Indian Journal of Animal Sciences **82** (8): 854–855, August 2012

Evaluation of DNA integrity of caprine cauda epididymal spermatozoa

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Received: 21 December 2011; Accepted: 10 April 2012

Key words: Caprine Cauda epididymal sperm, Comet assay, DNA integrity, Storage time interval, Supra vital staining

The epididymis of dead animals is a potential source of the viable sperms which can be used in the assisted reproductive technology (Fahring 2000). Domestic goat serves as a good ruminant model for the study of the sperm attributes in relation to wild ruminants. In this context, the present experiment was designed to study the possible recovery of the cauda epididymal sperms as well as the longivity of the caprine cauda epididymal spermatozoa *in vitro* at 4°C with special reference to the DNA integrity.

Testes (30) were collected immediately after slaughter of the animal from the local slaughter house of Mathura. Testes were kept in chilled normal saline and transferred to the laboratory in a thermox. The laboratory processing of testes was carried out within 30 min of reaching the laboratory. Testis were washed and cleaned with phosphate buffer saline (PBS, pH-7.4). Mechanical removal of fascia, blood vessels and sheath of testes was carried out. Care was taken to prevent the damage to epididymis.

The testes were divided into 3 groups and each group was having 10 testes. A set of testes were evaluated immediately after processing (0 h), 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. By removing the fascia and covering, the epididymis was exposed. In each step of processing the testes were thoroughly washed with PBS. After complete removal, the epididymis was kept in PBS for further processing at a pH of 7.4. Morpho-anatomically, different segments of epididymis were identified. The same procedure was followed for the second and third group of testes after 12 and 24 h of storage at 4°C respectively. Total 5 pairs of testis were used for the collection of epididymis and evaluation of sperm attributes under each group.

The cauda part was separated from the testes for the collection of sperms for the comet assay. The sperm samples were transferred to the ependorff tube and kept in refrigerator at 4°C as sperm stock sample for evaluation. Supravital staining was carried out with nuclear specific stain at 0, 12 and 24 h of sperm collection (Esteves *et al.* 2010).

The single cell gel electrophoresis (Comet assay) was performed according to Singh *et al.* (2003) with minor modifications. The comet assay was performed in the cauda epididymal sperms. In brief, 10 µl of sperm 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 pre-warmed (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 (500mM NaCl, 0.1M tris-base, 1mM EDTA, 0.2% DMSO, pH 9.0) for chromatin decondensation followed by electrophoresis at 24 V for 1 hour. The slides were treated with neutralization buffer (20 mM Tris, pH 7.4) and dried in incubator at 37°C. The slides were then incubated in PBS buffer containing ethidium bromide (0.01%) for 10 min. After wiping the stain, the 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. The obtained results were analyzed by using the SPSS software version 14 for the Comet positive spermatozoa by performing analysis of variance (ANOVA).

In this study we reported a significant difference ($P<0.05$) in supra vital negative sperms between the 3 intervals of time. We got 87.58 ± 0.94, 30.18 ± 0.94 and 7.18 ± 1.94% of supra vital negative sperms at 0, 12 and 24 h respectively. Similarly we got a significant difference ($P< 0.05$) in comet positive sperms between the 3 intervals of time. We reported 12.68 ± 1.54, 44.64 ± 2.94 and 87.18 ± 2.64% of comet positive

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sperms at 0, 12 and 24 h respectively. From the data, it was evident that with the rise in storage interval of time there was a significant reduction in the viable sperm count as well as increase in the number of sperms having damaged DNA.

The supravital negative sperms indicated the viable sperms (live) who did not take the nuclear stain. The data were taken as the base data to correlate with the Comet positive sperms. We did not get any relevant literature in goats to compare our results. However, Swain *et al.* (2011) reported a steady decline in the percentage of the live sperms with the passage of time in canines. 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*. 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 and the viability of the sperms will remain intact.

The major part of the study was focussed on the evaluation of the DNA integrity of the cauda epididymal sperms at various intervals of time. Sperm nucleus contains highly folded and compacted DNA (Sringam *et al.* 2011). This compaction in the DNA is associated with the formation of disulfide bond between the DNA and the protamines. Epididymal transit mediates the chromatin condensation as well as DNA compaction. This compaction mediates easy passage of the sperms in the female genital tract (Aitken *et al.* 1998). In our study, we aimed at the evaluation of sperm DNA integrity *in vitro* after recovery from the epididymis. As the time progressed, we noted that the DNA structure was compromised in the sperm cells and the degree of DNA damage was increased. In literature, we got many studies related to loss of DNA integrity in cryopreserved semen of various animals, but we did not get any information regarding the cauda epididymal sperms in goats.

With the progression of the storage time, the antioxidant defence to the sperms got compromised and that may be the major factor which induced damage to the DNA (Aitken *et al.* 1998). It was evident from our study that supra-vital negative sperms were decreased with the progression of the time. The exact mechanism was not elucidated in the study but we assumed that the long time storage of testes in the refrigerator without any preservative and extender caused a physiological disturbance in the sperm functions and hence the reduction in the viability of the sperms.

SUMMARY

Sperm DNA integrity of the sperms recovered from cauda epididymis was evaluated by Comet assay to study the sperm DNA integrity and viability of the sperms through highly efficient fluorescent staining. With the advancement of the storage time, the viability and intactness of the sperm DNA got compromised. Our findings will strongly form a baseline data for further studies about the cauda epididymal sperms in terms of DNA integrity. We reported that cauda can be a source of retrieval of sperms for assisted reproduction after the death of the animal. We strongly argue for the development of suitable extenders with antioxidants for the reduction of free radical mediated damage to the sperms as well as optimal cryopreservation technique. The sooner the recovery of the sperms from the cauda the better the viability and an increased probability for fertilisation. Further studies are required to elucidate the key cellular and molecular mechanisms which are associated with the loss of quality of the epididymal sperms.

ACKNOWLEDGEMENTS

We are thankful to Dr Rakesh Goel, Assistant Professor, Department of Genetics and Biostatistics, DUVASU, Mathura for analysing our data.

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