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Evaluation of *in vitro* longevity of caprine cauda epididymal sperms at different storage intervals of time

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Genome banking has become an important management strategy for the conservation of endangered or exotic species. The interest in preserving endangered species and valuable genetic material has resulted in increased attention to the possible recovery of viable sperm from the epididymis of dead animals (Loskutoff *et al.* 1996). Cauda epididymis serves as a store house of the sperms in all mammalian species of animals and serves as a potential source of the viable sperms in dead animals which can be used in the assisted reproductive technology (Foote 1999). Literature is scanty regarding the evaluation of *in vitro* longevity of caprine cauda epididymal sperms. With this overview, the present study was designed to study the possible recovery of viable sperms from the cauda epididymis at 4°C and at room temperature and evaluation of the physical seminal attributes at different intervals of collection.

Testes (30) were collected immediately after slaughter of the animal from the local slaughterhouse of Mathura between 4.00 – 5.00 AM and were kept in chilled normal saline (15 in numbers) and 15 testes were transferred in normal saline of room temperature (27– 30°C) and transferred to the laboratory in a thermox. The laboratory processing of testes was carried out within 30 min of reaching the laboratory.

The testes were divided into 3 groups and each group was having 5 testes. A set of testes were evaluated immediately after processing (30 min), second group of testes were evaluated at 12 h of storage and the third group of testes were kept in chilled normal saline in the bio-refrigerator and were evaluated after 24 h of storage for 4°C. Along with this, the testes were kept in normal saline at room temperature for the evaluation of sperm attributes at different intervals of time. Sperms were harvested by flushing the cauda epididymis with a 5 ml syringe containing 2 ml of Tyrode–lactate solution into a petri-dish. The epididymal sperm solution was collected and centrifuged at 3000 rpm for 30 min. The supernatant was aspirated and discarded and the pellet was resuspended in fresh Tyrode- lactate solution and the volume was made up to 1 ml. The sperm samples were transferred to the ependorff tube and kept in incubator at 37°C as sperm stock sample for evaluation.

The harvested sperms were evaluated for parameters like sperm motility (%), supra vital negative (%) as per Esteeves *et al.* (2007), abnormal sperm count (%) was evaluated by eosin-nigrosin staining, sperms with intact acrosome (%) were evaluated by FITC-PSA staining (Mendoza *et al.* 1992), and HOST positive spermatozoa (%) was carried by taking 125mOsm/L of hypoosmotic solution as per Fonseca *et al.* (2005) at both the storage temperatures as well as time intervals of collection to evaluate the *in vitro* longevity of the cauda epididymal sperms.

The obtained results were analyzed by using the SPSS software version 14 and means were compared by ANOVA for different sperm attributes at both the storage temperatures as well as the 3 intervals of collection of sperms. The results obtained from the study are presented in Table 1.

There was significant difference ($P< 0.05$) between all the time intervals of sperm collection and storage in terms of sperm motility which gradually decreased as the time of storage increased exhibiting a highest motility at 0 h of collection and a lowest at 24 h of collection at both the storage temperatures of 4°C and at room temperature. We did not get any relevant information in the goat model to corroborate our findings of the study. On the other hand we compare our results with that of the canines and observed the similar results as reported by Rota *et al.* (1995) and Fahring (2000). Swain *et al.* (2011) reported the decrease in the sperm motility of the epididymal sperms with the increase in time in canines at 4°C. Similarly Tarai *et al.* (2010) reported similar results while evaluating the cauda epididymal sperms in dogs at 25°C.

The loss of sperm motility may be due to lack of secretions of cauda epididymis. Cauda part of the epididymis induces

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Table 1. Mean of average of seminal attributes of cauda epididymal sperms collected at different intervals of storage time (4°C and 27–30°C)

Sperm parameters (%)	4°C (processing and storage)			27–30°C (processing and storage)		
	0 h	12 h	24 h	0 h	12 h	24 h
Supravital negative sperm	$87.58^{c}\pm0.94$	$30.18^{b\pm}0.94$	$9.56^{a\pm}0.46$	$80.17^{C}\pm0.64$	$20.43^{B}\pm1.94$	$8.98^{A\pm}0.82$
Sperm motility	$76.60^{c}\pm1.11$	$26.66^{b}\pm2.14$	$6.66^{a}\pm1.18$	$70.68^{C}\pm1.16$	$8.69^{B}\pm2.19$	$3.60^{A\pm}0.16$
Abnormal sperm count	$22.42^{a}\pm0.94$	$46.32^{b}\pm1.94$	$82.82^{c\pm}1.90$	$56.17^{A}\pm0.84$	$76.12^{B}\pm1.04$	$92.62^{C}\pm1.98$
HOST positive sperm	$72.26^{c\pm}1.59$	$22.22^{b}\pm1.68$	$9.76^{a}\pm0.46$	$46.18^{C}\pm1.09$	$12.56^{B}\pm1.78$	$7.24^{A}\pm0.18$
Intact acrosome	$68.73^{c}\pm2.16$	$28.79^{b}\pm2.17$	$10.84^{a}\pm0.66$	$48.42^{C}\pm1.63$	$8.76^{B}\pm1.13$	$8.16^{A}\pm0.66$

*a, b, c- Superscripts show the significant difference (P< 0.05) between the different time intervals at 4°C (processing and storage).*A, B, C- Superscripts show the significant difference (P< 0.05) between the different time intervals at 27–30°C (processing and storage).

motility by the secretion from the epithelium. The carnitine along with the forward motility proteins of the epididymis regulates the sperm motility. With the passing of the time and the time of storage of the sperms, the content of carnitine got reduced which instead reduced the sperm motility (Ghosh *et al.* 1993). When compared the motility of sperms at room temperature to that of 4°C, it was found that the motility is till lesser at room temperature and the degree of reduction in the sperm motility with increase in time is more as compared to the sperms stored at 4°C.

With the passage of the time the antioxidant defence of the sperms got reduced. The generation of free radicals got increased along with the storage intervals of time. The generated free radicals damaged to the sperms plasma membrane as well as the DNA and may be the probable factors for the loss of motility. Nair *et al.* (2006) reported a time dependent alteration in cauda epididymal sperms in canines due to the reduced antioxidant defence as well as free radical mediated damage to the sperm membrane. The results revealed a significant difference (P< 0.05) in percentage of live sperm when the testes were stored at 4°C and room temperature between different intervals of time. The livability of the sperms got reduced with the passage of the time. The live sperms did not stain with the fluorescent dye as it can only penetrate the dead cells (Esteeves *et al.* 2007). The dead sperm exhibited a strong fluorescence after binding to the nucleus specific Hoechest stain.

Live sperm (%) depends on many factors like intactness of membrane and defence from free radicals. Swain *et al.* (2011) reported a steady decline in the percentage of the live sperms with the passage of time in canine cauda epididymal sperms at 4°C and Tarai *et al.* (2010) reported the same at 25°C. In the present study, with the progression of time, the live sperm % decreased. It might be due to the lack of nutrition to the sperms, free radical damage due to loss of antioxidant defence, reduction in the energy metabolism of the collected sperms which were stored *in vitro* (Tarai *et al.* 2010). It can be concluded that the sperms collected from the cauda epididymis should be extended with diluents and antioxidants so that the live % will be maintained for a prolonged period. Kundu *et al.* (2001) reported an increase in the post thaw motility and livability of the caprine cauda epididymal sperms after cryopreservation. The extender medium was extended with amino acids alanine, proline, glycine and glutamine. Santos *et al.* (2007) reported that supplementation with both enzymatic and non enzymatic antioxidants significantly improve the post thaw quality of cryopreserved cauda epididymal sperms in Iberian Red deer in terms of livability and sperm motility. The sperms should be utilised as soon as possible after collection to get a better outcome in terms of fertility (Swain *et al.* 2011).

The percentage of abnormal sperms gradually increased with the advancement of the time. The results revealed a significant difference (P< 0.05) between all the 3 intervals of collection of sperms from the epididymis. Swain *et al.* (2011) reported similar results in the canines at 4°C. In the present study, gradually the number of normal spermatozoa decreased with the progression of collection time interval. Tarai *et al.* (2010) reported a time dependent increase in the abnormal sperm count at 25°C. Morphologically abnormal spermatozoa may have been due to rise in the process of oxidative damage, which resulted in membrane damage and leakiness. The free radical mediated damage was ascribed to the net reduction in the antioxidant defence as the testes were stored *in vitro*. Santos *et al.* (2007) reported that supplementation with both enzymatic and non enzymatic antioxidants significantly increased the post thaw quality of cryopreserved cauda epididymal sperms in Iberian Red deer in terms of normal sperm count.

Acrosomal integrity (%) was significantly different (P< 0.05) at different time interval of sperm collection and storage at 4°C and room temperature. Acrosome is the central regulator of fertilisation which mediates the sperm oocyte fusion as well as exocytosis of its constituents to bring out the process of fertilisation. That is why the intactness of the acrosome decides the fate of fertilisation. In the present study, with the progression of time, the percentage of intact acrosome gradually got compromised at both the storage temperatures and all the 3 intervals of sperm collection and evaluation. The decrease in the intact acrosome signified the

deterioration of sperm quality which instead raises question about the probable outcome of fertilisation. It can be assumed that with the passage of the storage time the free radical meditated oxidative stress was key player as reported by Swain *et al.* (2011) and Tarai *et al.* (2010). Our results are lower to the values reported by Fahring (2000) in dogs and it may be due to the difference in species and storage time intervals. Santos *et al.* (2007) reported that supplementation with both enzymatic and non enzymatic antioxidants significantly improve the post thaw quality of cryopreserved cauda epididymal sperms in Iberian Red deer in terms of intactness of the acrosome. The study strongly recommended the supplementation of antioxidants in the extenders to enhance the post thaw quality of the cryopreserved epididymal spermatozoa.

Results revealed a time dependent reduction in the HOST positive sperms which was significantly different ($P< 0.05$) at different intervals of time at both the storage temperatures. The degree of decrease in the sperm membrane integrity indicated a time dependent increase in the membrane leakiness. The findings of the present study were lower than the findings of Fahring (2000) and Zekariya *et al.,* (2004). This may be due to cauda epididymal sperm and experimental conditions whereas Swain *et al.* (2011) reported a similar trend in the cauda epididymal sperms of dogs at 4°C and Tarai *et al.* (2010) at room temperature. Malo *et al.* (2011) reported that supplementation with antioxidants markedly enhanced the post thaw HOST positive sperms in boar epididymal spermatozoa. From the discussion and review of the literature, it is quite evident that both the processing and storage temperature and time are critical in the deciding the fate of the epididymal sperms. The supplementation of the antioxidants in the extender will protect the sperm features as reported by various authors.

SUMMARY

The present study was designed to evaluate the effect of both temperature and storage time interval on the cauda epididymal sperm quality in bucks. The testes collected in normal saline at 4°C were exhibited improved sperm attributes at all periods of storage temperature as compared to the testes collected in normal saline at room temperature as well as preserved at the same temperature. In both cases, with the passage of time of storage the sperm features started deteriorating. That is why the study recommended that to maintain the sperm attributes for a longer period of storage, suitable antioxidants and amino acids may be supplemented. The sperms should be isolated as soon as possible after the collection of the testes to maintain the integrity and quality of the sperm from the cauda. It was evident that, as soon as possible, the sperms from the cauda should be recovered as well as storage temperature should be lesser than 4°C. Suitable strategies should be developed to cryopreserve the cauda epididymal sperms.

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