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Short Communication

Seasonal variations of cauda epididymal spermatozoa of bucks

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ABSTRACT

Objective: The study was conducted to evaluate the influence of season on cauda epididymal spermatozoa isolated from bucks.

Materials and methods: Testes of 30 mature bucks were collected from local slaughter house, and were processed for the retrieval of cauda epididymal spermatozoa for evaluation. Testes were collected in three seasons (winter, summer and rainy), and each season was having 10 pairs of testicles. Recovered spermatozoa from the cauda epididymis were processed immediately for evaluation of semen attributes (Spermatozoa motility, viability, plasma membrane integrity, acrosomal status and DNA integrity).

Results: Physiological effect of season was observed on progressive motility, percent of live spermatozoa, spermatozoal membrane integrity (HOST), acrosomal integrity, capacitation status and DNA integrity. Progressive motility, percent live spermatozoa, HOST positive spermatozoa, were found significantly ($P<0.05$) high in summer season, whereas, significantly ($P<0.05$) lower comet positive spermatozoa were found in summer season as compared to rainy and winter. Compromised acrosomal status was seen in winter and rainy seasons as compared to summer.

Conclusion: Compromised acrosome along with plasma membrane and higher percentage of spermatozoa with damaged DNA in cauda spermatozoa were observed during winter and rainy seasons as compared to summer season. Summer season was found to be the most suitable season for collection of cauda epididymal spermatozoa and can effectively be used for assisted reproduction with further investigations of associated mechanisms.

KEYWORDS

Cauda epididymis, Comet assay, DNA integrity, HOST, Season, Spermatozoa attributes

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INTRODUCTION

Epididymis serves as the site of spermatozoa transit, storage, maturation, acquisition of motility and fertility after testicular spermatogenesis (Cooper, 2007). Microenvironment of the epididymis provides suitable environment for spermatozoa where structural and functional changes take place in a segment specific manner and allow them to achieve functional competence (Aitken et al., 2007; Cornwall, 2009). The microenvironment of the epididymis induces number of changes in the spermatozoa in terms of membrane protein alteration, membrane composition and membrane remodelling (Cornwall, 2009).

Cauda serves the major site of spermatozoa storage as well as site of maximum concentration of spermatozoa before ejaculation (Blash et al., 2000). In recent past, cauda has been used for the retrieval of live and functional spermatozoa for the process of assisted reproduction and resulted in successful fertilisation. Cauda serves as the best possible source of live spermatozoa from the wild animals as well as the animals which show spontaneous unwanted death due to any means (Fickel et al., 2007). Successful and timely collection of the spermatozoa meets the requirement of cryopreservation as well as of *in vitro* fertilisation (Andrabi and Maxwell, 2007). Domestic goat serves as a good model for the wild ruminants and may be effectively used for the study of spermatozoa attributes.

Goat exhibits seasonal variations in circulating levels of testosterone, seminal plasma proteins (Chowdhury et al., 2002), and semen attributes (Swain et al., 2013). It is hypothesized that season may also influence the attributes of cauda spermatozoa, thus the present study was undertaken to evaluate the influence of season on spermatozoa attributes along with spermatozoal DNA integrity of goats.

MATERIALS AND METHODS

Collection of Epididymis: The study was carried out in matured buck of aged between 2 to 2.5 years of age as represented for slaughter at local slaughter house. The history was taken from the slaughter house as described by the butcher and confirmed by the pattern of dentition. The animals were apparently free from infections and were having good body condition score during slaughter. Testicles (N=30) were collected immediately after slaughter under strict hygienic conditions and transferred to the laboratory in chilled normal saline in a thermos flask. Collected testes were processed for collection of spermatozoa from cauda epididymis as per standardized methods (Swain et al., 2012a). Laboratory processing of testes was carried out within 30 min after collection.

Testes were washed and cleaned with phosphate buffer saline (PBS, pH-7.4). Fascia, blood vessels and sheath of testes were removed to expose the epididymis with cautions to prevent damage to epididymis. In each step of processing, testes were thoroughly washed with PBS. After complete removal, epididymis was kept in PBS (pH-7.4) for further processing. Morpho-anatomically, different segments of epididymis were identified and separated accordingly. Flushing was employed to collect the spermatozoa from the cauda epididymis.

Harvesting of Spermatozoa: Spermatozoa were harvested by flushing the cauda epididymis with a 5 mL syringe containing 2 mL of Tyrode–lactate solution into a petri-dish. Epididymal spermatozoa suspension was collected and centrifuged at 3000 rpm for 30 min in a refrigerated centrifuge at 4^0C. Supernatant was aspirated, discarded and the pellet was resuspended in fresh Tyrode- lactate solution and the volume was made up to 1 mL. The spermatozoa samples were transferred to eppendorf tube and kept in incubator at 37°C as spermatozoa stock sample for evaluation.

Evaluation of spermatozoa attributes: The period of study was divided into three seasons *viz*., rainy season (July to September), winter season (December to February) and summer season (April to June). The classification of seasons was carried out on the basis of climatic variables of tropical environment in India and in specific to western parts of Uttarpradesh. The base of seasonal classification was temperature, humidity as well as day length as established by the meteorological parameters (**Table 1**). The harvested spermatozoa were evaluated for spermatozoa motility (%), supra vital negative (%), abnormal spermatozoa count (%), acrosomal intactness (%), and hypo osmotic swelling test (HOST) (%) as per the standard methods (Swain et al., 2012b; Swain et al., 2012c).

Acrosomal intactness was evaluated by sensitive fluorescent based technique FITC-PSA (Swain et al., 2012b). Briefly, spermatozoa at a concentration of (1×10^6/mL) were suspended in PBS (Ca^{2+} and Mg^{2+} free) and the spermatozoa samples were centrifuged at 1000 ×g for 5 min. Spermatozoa samples were washed three times in PBS (pH=7.4). After three washings, final spermatozoa pellet was resuspended in PBS (pH=7.4). Ten µL of the sample was taken and a thin and uniform smear was made on a clean grease free glass slide. Air dried smears were incubated with FITC-PSA (100 µg/mL in PBS, pH=7.4) for 30 min at room temperature in dark.

Further, smears were examined by using Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under FITC/blue filter. Four hundred spermatozoa were differentiated according to the fluorescence pattern of their acrosome (bright fluorescence acrosome- intact, no fluorescence or only fluorescence of the equatorial segment means loss of acrosome).

Comet assay of cauda spermatozoa: Single cell gel electrophoresis (Comet assay) was performed according to the standard method (Swain et al., 2012a). In brief, 10 µL of spermatozoa suspension from the stock solution presenting more than 85% live cells was mixed with 125 µL of low melting point agarose (0.75%) and applied as a coat on normal agarose pre coated frosted slides. After setting of gel, normal melting point agarose (1%) was applied as third coat. Slides were immersed in a prewarmed (37°C) lysing solution-I (1.25 M NaCl, 50 mM EDTA, 100 mM tris base, 2 mg/mL reduced glutathione and 0.05 mg/mL DNAse free proteinase K, pH 10.0) for 3 h at 37°C followed by lysis with lysing solution-II (1.25 M NaCl, 50 mM EDTA, 100 mM tris base, 1% α-mercaptoethanol, 1% Triton X-100, pH 10.0) for 5 h at 4°C. After lysis the slides were kept 1 h in chilled electrode buffer (500 mM NaCl, 0.1 M tris-base, 1 mM EDTA, 0.2% DMSO, pH 9.0) for chromatin decondensation followed by electrophoresis at 24V for 1 h. The slides were treated with neutralization buffer (20 mM Tris, pH 7.4) and dried in incubator at 37°C. Slides were then incubated in PBS buffer containing ethidium bromide (0.01%) for 10 min. After wiping the stain, slides were observed under upright microscope at 400X magnification and images were captured using the integrated camera. Three slides were prepared from each age groups of buck and at least 100 comets were observed.

Statistical analysis: The obtained results were analyzed by using the SPSS software version 14 (Chicago, USA) and means were compared by ANOVA for different spermatozoa attributes and their variations in the three seasons. Significance was tested at 5% level ($P<0.05$).

RESULTS AND DISCUSSION

Results obtained in the study have been presented in **Table 2**. The results revealed significant ($P<0.05$) difference in individual spermatozoa motility in three seasons. The highest individual motility was found in summer season and lowest being found in winter season. Progressive motility is one of the significant parameters associated with the fertility of the spermatozoa. This helps the spermatozoa to reach the site of fertilisation and allows successful movement in the female genital tract (Barkawi et al., 2006). In the present study, significantly ($P<0.05$) higher motility of the spermatozoa was observed in summer compared to other seasons. The extended photoperiod in summer is responsible for increasing circulating concentrations of testosterone that may be one of the factors responsible for increasing the progressive motility of spermatozoa (Perez and Mateos, 1996; Barkawi et al., 2006; Chemineau et al., 2008).

Highest live spermatozoa percent was observed during summer months and no significant difference was found between winter and rainy seasons. Abnormal spermatozoa count was found significantly ($P<0.05$) higher in rainy season and all the three seasons exhibited a significant ($P<0.05$) difference in abnormal spermatozoa count. Viability of the spermatozoa is prerequisite for the process of cryopreservation as well as for optimal fertility. The more the viability of the spermatozoa and less the number of abnormal spermatozoa, the better is the quality of the spermatozoa (Barkawi et al., 2006). The influence of season on viability may be due to photoperiodic effects that affect the quality of the spermatozoa isolated from the cauda epididymis in different seasons. During summer months, the cauda epididymal luminal diameter as well as epithelial cell height increases that favors the improvement in spermatozoa livability as well as other physical attributes compared to winter and rainy seasons simulated the findings of present study (Bitto et al., 2008).

The retrieved spermatozoa were evaluated for the presence of cytoplasmic droplets. The results revealed a significantly ($P<0.05$) lower number of immature spermatozoa in the summer season as compared to the winter and rainy seasons. Effect of season on the spermatozoa maturation has been studied in the testes and it is well established that season regulates the circulating levels of testosterone and promotes optimal maturation of spermatozoa (Chowdhury et al., 2002; Barkawi et al., 2006). In the present study, this may be a probable factor which has regulated the spermatozoa maturation in the cauda epididymis. Similar trend was seen for the decapitated spermatozoa % in three seasons.

With the change in the season, alterations in morphometric characteristics of bucks occur in testes along with epididymis. The epithelial cells become prominent along with tall columnar shape. Circulating concentrations of hormone testosterone increases and this leads to change in the spermatozoal physiology as well as dynamics. This may be another factor, which explains our results of the present study (Bitto and Egbunike, 2006).

Table 1: Seasonal variations of Environment Temperature (^{0}C) and Relative Humidity (%)

Parameters	Summer season		Rainy season		Winter season	
	Range	Mean±SE	Range	Mean±SE	Range	Mean±SE
Environment temperature (°C)	34.50-36.50	35.16±0.13^c	28.50-30.00	29.47±0.07^a	6.00-17.50	12.15±0.63^b
Relative Humidity (%)	40-61	49.66±1.30^c	75-85	80.77±0.70^a	75-94	83.38±0.83^b

Different superscript in a row represented significant (P<0.05) difference.

Table 2: Physical attributes of cauda epididymal spermatozoa (Mean±S.E.) of bucks in three seasons.

Character	Summer	Rainy	Winter
Individual spermatozoa motility	77.16^c±0.82	64.60^b±1.11	47.81^a±0.85
Live spermatozoa count	88.98^b±0.94	82.42^a±1.14	80.12^a±0.97
Abnormal spermatozoa count	12.56^a±0.44	36.13^b±0.14	25.18^c±0.47
Proximal droplet	4.91^a±0.59	12.41^b±0.76	18.46^c±1.19
Distal droplet	81.93^c±4.57	78.86^b±0.99	74.02^a±0.18
No droplet	72.43^c±1.06	68.11^b±0.68	62.76^a±1.04
Decapitated spermatozoa	1.73^a±0.30	6.66^c±0.97	4.26^b±0.81
Host (+ve) spermatozoa	72.26^c±1.59	67.93^b±0.41	62.53^a±0.52
Acrosomal integrity	78.73^c±2.16	66.20^b±0.44	59.26^a±0.54
Comet (+ve)	9.78^b±0.84	13.32^a±0.92	14.66^a±0.68

Different superscript in a row represented significant (P<0.05) difference.

The isolated cauda epididymal spermatozoa exhibited a significantly (P<0.05) higher HOST positive spermatozoa in the summer season as compared to winter and rainy seasons. The HOST positive spermatozoa were found highest in summer season followed by rainy and winter seasons. HOST is the determinant of both intactness and quality of the spermatozoa (Fonseca et al., 2005). Hypoosmotic swelling test OS also determines the osmolar resistance conferred by the spermatozoa both *in vivo* and *in vitro* (Jeyendran et al., 1984). Variations in the HOS positive spermatozoa in the three seasons were reported in the study but no probable mechanism was assigned for this variation and need further investigation.

The acrosomal status of the isolated spermatozoa was determined by using the sensitive FITC-PSA fluorescent technique and the results obtained have been depicted in **Table 2**. The results revealed a significant increase in the percentage of spermatozoa with intact acrosome in summer season as compared to winter and rainy seasons. Acrosome plays significant role during fertilisation by bringing out acrosome reaction. The results could not be validated due to lack of information regarding the seasonal variations in the acrosomal status in cauda epididymal spermatozoa. Studies in ejaculated spermatozoa have already been established in literature showing more number of spermatozoa with intact acrosome during summer as compared to winter and rainy seasons (Kumar et al., 2014).

The DNA integrity was evaluated by using the single cell gel electrophoresis, the comet assay. The assay is considered as a sensitive assay for the determination of DNA fragmentation (Swain et al., 2012c). The cells having fragmented DNA form comets when they are exposed to an electric field in the gel. In the present study, higher percentage of comet positive cauda epididymal spermatozoa were detected in winter compared to rainy and the lowest in summer season. It is the first study to report the DNA integrity of cauda epididymal spermatozoa in different seasons. The ranges of comet positive spermatozoa in the cauda epididymis were similar to our earlier work (Swain et al., 2012c) but due to lack of literature regarding seasonal effect and spermatozoa attributes, the results could not be compared.

CONCLUSION

In this study, the best quality spermatozoa in cauda were observed in the summer compared to winter and rainy seasons. It can be concluded that summer season is the best season for the retrieval of spermatozoa from the cauda epididymis of bucks. However, further studies are required to investigate the mechanisms associated with the changes in seminal attributes of cauda epididymis in different seasons to develop a potential system for retrieval of spermatozoa from cauda for successful assisted reproduction in emergency.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest with any other people or organizations in any financial or personal relationship.

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