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Effect of cold storage period on the quality of ram cauda epididymal spermatozoa recovered postmortem

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Abstract: Postmortem spermatozoa recovery is an important tool for harvesting germplasm from genetically meritorious animals and endangered species. The principal motive of this study was to find out whether ram spermatozoa within epididymides kept at 4 °C for 48 h retain their quality in terms of progressive motility, viability, and acrosomal status. Twenty-four testicles with attached epididymides were collected with 8 samples each processed at 0, 24, and 48 h of storage. A good number of motile spermatozoa could be recovered from epididymides by incision method with up to 48 h of storage, although their quality declined significantly ($P < 0.05$) as postmortem storage time increased (motility: 83.33 ± 1.05%, 63.33 ± 2.47%, and 44.17 ± 1.53%; and epididymal sperm output (millions): 3500.00 ± 73.97, 1220.00 ± 1.01, and 666.67 ± 19.78 for 0 (control), 24, and 48 h, respectively). Viability and intact acrosomes (%) were also acceptable, although they decreased significantly with up to 24 h of storage. In conclusion, ram epididymides could be stored at 4 °C for 48 h when epididymal spermatozoa cannot be immediately harvested and cryopreserved.

Key words: Cauda epididymides, spermatozoa, ram, cold storage

Introduction

Preservation of cauda epididymal sperm is an important tool to conserve biodiversity. Since the number of endangered species is increasing, it is therefore mandatory to develop an effective methodology for their germplasm to be harvested after death and then preserved with acceptable quality. People around the globe have developed protocols to preserve the cauda epididymal sperm that are harvested immediately after slaughter or death of an animal, but there is less literature available about the holding of testicles after death at 4 °C for some time before spermatozoa are harvested. Lone et al. (1) reported better quality of sperm when testicles were transported at 4.9–6 °C as compared to ambient temperature from the abattoir to the processing laboratory, and greater numbers of motile and viable spermatozoa were recovered with the incision method as compared to the mincing method (2). However, it is very important to determine the maximum number of hours for which the testicles can be stored at 4 °C while maintaining acceptable quality, because at times the death of an animal is unpredictable and the processing laboratory might also be situated far away from the location at which the death has taken place.

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Foote (3) reported that motile sperm can be recovered from the epididymides of slaughtered bulls after up to 60 h at 5 °C. Kikuchi et al. (4) in pig and Kishikawa et al. (5) in mice also suggested that when a meritorious male animal dies unexpectedly and sperm processing cannot be done immediately, temporary holding of testicles at 5 °C may help to conserve the genetic merit of the animal. Therefore, the objective of the present study was to determine the effect of the time interval between death and sperm collection (0, 24, and 48 h) on the quality of spermatozoa evaluated on the basis of progressive motility, viability, and acrosomal integrity.

Materials and methods

The present study was conducted during the spring season at the Faculty of Veterinary Sciences and Animal Husbandry in the Division of Animal Reproduction, Gynaecology, and Obstetrics of the University of Agricultural Sciences and Technology, Jammu and Kashmir, India, situated at an altitude of 1585 m above sea level. The objective was to determine the effect of holding conditions of cauda epididymides, i.e. time and temperature, on progressive motility, epididymal sperm output, viability, and intact acrosomes of spermatozoa harvested from cauda epididymides of testicles obtained from local abattoirs. Twenty-four ram testicles were collected from 2 local slaughter houses in the city of Srinagar as early as possible and transported in an ice chest. The duration of transportation was 1 h from both slaughter houses. Eight testicles were processed immediately after reaching the laboratory and served as the control. Another 16 testicles were kept at 4 °C in a refrigerator with 8 testicles processed at 24 h and 8 processed at 48 h. The testicles were measured for testicular weight, cauda epididymal weight, and diameter. The weights were determined by electronic balance and diameter by vernier calliper. It is important to mention here that only testicles weighing ≥100 g were selected for this study.

The incision method was applied to recover spermatozoa from the cauda epididymides, in which cauda epididymides were detached from the testicles and then several longitudinal incisions were made on the lower end of the cauda epididymides to expose the spermatozoa to the external environment. Each cauda epididymis was kept in a 35-mm petri dish for 20 min so that the sperm would swim out into the 3 mL of 2.9% sodium citrate buffer. Finally, the cauda epididymides were rinsed with an additional 1 mL of buffer. After the spermatozoa were harvested in petri dishes, the concentration of each sample was found by haemocytometer method. The quality of spermatozoa was determined by measuring progressive motility, live sperm count, and intact acrosome percentage.

The progressive motility was determined following the method described by Zemjanis (6). A drop (100 µL) of sperm sample was kept on a prewarmed, grease-free slide, a coverslip was put over the drop and examined under the high-power objective (40× magnification) of a microscope, and the percentage of progressive motility was determined.

Viability was determined as per the method described by Blom (7). A drop (100 µL) of sperm sample was placed on a clean, grease-free slide and mixed with a single drop of eosin-nigrosin stain. The spermatozoa were allowed to interact with the stain for at least 2 min and then a smear was prepared. The prepared smear was air-dried and examined under oil immersion objective, i.e. at 100× magnification. Spermatozoa that were stained either partially or completely were considered as dead, and spermatozoa that appeared colourless were considered as alive. Two hundred spermatozoa were examined and the percentage of live sperm was determined.

Watson (8) described a procedure for determining acrosomal status in which a small (100 µL) drop of each sample was placed on a clean slide and a smear was made. The smears were air-dried and kept in Hancock's fixative for 15–20 min in a coupling jar. After the prescribed time interval, the slides were washed under slow running tap water for another 15–20 min and finally rinsed with distilled water. The slides were kept in a coupling jar containing Giemsa working solution overnight. The next day, the stained slides were rinsed with slow running tap water, air-dried, and observed under oil immersion objective (100× magnification). Two hundred spermatozoa were examined and the percentage of intact acrosomes was determined.

The data obtained were statistically analysed by one-way ANOVA using SPSS 16 and are expressed

as mean ± standard error of the mean (SEM). The significance of difference between means was analysed by post hoc analysis. The level of significance was set at 5%.

Results

The mean testicular weight and cauda epididymal weight of samples processed at 0, 24, and 48 h, along with biometry, are presented in Table 1. There was no significant difference between the means of the different groups (0, 24, and 48 h) in terms of testicular weight, cauda epididymal weight, and cauda epididymal diameter.

The epididymal sperm output and motility decreased significantly from 0 to 48 h of storage (Table 2). However, the viability and intact acrosome percentage decreased significantly from 0 to 24 h of storage, and there was no significant decline from 24 to 48 h.

Discussion

The cauda epididymides of live animals provide a suitable environment for the immature spermatozoa to become mature and acquire motility. The spermatozoa remain in a quiescent state and exhibit motility only when they come into contact with an external environment like seminal fluid or some media (9–11). The spermatozoa within the body of a dead animal degenerate faster, but many studies have indicated that spermatozoa recovered in some media from cauda epididymides, even many hours after death, remain functional (12–14). The duration and the temperature at which the dead animal was held before the testicles are harvested must exert some negative effect on the viability of spermatozoa (15,16).

In this study, there was a significant ($P < 0.05$) decrease in epididymal sperm output (millions) and progressive motility (%) from 0 to 48 h of storage at

Table 1. Weight and biometric measurements of testicles and their parts (mean ± SEM).

Storage hours	Parameters							
	Weight (g)			Testicle (cm)			Cauda epididymides (cm)	
	Testicle	Epididymis	Cauda epididymides	Length	Breadth	Width	Length	Breadth
0	141.75 ± 12.63	18.36 ± 1.46	8.98 ± 0.74	10.97 ± 0.59	6.1 ± 0.24	5.55 ± 0.22	4.3 ± 0.23	2.53 ± 0.14
24	164.05 ± 12.89	19.31 ± 1.42	8.88 ± 0.83	11.43 ± 0.43	6.3 ± 0.33	5.5 ± 0.18	3.83 ± 0.22	2.59 ± 0.19
48	155.08 ± 11.34	19.5 ± 1.37	9.49 ± 0.4	10.93 ± 0.44	6.12 ± 0.14	5.48 ± 0.13	4.08 ± 0.21	2.63 ± 0.13

Table 2. Effect of cold storage on quantity and quality of ram cauda epididymal sperm during different hours of storage (mean ± SEM).

Storage hours	Parameters			
	Epididymal sperm output (millions)	Progressive motility (%)	Live sperm count (%)	Intact acrosomes (%)
0	3500.00^{a} ± 73.97	83.33^{a} ± 1.05	93.85^{a} ± 1.98	96.29^{a} ± 1.39
24	1220.00^{b} ± 1.01	63.33^{b} ± 2.47	77.11^{b} ± 3.42	91.44^{b} ± 0.89
48	666.67^{c} ± 19.78	44.17^{c} ± 1.53	78.96^{b} ± 1.77	89.92^{b} ± 0.90
Overall	**1795.60 ± 3.00**	**63.61 ± 3.99**	**83.31 ± 2.27**	**92.55 ± 0.88**

Means bearing different superscripts within a column differ significantly ($P < 0.05$).

4 °C. However, the live sperm count (%) and intact acrosome percentage decreased significantly ($P < 0.05$) only up to 24 h of storage, with no significant decrease from 24 to 48 h of storage. The decrease in epididymal sperm output might be ascribed to the decrease in the motility of spermatozoa, because the sperm in the present study were recovered by the incision method, in which sperm are allowed to swim on their own into the medium. Karja et al. (17) also reported a significant decrease of motility and viability of ram spermatozoa collected from epididymides stored at 4 °C for 48 h. However, the values of different sperm quality parameters of this study might be still acceptable even at 48 h of storage at 4 °C (motility = 44.17%, live sperm count = 78.96%, and intact acrosomes = 89.92%).

Lubbe et al. (18) emphasised that the advantage of cold storage for various parameters of sperm quality, especially motility, could be due to the reduced metabolic rate of sperm cells when testicles are kept at 5 °C. The reason for the survival of spermatozoa in epididymides at 5 °C is unclear. However, it was assumed that epididymal fluid could contain unknown cold shock protective factors such as lecithin (19). Sankai et al. (13) suggested that the motility of mouse epididymal spermatozoa decreasing with an increase in storage temperature was related to changes in spermatozoa metabolic activity.

Aguado et al. (20) in ram and Garde et al. (21) in red deer and moufflon found that viability of sperm collected from the cauda epididymides decreased progressively as the time interval from postmortem to sperm harvesting increased. Yu and Leibo (14) reported that the motility of dog spermatozoa recovered from epididymides stored at 4 °C decreased significantly ($P < 0.05$) within the first 48 h of storage, but there was no significant decrease in membrane integrity and acrosome integrity within that time period. However, in the present study, the percentage of intact acrosomes decreased significantly ($P < 0.05$) from 0 to 24 h and nonsignificantly from 24 to 48 h. This variation could be attributed to the differences between species in respect to maintenance of sperm quality within the epididymides after death.

In the present study, even if the quality of the sperm samples decreased significantly due to long storage, the motility and viability of storing spermatozoa might still be acceptable, and assisted reproductive techniques like artificial insemination, in vitro fertilisation, or intracytoplasmic sperm injection could be used to achieve pregnancy with the germplasm of meritorious males or to conserve endangered species. Kikuchi et al. (4) in pig and Kishikawa et al. (5) in mice also suggested that when a meritorious male animal dies unexpectedly and sperm processing cannot be done immediately, temporary holding of testicles at 5 °C may help to conserve the genetic merit of the animal. In conclusion, the testicles of meritorious animals that die unexpectedly may be held at 4 °C for up to 48 h until further processing.

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