle pressure on the excised tissues into the medium with separate clean glass rods. The resultant suspensions were aspirated and filtered through individual nitex membrane (150 mm pore size) glass test tubes, centrifuged at 500g for 10 minutes and the supernatants were discarded. Finally, each sperm pellet was re-suspended with 2ml of PBS separately, vortexes for 3 seconds and kept at 37^0C into an incubator provided with 5% CO2 in air for 20 minutes allowing sperm cells to swim –up into the medium.

2.1 Assessment of spermatozoan motility

Spermatozoan motility evaluation is based upon subjective estimates of the percentage of sperm cells exhibited any kind of movement and the proportion of spermatozoa moving progressively forward.

Spermatozoan motility was assessed by standard subjective ranking method (WHO, 1997) [36]. Sperm suspension, 10µl, were aspirated carefully from upper layer of each test tube and placed on clean, dry microscopic glass slides separately by different micropipettes and covered with cover slips, and the slides were placed on a warm stage 37^0 C. Observations from a minimum of ten microscopic fields were made randomly under Leitz Phase contrast microscope (× 100; × 200). Sperm were assessed as showing movement that was either linear and progressive or non-progressive and those that were non-motile. An arbitrary scale (Kinetic rating) of ‘‘0 (lowest /absent) to 5 (highest)’’ was used to determine the spermatozoan overall motility subjected from each sample obtained from caput, corpus, and cauda epididymis respectively and their mean results were expressed in % motility.

2.2 Collection and biochemical estimation of epididymal luminal fluid

Each of portion epididymis was soaked carefully with tissue paper. Each portion of the ligated caput, corpus and cauda epididymis was cut gently and placed individually sterile dry watch glasses and weighted. A total 3.5 gm of each caput, corpus and cauda from each epididymis was weighted. Each portion of, the caput, corpus and cauda were then minced carefully by razor blades. 2 ml of chilled PBS was then poured into each glass. Individual glass rods were used to give gentle pressure on the excised tissues, and tissues were thoroughly flushed several times with same PBS solution remaining into each glass separate syringes. Whole content of each segment including the PBS solution were transferred to coded sterile test tubes and cold centrifuged at 15,000 g for 30 min. After centrifugation supernatant from each test tube was collected very carefully and alliqoted, in 1.5 ml Eppendorf tubes. All the tubes were then properly labelled, and kept at -20 ℃ till experimentation.

2.3.1 Biochemical estimation

For estimation of ACP and ALP, all the sample were thawed prior to estimate the concentration of ACP and ALP from each segment of the epididymis and were measured by commercially available kit. ACP and ALP concentration was estimated by the method of Tietz, 1955 [32].

2.4 Statistical Analysis

All the data were subjected to analysed by SPSS 26 (Statistical Package for Social Science) and Pearson’s correlation coefficient. The mean were compared using Duncan Multiple Range tests (Duncan, 1955) [13].

3. Results and Discussions

The mean percentage of spermatozoan motility was highest in the cauda segment (81.1 ± 1.23) followed by corpus (21.9 ± 0.84) and totally absent in caput region. (Table 1, Graph 1a). Analysis of variance (Tukeys HSD test) revealed highly significant difference ($p<0.01$) in the percentage of motility in between regions. Correlation of spermatozoan motility between corpus and cauda was related (r = 0.812) (Table 2), which was highly significant ($p<0.01$). Highly positive correlation ($p<0.01$) was found between spermatozoan motility with ACP (r = 0.432) and ALP (r = 0.538).

The level of ACP recorded were (6.03 ± 0.35), (6.14 ± 0.43), (6.95 ± 0.77) U/L in caput, corpus and cauda epididymis respectively. Concentration of ACP was highest in caudal

segment which was significantly differed ($p < 0.01$) from level of corpus and caput epididymis. The relationship between caput and corpus (r = 0.429), between caput and cauda (r = 0.513) varied significantly ($p<0.05$) and between corpus and cauda (r = 0.305) varied non significantly.

The level of ALP recorded were (6.72 ± 0.46), (6.64 ± 0.39), (7.97 ± 0.82) U/L in caput, corpus and cauda epididymis respectively. Concentration of ALP was highest caudal segment which was significantly differed ($p < 0.01$) from level of corpus and caput epididymis.

The relationship between caput and corpus (r = 0.624), between caput and cauda (r = 0.785) varied significantly ($p<0.05$) and between corpus and cauda (r= 0.396) varied non significantly.

Table 1: Level of Sperm motility, ACP and ALP of Spermatozoa from different region of epididymis,

Parameter	Epididymis region		
	Caput	Corpus	Cauda
Sperm Motility (%)	0^{c}	21.9 ± 0.84^{b}	81.1 ± 1.23^{a}
Acid Phosphate (U/L)	6.03 ± 0.35^{b}	6.14 ± 0.43^{b}	6.95 ± 0.77^{a}
Alkaline Phosphate(U/L)	6.72 ± 0.46^{b}	6.64 ± 0.39^{b}	7.97 ± 0.82^{a}

Table value expressed as Mean± SE: n=60, Different superscripts (a, b and c) differ significantly ($p<0.01$) row-wise according to Tukey's HSD test.

Table 2: Pearson's Correlation coefficient between different epididymal regions.

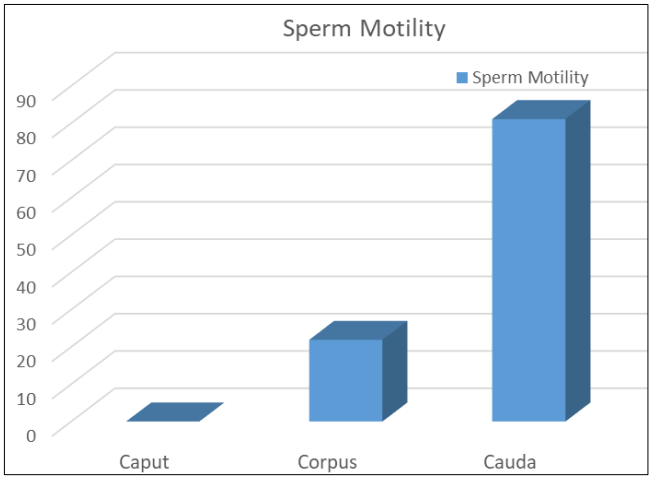
Parameters / Variable	Sperm Motility (%)	Acid Phosphate (U/L)	Alkaline Phosphate(U/L)
Caput and Corpus	-	0.429^{*}	0.624^{**}
Caput and Cauda	-	0.513^{*}	0.785^{**}
Corpus and Cauda	0.812^{**}	0.305	0.396

-absent, *Significant at 5% level ($p<0.05$), **Significant at 1% level ($p<0.01$).

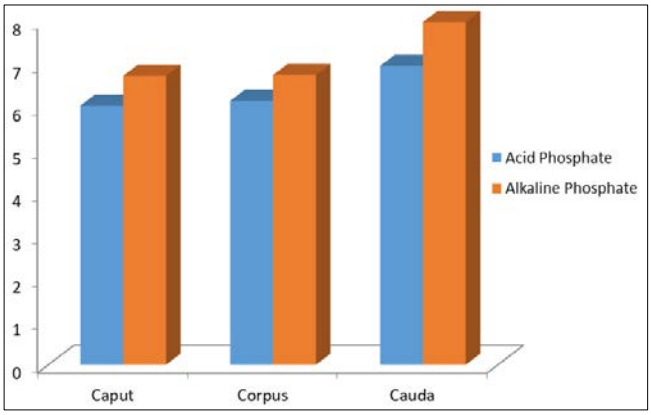
Table 3: Pearson's Correlation Coefficient between Sperm Motility (%) with different parameters

N=60	Acid Phosphate	Alkaline Phosphate
Sperm Motility	0.432^{**}	0.538^{**}

**Significant at1% level ($p<0.01$)



Graph 1a: Level of Sperm motility from different region of epididymis



Graph 1b: Level of ACP and ALP of Spermatozoa from different region of epididymis

The present study reveals that during passage of spermatozoa through the epididymal segments, the motion was strongest in the caudal region and was absent in the caput which simulate with the observation of Jindal and Panda (1980) [21] and Dutta *et al*., 1989 [12] in Goat, Oyeyemi and Ubiogoro (2005) [27] in boar. The findings in the present investigation are very much in agreement with that of Ansari *et al*., 2016 [1] who reported that the percentage of motile spermatozoa was found to be maximum in the cauda, followed by corpus and in caput the motility was absent.

The gradual increase in spermatozoan motility in the epididymal region might be due to an elevation in the content of intra sperm CAMP during transit and production of forward motility protein in bovine epididymis (Hoskins *et al*., 1979) [19], variation in chemical composition and local steroid hormone concentration in different parts of epididymis and with the age of maturing sperm (Hamilton, 1972) [17], and elevation of carnitine level reported in boar and human (Volgmayr, 1975) [35]. Roussel and Stallcup (1966) [31] reported that the percentage of motile spermatozoa was significantly correlated with ACP and ALP in seminal plasma, whereas the percentage of abnormal spermatozoa was negatively correlated with ALP and ACP in bull semen.

The value of ACP in present Black Bengal buck was also in conformity with the work of Goyal and Dhinga (1984) in calves and Einarsson *et al*., 1976 [14] in boar. Mahapatra (1988) revealed that the level ACP of diseased condition was significantly higher than normal ($p<0.01$) except cauda. The level of ACP was significantly lower ($p<0.01$) in corpus and vasdeferens in pathological condition than normal and in testis significant difference.

Concentration of ALP was highest in caudal segment which agreed with the report of Chauhan and sharma 1988 [6]. Beu *et al*., 2007 reported that ALP seems to be an essential enzyme related to the epididymidis metabolism, acting on the maintenance of spermatozoa stored in the luminal compartment of epididymal tail of Golden hamsters, and perhaps also in other mammalian epididymidis. Chauhan & Sharma (1988) [6] observed the level of ALP was increased as sperm traversed through the caput, corpus and cauda, which corroborated with the present findings. However, the difference in value found in corpus might be due to breed difference. It would be evident from the co-efficient of correlation that ACP & ALP activities are positively

correlated with each other in caput and corpus. Einarsson *et al*., 1976 [14] reported higher level of these enzymes in cauda region which similar to the findings obtained in the present study and these enzymes are positively correlated in cauda as indicated in present study. These observations may be correlated with reports that spermatozoa mature in the upper portion of the epididymis to suggest a maturational function for the enzyme.

4. Conclusion

The result obtained in the present work could be a basis of establishing physiological norms of different levels of enzymes and other components in physiological environment of epididymis of black Bengal buck where normal maturation of spermatozoa is taking place leading to acquiring of fertilization capacity. The norms can indicate any deviation in pathological condition of epididymis. The level could be compared and the deviation can suggest the type of abnormality in metabolism and maturation of spermatozoa. This will help to undertake measure and prevent reproductive failure in male animals. Present investigation confirms that acid and alkaline phosphatase levels and sperm motility, which exhibited a good correlation with all the parameters studied, might be used as a simple analytical tool to monitor as one of the indicator for sperm maturation process in comparison with other conventional evaluations which require further analysis.

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