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Qualitative ultrastructural changes and morphometry of deccani sheep spermatozoa preserved with egg yolk citrate extender

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Abstract: The present investigation was aimed to study the sequential changes in the sperm cell deterioration during liquid storage of Deccani sheep breed semen from dilution to 48 h of storage along with its seminal characteristics and sperm morphometric measurement. Thus the two Deccani adult rams (aged 2 years), were selected (six ejaculates/each ram) and the collected semen was diluted with Egg yolk citrate extender (EYC) (final concentration - 400 million spermatozoa/0.2 ml semen).Seminal characteristics were assessed along with sperm morphological changes by Electron microscopy immediately after dilution, at 24 and 48 h of storage, respectively. Sperm morphometry was analysed by Image analysis.The percentage of Individual motility, Live spermatozoa, Acrosomal integrity and Hos-test reactive sperm decreased significantly ($P<0.05$) from 80.41 to 49.16%, 82.75 to 51.25%, 94.16 to 83% and 76 to 48.58%, respectively during liquid storage of semen from initial dilution to 48 h of storage. The sperm head length (µm), Head width, sperm head area (µm^2), sperm head perimeter (µm), mid piece length (µm), proximal mid piece width (µm), distal midpiece width (µm), volume of mid piece (µm^3) and acrosomal cap length (µm) were 7.80, 4.33, 26.84, 20.63, 14.03, 0.74, 0.51, 4.54 and 5.24, respectively. Electron microscopic qualitative evaluation revealed that the main site of injury is the apical ridge of ram spermatozoa when stored at 5ºC. The electron density of the mitochondria reduced indicating concomittant depletion of ATP and loss of motility resulting in reduction of fertility.

Keywords: Liquid preservation, Sperm cell deterioration, Sperm morphometry, Electron microscopy, Mitochondria

INTRODUCTION

Artificial insemination has yielded lower conception rates than natural mating in sheep population due to failure of Artificial Insemination (A.I) that can provide sufficient number of active sperm in the oviduct at the time of fertilisation (Marai and Owen, 2013) as these species were highly sensitive to cold shock (4ºC) compared to bull, rabbit and humans causing subsequent loss of membrane integrity of the acrosomal region (Fiser and Fairfull, 1989; Aitken and Fisher, 1994). When ram semen was stored at 5°C, the rate of decrease in fertility was 10–35% per day of storage (Salamon and Maxwell, 2000) and it maintained acceptable motility and viability up to 48 hours due to increased proportion of capacitated and acrosome reacted spermatozoa contributing to lower viability. Eventhough, Light microscopy reveals general detail about different part of spermatozoa; electron microscopy can reveal much smaller changes in the spermatozoon than optical methods (Prinosilova *et al.*, 2012). So it is considered as an essential tool for the evaluation of physiolological changes occuring in a spermatozoon during liquid storage or chilling of semen at 4-5ºC. Moreover, TEM delivers insights in the inner and outer fine structures of spermatozoa (Pesch and Bergmann, 2006). The present study was designed to study the seminal characteristics of Deccani sheep along with qualitative assessment of sequential changes in the deterioration of spermatozoa when stored at 5ºC. Thus, to conclude the reason for reduction in motility and fertility rate after liquid storage of semen.

MATERIALS AND METHODS

Ethical approval: The present study was conducted after approval by the Institutional Animal Ethics Committee.

Experimental animals and management: Two Deccani sheep (aged 2 years), with body weight of 45 kg having good libido and adaptability to Artificial Vagina (AV) semen collection technique were selected. The rams were maintained under intensive management in loose housing system. The animals were fed

daily with concentrate feed at the rate of about 300 gm per day per head in addition to adlibitum quantity of green fodders.

Semen collection and dilution: The semen ejaculates (n=12) was collected from two rams located at Livestock Farm Complex (LFC), College of Veterinary science, Rajendranagar (longitude: 78.4018° E, latitude: 17.3203° N), Hyderabad after training for 2 weeks prior to the study. Semen was collected from the rams twice in a week with the help of standard artificial vagina (AV) (45°C). Semen was divided into 2 aliquots - one part for assessment of fresh semen characteristics and other part was diluted with Egg yolk citrate extender (EYC) [2.9% aqueous solution of trisodium citrate, 20% (v/v) of egg yolk, streptomycin (1000 µg/ml) and pencillin (1000 IU/ml)] (1:4 ratio) to a final concentration of 400 million spermatozoa/0.2 ml semen.

Fresh semen characteristics

Semen volume, colour and consistency: The volume (ml) of ejaculate was recorded directly from the graduated semen collection tube (Mortimer, 2000). Colour and consistency of semen were also noted by visual observation directly from the graduated semen collection tube (Elsharif, 2010).

Mass activity: Mass sperm motility score is a rapid test and has the advantage of being easy to perform, inexpensive from an economic perspective and predictive of sperm fertilizing capacity (David *et al*., 2008). Immediately after collection, a drop of neat semen was placed on a dry, clean glass slide kept on biotherm stage maintained at 37°C and examined under low power (100X) without a cover slip. The scoring of mass activity of the semen samples was done as per the wave pattern (Herman *et al.,* 1994).

Sperm concentration: Sperm concentration was determined by using improved Neubauer counting chamber after dilution with semen diluting fluid (1:200).

The sperm concentration was calculated using the Equation No.1 (Saxena, 2000):

Sperm concentration = N X D X 4000/n per mm^3

Where N = Number of spermatozoa counted; D = Dilution rate; n = number of tertiary squares counted.

Diluted semen characteristics: The semen diluted with EYC was evaluated immediately after dilution (0 h) and after 24 and 48 h of storage at 5ºC as follows:

Individual Motility: 10µl semen mixed with 200µl normal saline was used for fresh semen evaluation. The individual motility of spermatozoa was assessed by placing a cover slip on a drop of diluted semen on a clean glass slide under the microscope (10X) with biothermstage attached. The motility was observed under high power at 400X magnification and expressed in terms of percentage of progressively (0-100) motile sperms.

Live spermatozoa percentage: To ascertain the percentage of live spermatozoa, a smear was prepared by mixing semen (1 drop) with Eosin-nigrosin (2 drops) (Eosin 1.67 gm, Nigrosin 10 gm, Distilled water 100 ml) and examined under high power (1000X) objective (Campbell *et al*., 1956). All stained spermatozoa were considered dead and the unstained spermatozoa as live.

Sperm abnormalities: 3%Rose Bengal stain (3 gm Rose Bengal powder, 1ml Formalin added to 100 ml Distilled water) was used (Shukla, 2011). Semen smear was stained with rosebengal stain for 5-6 minutes and 100 sperm cells were counted at high magnifications (1000X).

Acrosomal Integrity: The acrosomal integrity was evaluated by Giemsa staining method described by Watson (1975). The semen smear slides were put into 5% formaldehyde (fixation) at 37^0C for 30 min and then stained with Giemsa working solution [Giemsa's stock solution -3 ml, phosphate buffer (pH-7) -2 ml and dist. water- 45 ml] and kept at 37ºC for 3 hrs. 100 sperm cells were counted at 1000X magnification.

Hypo-osmotic Swelling (HOS) Test: Plasma membrane integrity was assessed by this test and was done according to the procedure of Jeyendran *et al*. (1984). 1ml of pre-incubated (at 37ºC) hypo-osmotic solution (7.35 g trisodium citrate, 13.51 g fructose, distilled water to 1,000 ml; 150 mOsm/L) was mixed with 0.1 ml of diluted semen in a small test tube and then incubated in water bath at 37°C for 30 minutes. A drop of incubated semen was examined under phase contrast microscope at 400X magnification for swelling (ballooning or curling) of sperm tails.

Methylene Blue Reduction Test (MBRT): This test was done according to method adopted by Herman and Madden (1953). To 1ml of diluted semen, 100 µl of 1% methylene blue solution (50mg of methylene blue dissolved in 100ml of 2.9% sodium citrate dihydrate solution) was added and incubated in water bath (47° C) after sealing it with 1cm thick mineral oil layer to ensure anaerobic condition.Time required for the colour change was recorded.

Semen samples processing protocol for scanning electron microscopy (SEM) and transmission electron microscopy (TEM): Samples were processed according to the procedure given by Boonkusol *et al.* (2010) with slight modification. Semen ejaculates (n=6) from two rams were collected using artificial vagina. Immediately after collection, samples were segmented into 0, 24 and 48 h and diluted with Egg yolk citrate extender at 1:4 ratio. Samples at 0 h were immediately fixed in 2.5% gluteraldehyde ($C_5H_8O_2$) in 0.1 M phosphate buffer (pH 7.2) for 24 hrs at 4°C. Remaining samples (24 and 48 h) were preserved at 4ºC and were fixed on day 1 and 2 respectively. Samples for SEM were post fixed in 2% aqueous osmium tetroxide for 4 hrs. The processed samples were scanned under scanning electron microscope (SEM – Model: JOEL –JSM 5600) at required magnifications. For TEM, samples were washed with PBS for 4 times

each 45 minutes after pre-fixation with gluteraldehyde and then post fixed in 1% aqueous Osmium tetroxide for 2 h.. Ultra thin (60 nm) sections were mounted on copper grids and stained with saturated aqueous Urenyl acetate (UA) and counter stained with Reynolds lead citrate (LC). Viewed under TEM (Model: Hitachi, H- 7500 from JAPAN) at required magnifications as per the standard procedures at RUSKA lab's college of Veterinary science, P.V. Narsimha Rao Telangana Veterinary University, Rajendranagar, Hyderabad, India.

Sperm morphometry assessment: Images obtained (Fig. 1 to 5) from Electron microscopy were used for sperm morphometry assessment. Sperm measurements were assessed using ImageJ software (National Institutes of Health, USA; version 1.45e).The following sperm morphometry parameters were determined: head width, head length, area, perimeter, proximal midpiece width, distal midpiece width, midpiece length. Each sperm head was measured for four primary parameters [area (A, in μm^2, as the sum of all pixel area contained within the boundary), perimeter (P, in µm, as the sum of external boundaries), length (L) and width (W) (in µm) (Yaniz *et al*., 2012). From these measurements, estimation of sperm midpiece volume was done using the Equation No. 2 for the volume of a truncate cone (Ros-Santaella *et al*., 2014):

Volume = $(\pi \times L/3) \times (R^2 + r^2 + R \times r)$

Where, L is the length of the midpiece, R is the half proximal midpiece width, and r is the half distal midpiece width.

Statistical analysis: Data were statistically analyzed by using Statistical Package for Social Science (SPSS, version 16). ANOVA (Analysis of variance) was used to study the effect of duration of storage on the diluted and chilled (5ºC) semen. The significance of all the parameters were measured at $P < 0.05$ level significance. The comparison of the means was done by Duncans test at a probability level of 5%.

RESULTS AND DISCUSSION

The mean (±S.E) seminal traits of fresh semen and diluted semen of Deccani Rams from immediate dilution with EYC (0 h) to 24 and 48 h of storage (5ºC) were presented in the Table No. 1 and 2. The mean semen volume in the Deccani ram species was 0.75 ± 0.07 ml which was comparable with the volume (0.65 ml) of Garut rams (Boediono *et al.*, 2004), and Chottanagpuri rams (Bharti *et al.*, 2009) while higher semen volume (1.5 ml) was recorded in Pleven blackhead rams (Yotov *et al.*, 2011) and Baluchi x Moghani, Ghezel x Baluchi, Ghezel x Merino, Merino x Moghani rams (Asadpour *et al.*, 2012). These differences between various reports could be due to season of the study, breed, age of rams, environmental factors (Moghaddam *et al*., 2012). In other breeds, more volume was due to the higher body weight and scrotal circumference. Moreover, highest ejaculate volumes were recorded in October (Autumn) and the lowest in June (Summer), and the overall mean ejaculate volume was highest in autumn (Gundogan *et al.*, 2007).

Table 1. Deccani sheep seminal traits.

Seminal traits	Mean ± S.E (n=12)
Volume	0.75±0.07
Concentration	10386±537
Mass activity	4.00±0.24
Colour	Creamy white
Consistency	Thick

Values in parenthesis are the no. of ejaculates collected from the rams.

The semen colour and consistency of Deccani ram was creamy white and thick in the present study which was similar to that of Ossimi, Barki, Rahmani, Awassi, Finnish ram semen (creamy and thick) (Kayali *et al.*, 2014). The average semen consistency of Deccani ram was thick in the present study while moderate consistency was reported for Garut ram semen (Nalley *et al.*, 2013). The variation may be due to sperm concentration (Youngquist, 2007) and in the present study the breed produced average semen concentration of 10386 ± 537 millions/ml which was much higher compared to the concentration of Garut rams (Boediono *et al.*, 2004). The mean mass activity (0-5 scale) of Deccani ram semen was 4.00 ± 0.24 which was similar to the mass activity of Kivircik and Awassi rams (Alcay *et al.*, 2014) and lower compared to that of Ossimi, Barki, Rahmani, Awassi, Finnish rams (Kayali *et al.*, 2014). The variation might be due to genetic and environmental changes (Abdel-Rahman *et al.*, 2000), nutritional and physical changes (Toe *et al.*, 1994).

The percentage of Individual motility, live spermatozoa, Acrosomal integrity and Hos-test reactive sperm decreased significantly ($P<0.05$) from 80.41 to 49.16%, 82.75 to 51.25%, 94.16 to 83% and 76 to 48.58% respectively (Table No.2) as the duration of storage increased from 0 h to 48 h (5ºC). The decrease in these parameters might be contributed to the production of ROS species (Radical oxygen species) because the sperm plasma membrane is rich in polyunsaturated

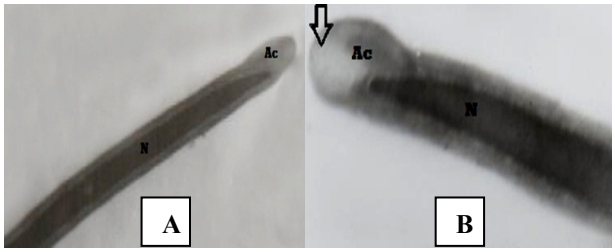

Fig. 1. *Sagittal sections of anterior end of Deccani ram spermatoozoon observed under Transmission Electron Microscopy (TEM). A. Undamaged normal head of ram spermatozoa showing Nucleus (N) and Acrosomal substance (Ac) after dilution (0 h) with EYC. x 10,000. B. 24 h after chilling (5C). (↑) indicates reduced acrosomal substance (Ac). x 30,000.*

Table 2. Mean (± S.E) of Deccani Ram seminal parameters after dilution at different durations of storage.

Parameters	Duration of storage (n=12)		
	0 h	24 h	48 h
Individual Motility (%)	80.41±0.41[a]	64.16±1.48[b]	49.16±1.60[c]
Live spermatozoa (%)	82.75±1.81[a]	72.83±1.43[b]	51.25±0.89[c]
Acrosome Integrity (%)	94.16±0.96[a]	88.83±1.40[b]	83.00±1.70[c]
Sperm Abnormalities (%)	4.25±0.27[a]	4.33±0.39[a]	4.83±0.27[a]
HOS test Reactive sperm (%)	76.00±1.79[a]	66.58±1.18[b]	48.58±1.43[c]
MBRT (min)	1.36±0.08[a]	3.45±0.08[b]	6.02±0.13[c]

Values are mean ± S.E and values in parenthesis are the no. of ejaculates collected from the rams. Means with different superscripts (a, b and c) in the same row differ significantly (P<0.05).

Table 3. Deccani ram sperm morphometric parameters (Mean ± S.E) measured under Electron microscope using image analysis.

PARAMETERS	Overall Mean ± SE (n=48)*
Head Length (µm)	7.80 ± 0.15
Head Width (µm)	4.33 ± 0.04
Area (µm^2)	26.84 ± 0.33
Perimeter (µm)	20.63 ± 0.06
Midpiece Length (µm)	14.03 ± 0.37
Proximal Mid Piece Width (µm)	0.74 ± 0.02
Distal Midpiece Width (µm)	0.51 ± 0.01
Volume of Mid Piece (µm^3)	4.54 ± 0.22
Acrosomal Cap Length (µm)	5.24 ± 0.01

*n = No. of spermatozoa measured from six semen ejaculates.

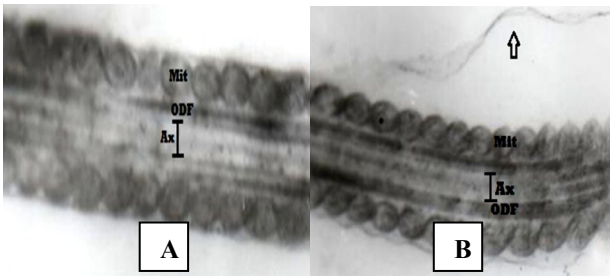

Fig. 2. *Sagittal sections of mid piece of Deccani ram spermatozoon observed under Transmission Electron Microscopy (TEM). (A) After dilution (0 h) showing intact plasma membrane and well-preseved mitochondria (Mit) with evenly distributed electron density of mitochondria x 25,000. (B) At 48 h of storage - loss of intactness of plasma membrane indicated by an arrow. x 25,000. (Ax: Axoneme; Mit: Mitochondria; ODF: Outer Dense fibers).*

fatty acids and therefore susceptible to peroxidative damage with pH changes and consequent loss of membrane integrity, decreased sperm motility and eventually loss in fertility, resulting from reactive oxygen species during aerobic incubation (Yaniz *et al.*, 2008). There was no significant difference (P≥0.05) between the sperm abnormalities reported during different durations of storage. The MBRT time (min) increased significantly (P≤0.05) as the period of storage increased from time of dilution to 48 h of storage indicating the reduced dehydrogenase activity (metabolic activity) of spermatozoa.

Sperm morphological changes assessed by SEM and TEM: The spermatozoal structure as observed immediately after dilution showed no significant changes observed by both SEM and TEM with intact plasma membrane (PM) of the head and the acrosome showed its intactness without much change (Fig. 1A). Mid piece was undamaged with well preserved mitochondria (Fig. 2A) which were in accordance with the findings of Kakar and Anand (1984). The electron density of the acrosomal substance (Ac) was normal and homogenous (Fig. 3A) which was inaccordance with the ultrastructure of the freshly collected ram spermatozoa reported by Jones *et al*. (1973).

At 24 h of storage at 5ºC, there was loss of intactness of the PM over the head of spermatozoa, vesiculation of the outer acrosomal membrane (OAM) and PM. The electron density of the Ac has been reduced at the apical ridge of the head (Fig. 1B) of the spermatozoa indicating damage of the OAM at the level of apical ridge which lead to loss of acrosomal contents. The electron density of the Mitochondria was slightly reduced (Fig. 3B) and the PM showed discontinuity at the level of midpiece (Fig. 2B).

At 48 h of storage at 5ºC, most of the spermatozoa showed loss of the acrosomal membrane, PM and damage to acrosomal cap (Fig. 4A). There was marked loss of intactness of PM over the midpiece and most of the spermatozoa showed no PM. The mitochondria showed marked signs of degeneration. The large intracristal spaces or translucent areas in the mitochondria of midpiece (Fig. 3C) indicated the depletion of sperm energy reserves (ATP) which lead to lower sperm motility. Sperm motility is lost as a consequence of lipid peroxidation not only due to ROS attack (Shimizu *et al*., 1983), but also due to the concomitant depletion of ATP (Lamirande *et al*., 1997). Vacuolation was found beneath the outer acrosomal membrane when visualized at 10,000X magnification. Spermatozoa even showed complete loss of Ac with acrosomal membrane showing wavy pattern at 48 h of storage (5ºC) (Fig. 4B). The signs of degeneration were severly marked at 48 h of storage in semen samples showing bare nucleus with adherent basal plate (Fig. 4C) and nuclear condensation in the spermatozoa.

Sperm morphometry: The morphometric measure-

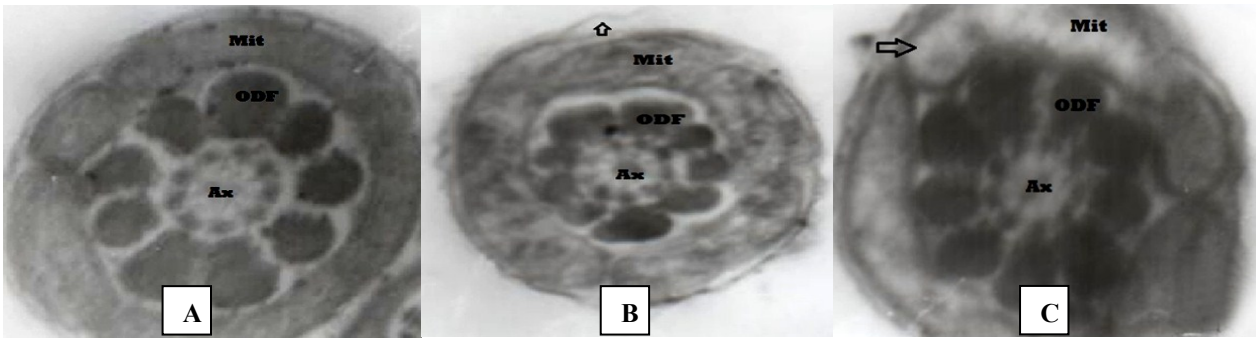

Fig. 3. *Cross section of Midpiece observed underTEM. (A) After diution (0 h) - Midpiece showing evenly distributed electron density of mitochondria. Note axial 9+2 fiber pattern. x 50,000. (B) After 24 h of storage - showing discontinuity in plasma membrane and decreasing electron density. x 40,000. (C) At 48 h of storage - showing condensed mitochondria with large intercristal spaces (↑). x 70,000. (Ax: Axoneme; Mit: Mitochondria; ODF: Outer Dense fibers).*

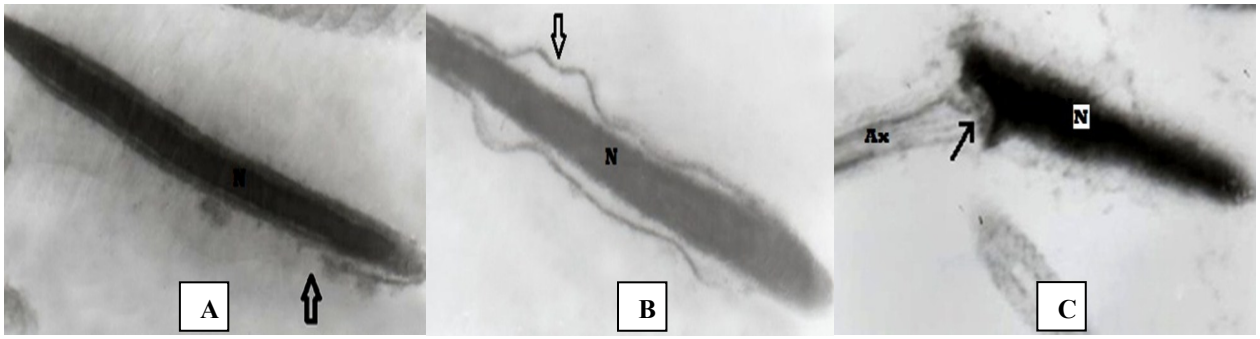

Fig. 4. *Sagittal section of spermatozoon head after 48 h of storage (5ºC) observed under TEM. (A) Showing complete loss of plasma mebrane. (↑) discontinuity in acrosomal membranes. x 20,000. (B) showing complete loss of acrosomal substance with acrosomal membrane showing wavy pattern . x 20,000. (C) Showing bare nucleus and the arrow indicates adherent basal plate. x 10,000. (Ax: Axoneme; N: nucleus).*

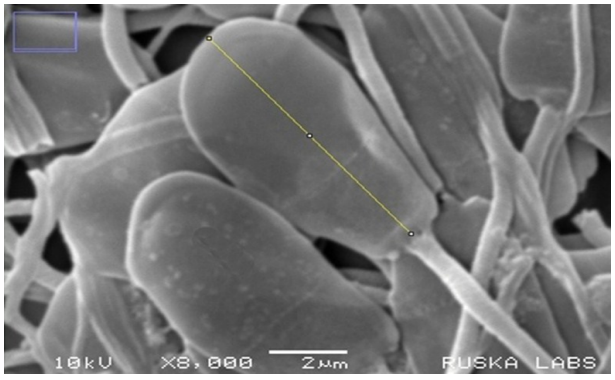

Fig. 5. *Measurement of Sperm head length by using ImageJ Software*

ments of Deccani sheep spermatozoa (n=12) where sperm head length (µm), Head width, sperm head area (µm^2), sperm head perimeter (µm), mid piece length (µm), proximal mid piece width (µm), distal midpiece width (µm), volume of mid piece (µm^3) and acrosomal cap length (µm) were 7.80, 4.33, 26.84, 20.63, 14.03, 0.74, 0.51, 4.54 and 5.24, respectively. The acrosomal cap covered 66.58% of sperm head surface accounting for 2/3rd part of sperm head surface as reported by Kakar and Anand, (1984). The sperm head length, width and area obtained in the present study was in accordance with that of Rasa Aragonesa ram sperm (7.65 µm, 4.58 µm and 29.93 µm^2) assessed by computerized sperm morphometric analysis (Marti *et al.*, 2012). The sperm head length and area in the present study was slightly lower than the measurements obtained in Merino ram sperm (8.11 µm and 39.43 µm^2) (Boshoff, 2014). The sperm head area, length and perimeter were lower than that of Rasa Aragonesa ram sperm (30.05 µm^2, 8.23 µm and 22.28 µm respectively) assessed by image analysis (Yaniz *et al*., 2012). The present study indicates that sperm morphometry of the Deccani breed is inagreement with the other breeds with a slight variation. The variation might be due to breed, season, and the quality of sperm ejaculates (Courot *et al.,* 1981). The present study indicates that image analysis is useful for morphometric measurement of spermatozoa. This software is free, a new automatic, objective and repeatable method to evaluate the morphometry of sperm heads and fulfils the conditions of an ideal method for the morphometric analysis of sperm heads (Yaniz *et al*., 2012).

Conclusion

Liquid storage (5ºC) of Deccani sheep semen deteriorated semen quality by 48 h of storage which has been revealed by optical microscopy but detailed investigation was provided by Electron microscopy. Loss of

intactness of plasma membrane and acrosome, decreased density of mitochondrial matrix, vacuolation (acrosome reaction like changes) beneath plasma membrane were clearly evaluated by EM. Sequential changes in the diluted semen indicate that the main site of injury is the apical ridge of ram spermatozoa and the liquid stored semen (5ºC) should be inseminated within 48 h of storage for better conception rates. The sperm morphometric measurements were within the normal limit.

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