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Saurabh
Department of Veterinary gynecology and obstetrics, College of Veterinary Science & Animal Husbandry, Narendra Dev University of Agriculture & Technology, Kumarganj, Faizabad, Utter Pradesh, India

Sushant Srivastava
Department of Veterinary gynecology and obstetrics, College of Veterinary Science & Animal Husbandry, Narendra Dev University of Agriculture & Technology, Kumarganj, Faizabad, Utter Pradesh, India

Pushkar Sharma
Department of Veterinary gynecology and obstetrics, College of Veterinary Science & Animal Husbandry, Narendra Dev University of Agriculture & Technology, Kumarganj, Faizabad, Utter Pradesh, India

Vijay Gautam
Department of Veterinary gynecology and obstetrics, College of Veterinary Science & Animal Husbandry, Narendra Dev University of Agriculture & Technology, Kumarganj, Faizabad, Utter Pradesh, India

Correspondence
Sushant Srivastava
Department of Veterinary gynecology and obstetrics, College of Veterinary Science & Animal Husbandry, Narendra Dev University of Agriculture & Technology, Kumarganj, Faizabad, Utter Pradesh, India

Effect of ascorbic acid on preservability of spermatozoa of buffalo bull after storage of epididymis at temperature 4 ^{0}C and -196 ^{0}C

Saurabh, Sushant Srivastava, Pushkar Sharma and Vijay Gautam

Abstract
The present study was carried out to evaluate the influence of ascorbic acid (@ 2mM after incorporation in on quality of spermatozoa after storage of epididymis at temperature 4 ^{0}C & -196 ^{0}C. Cyto-morphological characteristics of epididymal semen from forty testes stored at room temperature, refrigerated and and -196 ^{0}C were evaluated). Similarly, tris dillutor with ascorbic acid were infused inside the ligated epididymal part cauda and stored at 4 ^{0}C (T_2) and -196 ^{0}C (T_4) and further evaluated for cryoprotectant efficacy of ascorbic acid. A significant variation in initial motility, viability, and abnormal sperm count were observed among the groups. In present study the motility of spermatozoa was significantly (P<0.01) positively correlated with live count per cent and HOS reactive spermatozoa per cent, whereas, it is negatively correlated with sperm abnormality per cent. On histological examination of epididymis revealed that cryoinjury during storage at low temperature might be a cause of poor post-thaw recovery in T3 and T4 treatment. On the basis of present study it can be concluded that cryopreservation of whole epididymis may be utilized as one of the potential method for storage of epididymal spermatozoa and ascorbic acid as an additive have a promising results in cryopreservation of epididymal semen.

Keywords: Buffalo bull, epididymal spermatozoa, ascorbic acid and cryopreservation

1. Introduction
Artificial insemination (AI) is one of the major reproductive biotechnologies of the modern era through which rapid genetic improvement in livestock has been achieved in the developed countries [28]. To raise genetic potential of livestock, AI with frozen-thawed spermatozoa was introduced in most of the developing countries more than three decades ago, yet it has not been applied in buffaloes on a large scale [5, 6]. It is reported that viability or fertility of frozen thawed semen is lower in buffalo as compared to cattle [4, 5, 40].
The fertility of the spermatozoa taken from cauda epididymis is more or less the same as spermatozoa from the ejaculate [19]. If those gametes are recovered and used to produce embryos by assisted reproductive technique is possible to have progeny even after animal are dead [47]. Preservation of cauda epididymal sperm is an important tool to conserve biodiversity. Obtaining caudal epididymal sperm is an important technique in the propagation and conservation of animal specimens with high genetic values after serious injury or from dead animal specimens with high genetics values after serious injuries or from dead animals [15], endangered species and pets [16]. The recovery and freezing of viable sperm from epididymis of dead animal (post-mortem recovery) is an interesting option for preserving male gamete and thus for maintaining germplasm banks. This process has been reported in dog [32]; rabbit [37]; bull [17] and buffalo bull [28].
Cryopreservation of epididymal spermatozoa from accidentally dead animal and use of this germplasm by artificial insemination are the good options for the propagation of this breed of animal. Successful cryopreservation of the epididymal spermatozoa of murrah bull needs suitable cryo-diluent capable of protecting sperm from cryo-damage. During cryo-preservation buffalo bull spermatozoa are more susceptible to oxidative damage as compared to cattle bull spermatozoa [29, 30, 34]. It is believed that this difference is due to higher contents of polyunsaturated phospholipids present in plasma membrane of buffalo bull spermatozoa [41]. Freezing process accelerate the production of ROS molecules which may decrease the viability of buffalo bull spermatozoa during storage [18, 43].

Ascorbic acid (vitamin C) may act as an oxidant at low concentrations and as an antioxidant at high concentrations [1, 12]. Ascorbic acid increases the percentage of live, an acrosome intact spermatozoa during storage at 5°C. Ascorbic acid is a very efficient antioxidant and a scavenger of oxygen free radicals which are toxic products of many metabolic processes [15] and that observation also shows that addition of antioxidants such as ascorbate and alfa-tocopherol to dilutors improves sperm quality by exerting protective effect on both metabolic activity and cellular viability of frozen bovine semen [10, 36]. Cysteine is an amino acid having the ability to penetrate the cell and protect the cell bio-membrane system from the deleterious effects of free radicals by scavenging them directly [11]. On the basis of previous information on cattle spermatozoa1 [1, 9, 10], we hypothesized that addition of natural antioxidants (vitamins C) in the semen extender may also be a step closer to improve the frozen-thawed quality of buffalo spermatozoa.

2. Materials and Methods

2.1 Collection of Samples

Buffalo bull testes were collected from Arora (AOV Ltd.) and Mass agro food Pvt. Ltd. located at Unnao, Uttar Pradesh during October 2014-March 2015. The testicles were brought to the laboratory in a plastic bag containing normal saline solution (NSS), kept in air-tight sterile cryobox (4 ^{0}C) and were processed within 6 hrs after collection. Forty pairs (40) of testis were divided into five groups (T_0, T_1, T_2, T_3 & T_4,) and each group contain eight pairs testis. Tunica albugenia were removed from the testes. Fat-pad, blood vessels, adipose and connective tissues were cleaned carefully, and washed thoroughly with cold NSS. As per anatomical positions, ligatures were placed unilaterally at the cauda epididymis (i.e. posterior region of the corpus epididymis and anterior region of the vas deferens, respectively).

2.2 Inoculation, equilibration and freezing procedures

Cytomorphological characteristics were evaluated and semen was collected from the eight pairs (To) of testicle within 6 hrs of slaughter of animals. A tris dilutor was inoculated into the cauda epididymis of group T_1 & T_3 and dilutor containing Tris and ascorbic acid @ 2mM was inoculated into the cauda epididymis of group T_2 & T_4 with the help of 30 gauge needles. Each group of epididymis were wrapped separately with aluminium foils to prevent the deposition of ice crystals directly on epididymal surfaces and equilibrated at 5 ^{0}C in refrigerator for 2 hours. After equilibration, each group of epididymis was stored immediately at their respective temperature at 4 ^{0}C in group T_1 & T_2 and at -196 ^{0}C T_3 & T_4 groups for 24 hours [28].

2.3 Thawing and Evaluation of Cauda Epididymal Spermatozoa

Thawing of the epididymis was performed after 24 hrs of storage. Before thawing aluminium foil were removed from the testes and epididymis and were allowed to warm to room temperature (35 ± 2°C) for 2 min and immersed immediately into a water bath at 38.5 ± 1°C for 5 min till the outer surface of the epididymis were softened.

Thawed cauda epididymis was dissected carefully from both the ligated ends of vas deferens and corpus epididymis. Each cauda was kept into separate Petri dishes containing 5 ml of 0.15 M phosphate buffer saline (PBS) pH 7.4, previously warmed to 37°C. Caudal portions were minced carefully with razor blades and were allowed to suspend in the buffer for 5 min at 37°C. Gentle pressure on the excised tissues was given by separate glass rods.

The volume of flush was measured with micropipette, subtracting the volume of buffer used for flushing the cauda. Semen samples were immediately estimated for volume, colour, consistency, concentration/ml, and percent progressive motility, percent live spermatozoa count, sperm abnormalities and percent HOS reactive spermatozoa in frozen-thawed epididymal semen [28].

2.4 Initial Motility Percent

The initial motility as percent progressively motile sperm of freshly diluted caudal semen was recorded. A drop of semen was placed on a pre-warmed glass slide and covered with cover slip. The per cent of motile spermatozoa was assessed subjectively at 37 ^{0}C, using a heated stage, by using 5-6 fields per slide was observed under phase contrast microscope (40X). Spermatozoa that moved due to swimming regardless of its speed, was considered as to be motile. The motility was regarded as percentage of progressively motile spermatozoa after storage of testis at different temperature [41].

2.5 Viability (%)

The percentage of live and dead epididymal spermatozoa of bull testis was estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al.*, 1953). The composition of the stain included Eosin-y 1.67 g and 10 g of Nigrosin in 100 ml of 2.90% sodium citrate buffer. The stain was matured and then used. The smears were prepared in duplicate, after mixing; a small drop of neat semen with four drops of stain on clean grease free microscopic slide at 37 ^{0}C, a thin smear was prepared. Two hundred spermatozoa were counted under the oil immersion, objective (100X) of a phase contrast microscope for estimating the percentage [41].

2.6 Sperm abnormality percent

Abnormal morphology of spermatozoa was studied by examination of wet smear and dry smear. Wet smear were prepared by suspending semen in formal saline solution and dry smear were stained with eosin-nigrosin stain, percentage of abnormal sperm was recorded [41].

2.7 Histopathological study

Testis were flushed with cold normal saline and then fixed by complete immersion in 10% neutral buffered formalin, labelled and kept for two days, followed by preservation in 70% ethyl alcohol. They were dehydrated ascending grade of ethanol (50%, 70%, 95% and absolute alcohol), cleared in xylene and embedded in paraffin wax. Blocks were prepared and 4µm were cut by sledge microtome. The paraffin embedded section were deparaffinised, washed and stained with haematoxylin and eosin (H & E). The stained slides were examined under light microscope [44].

2.8 Statistical analysis

Statistical analysis was performed using Graph Pad Prism software (Graph Pad software, San Diego, CA, USA). Results were expressed as Mean ± S.E. and all statistical comparisons were made by means of the one–way ANOVA. P value <0.05 was considered significant.

3. Results and Discussion

3.1 Initial Motility (%)

The average per cent initial motility of epididymal semen of each groups are presented in table-1. Per cent motility differed

significantly (P<0.05) among the group. Present finding was in agreement with the observation as several researchers where the mean values of motility of fresh epididymal semen varied between 50 to 89.4% [21-24, 27, 32, 38].

Bovine spermatozoa extracted from cauda epididymis were fully motile and their ability to fertilize an ovum was similar to that of ejaculated spermatozoa [2, 3, 14]. Other researchers have evaluated the efficacy of epididymal sperm collection from various domesticated and wild animals at different time intervals after post mortem [31]. Preservation of caudal spermatozoa at 4 ^{0}C to 5 ^{0}C has been reported [16, 33] and caprine spermatozoa collected from cauda epididymis have been recorded to preserve successfully for 96 h at 4 ^{0}C and able to fertilize ova [26]. In canine, epididymal spermatozoa collected six hours after post-mortem were preserved up to 8 days at 5 ^{0}C [50], where spermatozoa progressive motility and membrane integrity were recorded as 50% and 80% respectively. These results compare quite well with the results reported in present study. It was documented that caprine caudal epididymal spermatozoa were showing progressive motility while stored in electrolyte free medium and indicated significant variation on different media which was in agreement with our findings [49]. Studies on Sika deer (*Cervus nippon*) [21] and Iberian red deer (*Cervus elaphus hwaspanicus*) [46], epididymal spermatozoa showed that viable sperm can be isolated up to 7 days and 3 days after cool storage respectively, but sperm motility decreased the storage period continued in both of studies. After incorporation of ascorbic acid as an additive, in the dilutor improved the motility of harvested spermatozoa at 4 ^{0}C (T2) and -196 ^{0}C (T4). Antioxidants have an important role in maintaining the motility and the genetic integrity of sperm cells against oxidative damage [25].

In present study, the motility of spermatozoa was significantly (P<0.01) positively correlated with per cent viability (%) (r= 0.82, 0.86, 0.91, 0.95 and 0.93) and per cent HOS reactive spermatozoa (r= 0.85, 0.69, 0.91, 0.95 and 0.76) in group T_0, T_1, T_2, T_3 and T_4, respectively whereas, it was negatively correlated (r= -0.81, -0.89, -0.98 and -0.96) with per cent sperm abnormality in groups T_0, T_1, T_3 and T_4, respectively.

3.2 Sperm abnormality

The mean ±S.E. sperm abnormality of T_0, T_1, T_2, T_3 and T_4 is presented in the table 1 and figure no- 2 & 3. Significantly lower Per cent sperm abnormality in epididymal semen was reported in group T_0 differ as compared to T_0, T_1, T_2, T_3 and T_4. Sperm abnormality differed significantly (P<0.05) among the groups.

Higher abnormality during cryo-storage was observed in present study was self-explanatory that epididymal spermatozoa had severely affected and losses their functional and structural integrity during cryostorage. During freezing, a substantial portion of the sperm cells undergo irreversible damage due to cryo, mechanical, chemical and osmotic stress. There occurs significant differentiation in sperm biophysical characters, such as cell surface area, cell volume, and water volume and membrane permeability to water. The sperm plasma membrane was of crucial importance to freeze survival of spermatozoa and was regarded as primary site of freezing injury. Ice crystal mediated damage of SPM affects spermatozoa motility and viability seriously and was responsible to be a major cause of cell death due to freezing. A large portion of cell destruction has also been observed when the transition period from the liquid to solid phase or vice versa was prolonged. Cryo-damages of spermatozoa of group T_4 was lesser than that recoded in T_3, due to effect of ascorbic acid. Ascorbic acid is naturally occurring antioxidants of buffalo semen, to protect the spermatozoa from oxidative damage [41]. However, the indigenous antioxidant system to protect the spermatozoa integrity from ROS during freezing is insufficient; it was recorded that incorporation of vitamin C in TCA based extender improved the motility of liquid buffalo bull semen [8, 35, 39, 48]. The greater number of motile spermatozoa in sample frozen with natural antioxidants would increase the fertilizing potential of post thaw spermatozoa [12].

A negative correlation (P<0.01) sperm abnormality was observed with initial motility, HOS reactive sperm percentage and live count percentage. These findings were in agreement with observation [28].

3.3 Histology of Epididymis

Histology of testis was consists of seminiferous tubules and interstitial cell. Seminiferous tubule containing different stages of spermatogonic cell, sertoli cell and seminiferous lumen. Our histologicals picture was similar to finding of Singh and Bharadwaj [45] in one humped cameland Bacha and Bacha [7] in sheep & goat.

The epididymis group of T_0, T_1, T_2, T_3 and T_4 is covered by two layered pseudo stratified epithelium in caput, corpus and cauda. The sperm are showed in the lumen of cauda epididymis group T_0, T_1, T_2, T_3 and T_4.The epithelium is separated by basement membrane from the connective tissue wall which has smooth muscles cell. Main cell of epithelium was columnar cell that was extended from basal lamina towards the lumen of tubules. They have stereocillia which are long at head region (caput) and shorter at tail region (cauda). In group T_0 tubules were relatively more round, compacted, more thickness of interstitial tissues, columnar cell was tall, stereocillation number was more and expressed less degenerative changes as compared to group T_1, T_2, T_3 and T4. The tall columnar cells were become flattened and reduction in stereocillation number was observed in T_3 and T_4 group (Fig 3-8). Cryo-injury was more noticed in the epididymal stored at -196 ^{o}C without any treatment in group T_3 as compared to T_4 group. Ascorbic acid had showed their potential antioxidant by normalizing such structure during storage of epididymis at 4 ^{o}C and -196 ^{o}C. Non-enzymatic antioxidant ascorbic acid (vitamin C) has been proposed as electron donor for some transplasma membrane redox systems and protect cell from ROS damages. No such type of research cited in available literature. Higher cryodamages was recorded in treatment T_3 and T_4 as compared to group T_1 and T_2 these damages might be due to formation of ice crystal in extra and intracellular environment, increasing solute concentration.

Table 1: Mean (±S.E) of cytomorphological characteristics and HOS reactive spermatozoa per cent epididymal semen of murrah bulls in stored different cryo-temperature (4 ^{0}C & -196 ^{0}C) and ascorbic acid treated groups.

Parameters	T_0	T_1	T_2	T_3	T_4	Pool Mean
Motility (%)	78.50±1.70^a	62.00±0.71^b	68.88±0.67^c	33.00±0.53^e	43.88±0.77^d	57.25±2.69
Viability (%)	86.13±1.09^a	70.75±0.65^b	75.38±0.69^c	42.88±0.93^e	55.13±0.83^d	66.05±2.49
Sperm abnormality (%)	15.50±0.93^e	29.63±0.57^{bd}	27.25±0.98^d	40.38±1.39^a	28.75±0.45^c	28.30±1.33

Mean bearing different superscript (a, b, c, d, and e) in a row significantly differed repeatedly for each attributes.

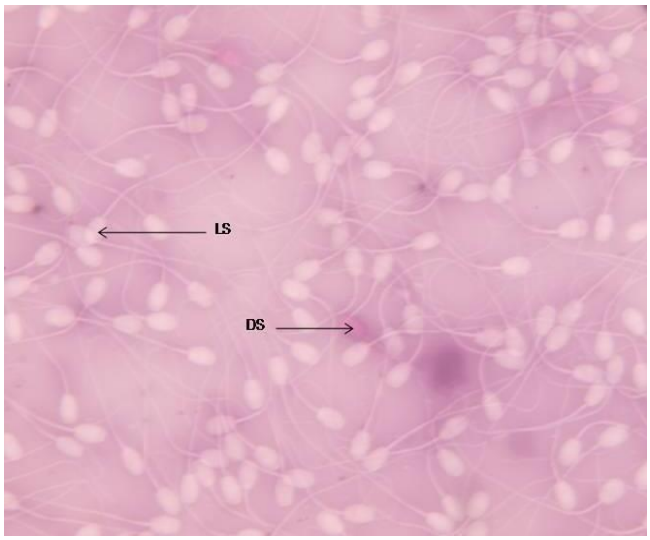

Fig 1: Figure showing Live and Dead sperm, Live Spermatozoa (LS) and Dead Spermatozoa (DS).

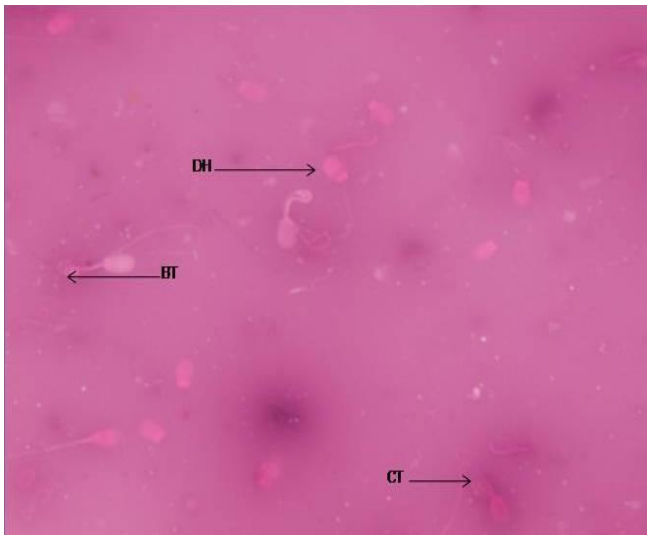

Fig 2: Figure showing different type of sperm abnormality, Decapitated Head (DH), Bent Tail (BT) and Coiled Tail (CT).

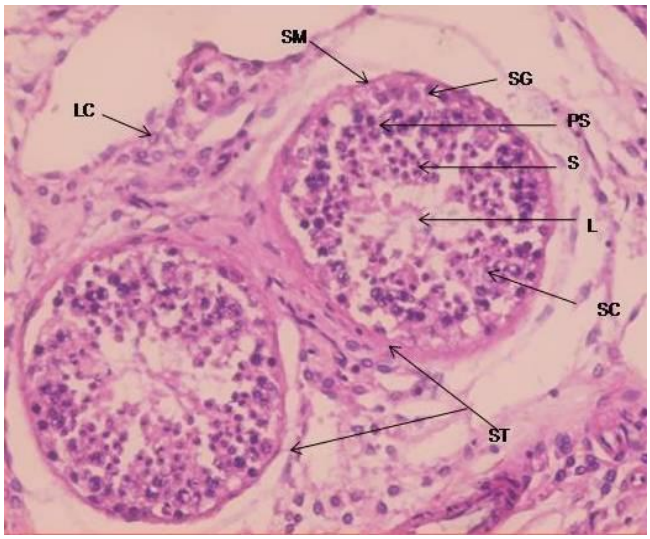

Fig 3: Histology of testis (T.S.) showing- Seminiferous Tubules(ST), Smooth Muscle (SM), Spermatogonia (SG), Primary Spermatocyte (PS), Spermatid (s), Sertoli Cell(SC), Leydig Cell (LC).

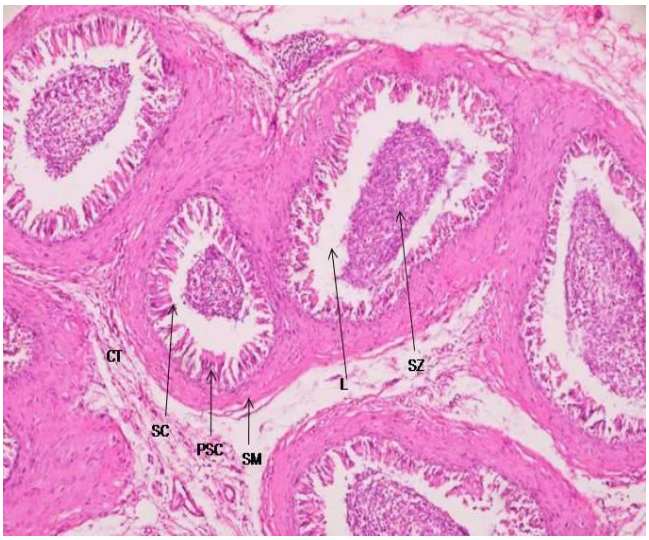

Fig 4: Histology of Cauda Epididymis within 6 hrs of slaughter (group TO) showing- Smooth Muscle (SM), Pseudostartified Columnar Epithelium (PSC), Stereocillia (SC), Lumen (L), Spermatozoa (SZ), Connective tissue(CT).

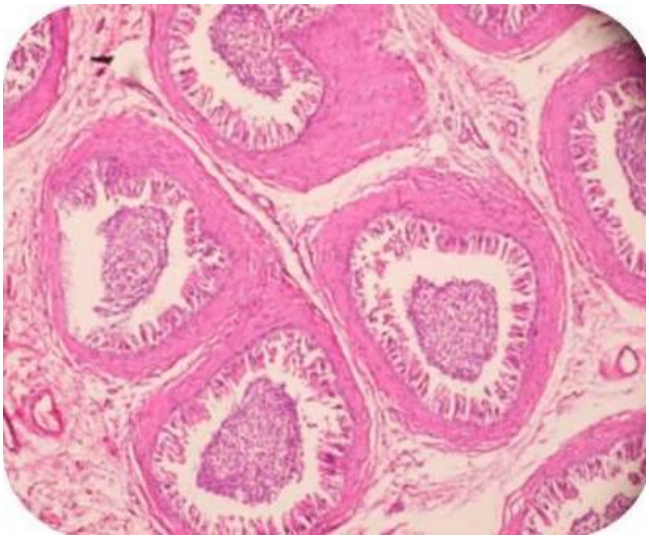

Fig 5: Histology of Cauda Epididymis of group T_1 stored at 4 ^{o}C.

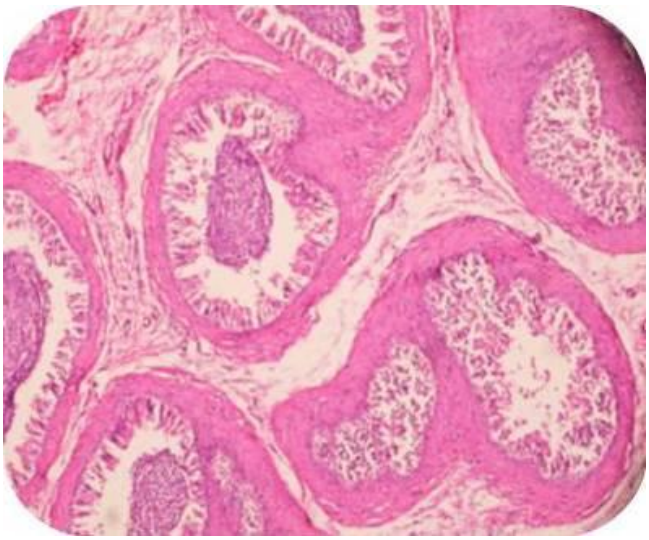

Fig 6: Histology of Cauda Epididymis of group T_2 stored at 4 ^{o}C.

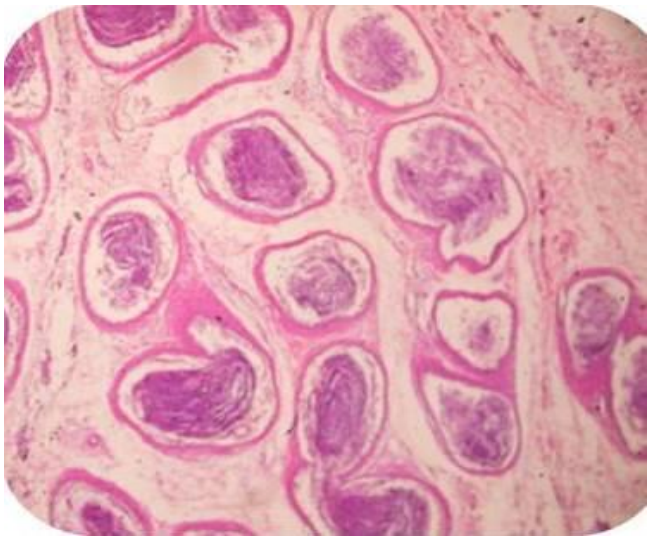

Fig 7: Histology of Cauda Epididymis of group T_3 stored at -196 ^oC.

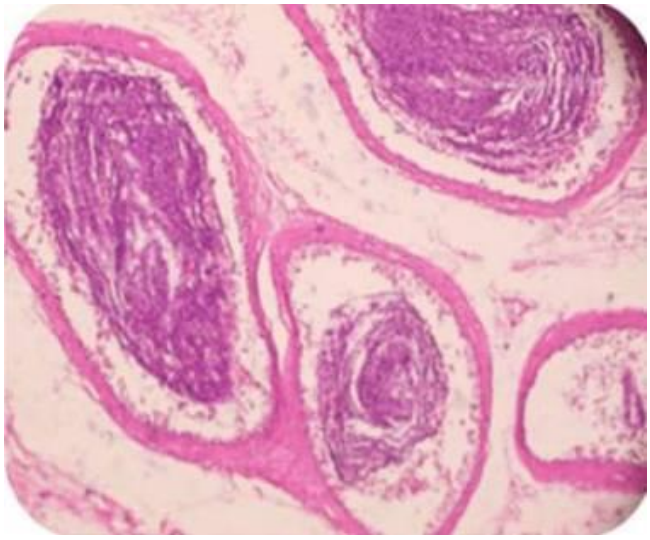

Fig 8: Histology of Cauda Epididymis of group T_4 stored at -196 ^oC.

4. Conclusion

On the basis of present study it can be concluded that cryopreservation of whole epididymis may be utilized as one of the potential method for storage of epididymal spermatozoa. Incorporation of ascorbic acid @ 2 mM in tris diluter resulted in maximum increase in post thaw motility, viability and per cent HOS reactive sperm with minimum abnormality. It could be inferred that ascorbic acid is a promising semen additive which can be used further for improvement of quality of post thawed epididymal semen and it fertilizing capacity from the response of sperm cell to hypo-osmotic solution which, intern reveals about the structural normality of sperm cell.

5. Acknowledgement

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