EFFECT OF DILUENTS AND TEMPERATURE ON THE VIABILITY OF BOVINE SPERMATOZOA FROM CAUDA EPIDIDYMIS WHILE STORED AT 4°C, 25°C AND 39°C

F. K. Njonge and M. Mutugi
Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
E-mail:francisnjonge@yahoo.com

Abstract
The objective of this study was to evaluate the effect of commonly used diluents in storage of caudal epididymal spermatozoa under different temperature treatments. Eighteen pairs of bull epididymides were obtained from a local abattoir and caudal epididymal spermatozoa were harvested and processed in the laboratory. Epididymal spermatozoa were pooled from groups of three bulls to give six pooled samples for this study. The effect of three diluents namely, tris, egg yolk citrate and Skim milk were tested under three temperature treatments of 4°C, 25°C and 39°C on their effect on viability of the caudal epididymal spermatozoa. The percentage motility and the livability of the spermatozoa were considered as a measure of their viability. On incubation for 96 hrs at 4°C tris maintained a high percentage of motile caudal epididymal spermatozoa than any of the other diluents, with 24.2±2.2 percent motile spermatozoa, while skim milk had 6.6±0.5 percent. The egg yolk diluent did not have any motile spermatozoa at 96 hrs of incubation ($P<0.05$). After the same period and temperature treatments, tris contained 25.9 ±0.4 percent live spermatozoa, skim milk 10.8 ± 1.2 percent and egg yolk citrate 6.8 ±0.2. The difference was statistically significant ($P< 0.05$). The temperature of 4°C and tris were found to maintain higher motile and live caudal epididymal spermatozoa than egg yolk citrate or skim milk diluents. Caudal epididymal spermatozoa when stored in the commonly used diluents for longer period of time can be used for artificial insemination (A.I) especially where a very good bull cannot ejaculate due to one reason or another or in situations where the preservation of genetic material is paramount.

Key words: Caudal epididymis, bull viability, spermatozoa, in vitro

1 Introduction

Semen for artificial insemination (A.I) is normally collected from live bull through artificial vagina or electro-ejaculation. Some very promising bulls may have difficulties in ejaculation due to one reason or another. Therefore collection of caudal epididymal spermatozoa postmortem is very important to preserve their genetic potential, insure the preservation of genetic variability of some endangered species. The preservation of such spermatozoa and assessment of their viavility in vitro is equally very important. The epididymis is part of the excurrent duct system consisting of a highly convoluted duct attached to the posterior border of the testis, into which spermatozoa leaving the testis via the efferent duct passes. It is about 80 m long in bulls and 20 m in humans (Mann and Lutwak-Mann 1981, Robaire and Hermo 1988). In most species this structure is known to consist of three main distinct regions namely, the head or caput, a narrow body (corpus) and the tail (Cauda), (Cooper 1986).

It has been very clear that mammalian spermatozoa undergo an essential and rather complicated process of maturation as they pass through the epididymis (Zimmerman *et al*., 1979, White and Voglmyr 1986, and Bedford and Hoskins, 1990). Each of the three regions of the epididymis has been shown to differ both morphologically and physiologically suggesting that each region has a specific function (Amann 1987). For instance the middle segment of the epididymis is known as the area where maturation of spermatozoa occurs and the terminal segment as the region where spermatozoa are stored awaiting ejaculation (Amann, 1987, Bedford and Hoskins 1990). Caudal epididymal spermatozoa has been reported to be motile and to possess some fertilizing ability (Igboeli and Foote 1968, Amann and Griel 1974, Amann 1987, Hammerstedt and Park 1987, Dong *et al*., 2008, Uttam *et al.,* 2009).

Studies have indicated that diluted epididymal sperm could survive for 20 minutes at 37^{o}C (Carr and Acott 1984) and 60 hrs when stored in tris diluent (Igboeli and Foote 1968), Other studies have also verified that caudal spermatozoa when diluted with extenders are viable even for longer period of time(Julie *et al*., 2004, Uttam *et al*, 2009). Yu *et al*., (2002), reported that in canine, caudal spermatozoa were motile after 8 days while stored at 4^{o}C.

Therefore this study was designed to compare the effect of commonly used diluents in spermatozoa preservation, when subjected to different temperature treatment in maintaining bovine spermatozoa *in vitro* after collection directly from the cauda epididymis.

2 Materials and Methods

2.1 Collection of Samples

Eighteen bull epididymides were randomly obtained from a local abattoir soon after stunning on the slaughtering line. They were kept in a polystyrene insulated box at ambient temperature during transportation to the laboratory in the school of tropical veterinary medicine Townsville. The epididymides were physically examined and found to be healthy without any external injuries or any other visible abnormalities.

The epididymides were transported to the laboratory and processed within 2 hours after collection from the slaughtering line. The whole epididymides were dissected free from the testis by use of a scalpel blade and a pair of scissors. The excess tissues were trimmed from each vas deferens and the most caudal portion of each epididymis cut and removed from the rest of the epididymis. The cauda epididymides were isolated by cutting the corpus at the distal portion.

Spermatozoa were collected by retrograde perfusion with paraffin oil. A 22- gauge needle was inserted into the lumen of the vas deferens and 5 ml of paraffin oil was flushed towards the cauda of the epididymis. This procedure was to facilitate the bulging of the tubules of the epididymis so that the collection of the samples was made easier. A small slit was made into the caudal epididymis with a scalpel blade and contents collected with a micropipette. The slit was carefully made to avoid cutting through any blood vessels. Several cuts into the cauda epididymis were made to enable a sufficient volume to be collected. Epididymal spermatozoa were pooled from groups of three bulls to give six pooled samples for this study. Samples from three bull's epididymis were pooled together.

2.2 Preparation of Diluents

2.2.1 Egg Yolk Citrate (EYC)

Buffered sodium citrate was prepared using a modification of the procedure of Davis et al, (1963a). Thirty grams of tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$, BDH Chemicals, Victoria, Australia) were weighed, dissolved in one litre of double distilled water and thoroughly mixed. To make 0.1 molar sodium dihydrogen orthophosphate (NaH_2PO_4, Ajax Chemicals, Australia), 14.196 g was weighed and dissolved in one litre of double distilled water, while 0.1 molar di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4, Ajax Chemicals) was prepared by weighing 15.601 g and dissolving in one litre of double distilled water.

The buffered sodium citrate solution was prepared by adding 128 ml sodium citrate to 16 ml sodium dihydrogen orthophosphate and 16 ml di-sodium hydrogen orthophosphate anhydrous. This was in a ratio of 8:1:1 respectively to constitute 160 ml solution. In the 160 ml of buffered sodium citrate, 2.5 g fructose (B–D–Levulose fruit sugar, Sigma Chemicals, St Louis, USA) was dissolved and mixed thoroughly on a stirrer (Chiltern Scientific, New Zealand).

Penicillin G (Benzyl penicillin, Sigma Chemicals) 0.119 g and 0.266 g Streptomycin sulfate (Streptomycin sesquisulfate) were added to the solution. This was equivalent to 1000 IU/ml of penicillin and 1 mg/ml of streptomycin as the recommended standard (Foote *et al,* 1960, de Jarnette *et al.,* 2004). Egg yolk (40 ml) was added to the solution and mixed thoroughly for about one hour before semen was diluted. The solution was maintained at 35^0C until the dilution of the semen was carried out.

2.2.2 Skim Milk (SMEY)

Skim milk powder (Diploma Instant, Bonlac Foods, Australia) was bought from a supermarket. Twenty-one grams of nonfat skim milk powder were added to 180 ml of double glass distilled water. Using a hot plate magnetic stirrer the solution was thoroughly mixed and heated at 95^0C for 10 minutes without boiling. The solution was allowed to cool to room temperature by placing it on the bench at 20^0C and held overnight at 5^0C before use in the diluent preparation (Karabinus *et al.* 1991, (Akhter *et al.,* 2007).

One hundred and eighty millilitres of reconstituted skim milk was used to dissolve 2.5 g fructose (B–D–Levulose fruit sugar, Sigma Chemicals). Egg yolk (20 ml) was added and mixed thoroughly. Penicillin G and streptomycin sulfate were added at the same rate as used in the EYC diluents.

2.3 Tris Diluent

The ingredients were mixed using a modification of the procedure of Grafer and Fjeld (1988). To prepare tris stock solution, 6.04 g tris (Hydroxymethyl, Methylamine [$NH_2.C(CH_2.OH)_3$]; Ajax Chemicals), 3.5 g citric acid (trisodium dihydrate [$C_6H_5O_7Na_3.2H_2O$], Sigma Chemicals) and 2.5 g fructose (B–D–Levulose fruit sugar, Sigma Chemicals) was dissolved in 180 ml of double glass distilled water and mixed thoroughly.

Penicillin G (0.119 g) and streptomycin sulfate (0.266 g) was added and the solution thoroughly mixed as described above for the EYC and the skim milk. At one hour before the semen was diluted, 40 ml of fresh egg yolk (20% v/v) was added to the diluent. All the diluents were maintained at 35^{0}C in a water bath until used to dilute the semen.

2.4 Dilution of Cauda Epididymal Spermatozoa

Three millilitres of each of the diluents (tris, skim milk and egg yolk citrate) were initially placed in petri dishes, which were maintained at a uniform temperature of 35^{o}C to initially have all the samples at the same temperature before further processing. This was used for the initial dilution of the samples because the freshly collected caudal epididymal spermatozoa were in very small quantities of approximately 10 ul and highly concentrated.

Three aliquots were made from each petri dish and the nine aliquots of diluted caudal spermatozoa were held in test tubes and placed in a water bath whose temperature was maintained at 35^{o}C. The spermatozoa concentration in each test tube was adjusted to contain approximately 15-20 x 10^6 spermatozoa by addition of each of the three diluents.

2.5 Motility Assessment of Caudal Spermatozoa

Using a micropipette a small drop of diluted caudal spermatozoa was placed on a microscope slide, prewarmed at 35^{o}C by a hot plate (Easy Veterinary Equipment, Sydney, NSW, Australia) and a coverslip applied to obtain a thin even layer.

The 4^{o}C samples were placed in a refrigerator while 25^{o}and 39^{o}C samples were placed in temperature-regulated water baths. A thermocouple (Fluke 51 K/J thermometer, USA) was used to ensure the set temperatures were maintained throughout the experiment. Motility assessment was undertaken immediately after dilution and thereafter on hourly basis up to 5 hours. Subsequent assessments were done after every 24 h interval up to 96 hours.

By use of a counter a 100 spermatozoa in different microscope fields were assessed The percentage of individual motile spermatozoa was estimated at 400X magnification under a phase contrast microscope as described by Foot 1970, Foot 2002, Akhter *et al*., (2007). Visual assessment of forward progressive spermatozoa motility by microscope is an important characteristic for the evaluation of the quality of semen and has been in use for a long timed (Tomlinson *et al,* 2001; Foot, 2002; Daader and Zeidan 2008 and Vyt, *et al.,* 2004). The counts were recorded as motile and non-motile spermatozoa. Duplicate samples were examined at each sampling time.

The motility of caudal epididymal spermatozoa was assessed immediately after dilution and there after on an hourly interval until 5 hrs. Subsequent sampling was done every 24 hrs. Those samples, which were subjected to the 4^{o}C treatment, were placed in a regulated refrigerator to maintain them at that temperature. The samples subjected to 25^{o}C and 39^{o}C treatment were place in temperature – regulated water baths.

2.6 Assessment of Caudal Spermatozoa Viability

Nigrosin-eosin stain was used to determine the number of live and dead spermatozoa. The nigrosin-eosin solution was prepared by mixing 75 ml of nigrosin solution with 15 ml of stock buffer solution and 10 ml stock glucose solution. Eosin yellow (2.5 g) (Fluke, Switzerland) was finally added and mixed thoroughly (Haycock, 1951). Five drops of the stain were mixed with one drop of semen and incubated for 3 minutes at 35^{o}C. Then a smear was made from the mixer and allowed to dry on a hot plate maintained at 35^{o}C. Two hundred sperm cells were counted randomly at least from 10 different microscopic fields under the light microscope ×400). Three individual stained slides from each treatment group were counted and their mean results were expressed as percent viable spermatozoa, (Dott and Foster1972, Mathews *et al*., 2003).

2.7 Statistical Analysis

The percentage of motile and live caudal epididymal spermatozoa at each point and temperature treatment were subjected to One-way Analysis of Variance (ANOVA) using the SPSS statistical program for windows (Version, 10, SPSS Inc, Illinois, USA). The tukeys honestly significant difference multiple range comparison of means was used to compare the motility and live/dead spermatozoa data. Comparisons with P value less than 0.05 were regarded as significant.

3 Results

The percentage progressive motility of the caudal epididymal spermatozoa was used as a measure of their viability coupled with the assessment of the live spermatozoa after eosin-negrosin staining procedure. Motility of spermatozoa declined quickly while incubated at 39^{o}C and within 5 h only tris diluent was maintaining spermatozoa motility of 34.4± 4.4 percent. At 25^{o}C incubation, the caudal epididymal spermatozoa remained more motile and within 24 h spermatozoa in EYC and skim milk had decreased in motility to 17.7± 3.9 and 12.0± 5.5 percent respectively (Figure 2). Tris diluent at the same period of incubation had 25.5± 4.7 percent progressively motile spermatozoa and at 72 h of incubation, it was only tris, which was maintaining 7.07± 3.5 percent progressively motile spermatozoa ($p<0.05$).

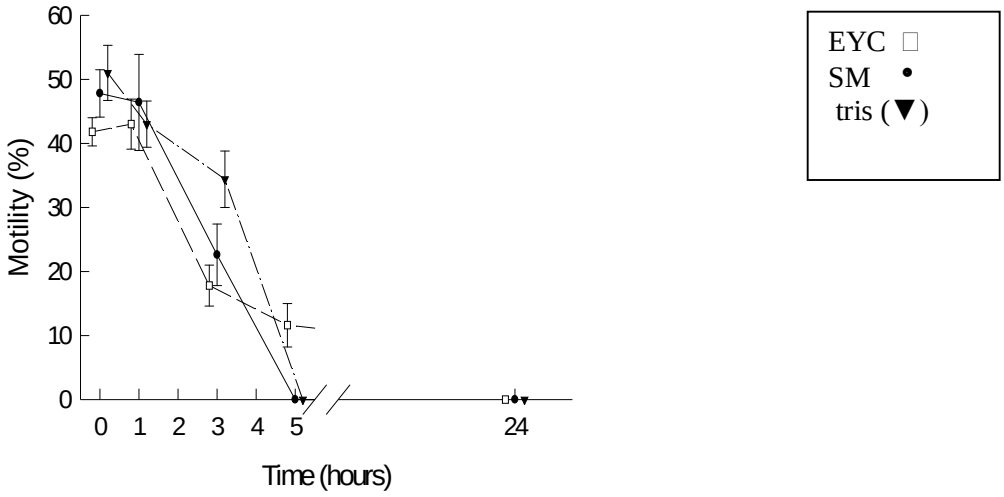

Figure 1: Percent (±SE) mean motility of caudal epididymal spermatozoa (n=6) when incubated at 39°C in egg yolk citrate, skim milk and tris

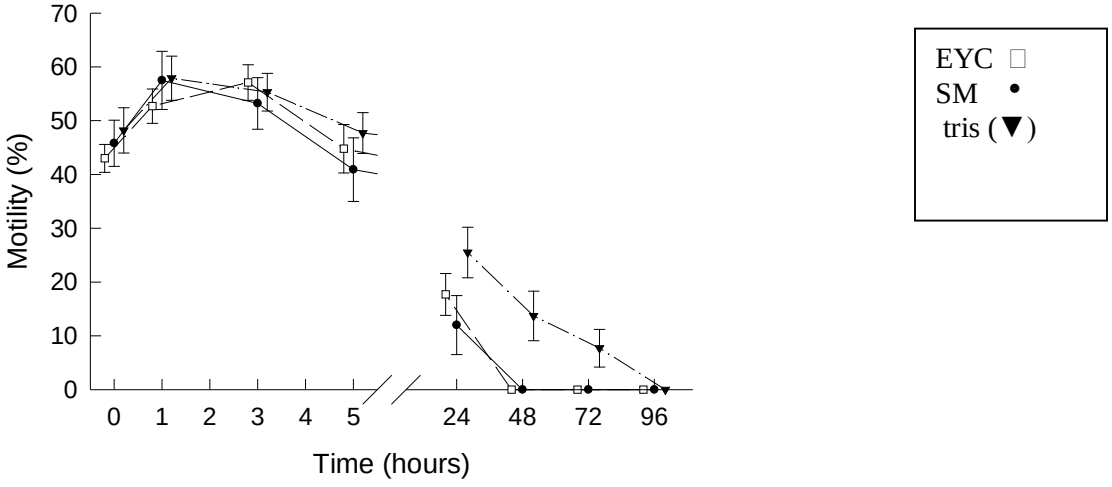

Figure 2: Percent (± SE) mean motility of caudal epididymal spermatozoa (n=6) at 25°C when incubated in egg yolk citrate, skim milk and tris

On further incubation at for 96 h tris maintained quite high number of motile spermatozoa at 4^{o}C incubation (Figure 3). Tris was maintaining 24.2± 2.2 percent progressively motile spermatozoa P<0.05). Other diluents had insignificantly low motile spermatozoa.

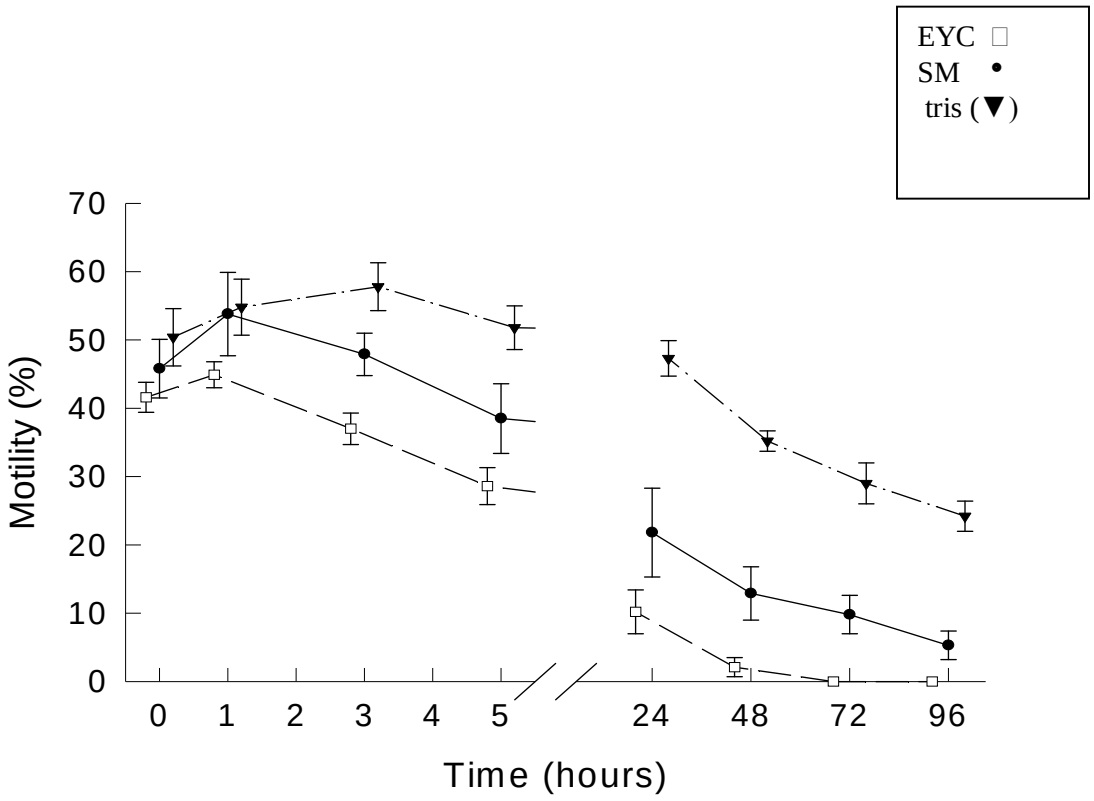

Figure 3: Percent (± SE) mean motility of caudal epididymal spermatozoa (n=6) at 4^{o}C when incubated in egg yolk citrate, skim milk and tris.

3.1 Live/Dead Spermatozoa

After 96 h of incubation at 4^{0}C. tris had 25.9±0.4, egg yolk citrate had 6.8±0.2 and skim milk 10.8±1.2. The diference was statistically significant (Table 1). The 25^{0}Cand 39^{0}C temperature treatments beyond 48 h of incubation, it is only tris which had 5.0±3.2 live caudal spermatozoa. The trend of motility and live spermatozoa was very similar confirming their viability.

Table 1: The percentage mean ((± SE) of live caudal epididymal spermatozoa at the specific time intervals when epididymal samples (N=6) when incubated in egg yolk citrate, skim milk or tris at 4°C, 25°C and 39°C

Temp and Time (h)	Egg Yolk	Skim Milk	Tris
4°C 1	73.5±2.9[a]	71.5±4.5[a]	69.0±5.2[a]
3	53.5±4.2[a]	52.7±5.7[a]	62.1±4.5[a]
5	47.6±3.6[a]	42.2±3.8[a]	54.5±4.4[b]
24	33.9±4.0[a]	30.6±4.2[a]	47.5±1.7[b]
48	17.9±3.3[a]	19.9±4.5[a]	36.2±2.2[b]
72	12.9±2.5[a]	16.6±3.8[a]	28.8±1.9[b]
96	6.8±0.2[a]	10.8±1.2[a]	25.9±0.4[b]
25° C			
1	70.5±4.7[a]	66.3±5.4[a]	70.9±4.7[a]
3	53.3±5.9[a]	55.7±3.2[a]	64.0±3.1[a]
5	51.6±4.9[a]	46.4±5.1[a]	58.3±3.4[a]
24	32.1±4.8[a]	24.6±5.4[a]	44.0±3.1[b]
48	23.6±7.1[a]	19.2±6.0[a]	32.0±1.7[a]
72	0.0	0.0	5.0±3.2
39°C			
1	68.5±3.6[a]	46.9±7.8[a]	60.8±6.0[a]
3	47.4±4.5[a]	32.0±3.0[b]	43.4±2.8[a]
5	28.9±2.3[a]	23.3±3.0[b]	31.2±3.6[a]
24	6.1±2.7[a]	2.1±1.4[a]	14.4±5.9[b]
48	0.0	0.0	0.0
72	0.0	0.0	0.0

Different superscripts in the same low indicate significant difference (P<0.005)

4 Discussion

The mammalian spermatozoa undergo an essential and rather complicated process of maturation as they pass through the epididymis(Amann and Griel 1974, Amann 1987). At the end of the cauda epididymis the spermatozoa are fully mature and has been reported to have the ability to fertilize an oocyst(Amann 1987) .Bovine spermatozoa extracted from cauda epididymis are fully motile and their ability to fertilize an ovum is similar to that of ejaculated spermatozoa (Amann and Griel 1974, Amann 1987, Carlos *et al*., 2002,)Other researchers have evaluated the efficacy of epididymal sperm collection from various domesticated and wild animals at different time intervals after post mortem (Lubbe, *et al*., 2000, James, *et al.,* 2002). Preservation of caudal spermatozoa at 4 to 5°C has been reported (Martinez-Pastor 2005, Dong et al 2008) and caprine spermatozoa collected from the cauda epididymides have been reported to preserve successfully for 96 h at 4°C and were able to fertilize ova (James *et al.,* 2002). In Canine, epididymal spermatozoa collected six hours after post-mortem were preserved for up to 8 days at 5°C (Yu, *et al*., 2002), where spermatozoa progressive motility and membrane integrity were 50 % and 80 % respectively. These results compares quite well with the results reported in this study.

Uttam Datta *et al*., 2009) also documented that caprine caudal epididymal spermatozoa were showing progressive motility while stored in electrolyte free medium and indicated significant variation on different media.

Their preservation at different temperatures and extenders was evaluated in this experiment. Earlier reports had indicated that caudal epididymal spermatozoa from rats, hamsters, mice, guinea pigs and man were immotile when undiluted (Morton *et al*., 1978), Usselman and Cones 1983, turner and Reich 1984), but later on Carr and Acott (1984) reported that upon dilution with buffer solution bull spermatozoa were highly motile. Garde *et al,* (1994) had demonstrated that ram caudal epididymal spermatozoa were motile when diluted in phosphate buffer solution at and kept at room temperature. This study reports the motility of caudal spermatozoa and also shows that they were alive at various temperatures compared in this study.

We therefore decided to compare the motility of bull's caudal epididymal spermatozoa when diluted in egg yolk citrate, skim milk and tris while incubated at 4^{o}, 25^{o} and 39^{o} C to determine which among the three diluents could be superior in maintaining the caudal epididymal spermatozoa's viability.

The temperature effect on caudal epididymal spermatozoa at 39^{o}C was compared to the effect of the normal room temperatures of 25^{o}C and when preserved at refrigeration temperature of 4^{o}C. Motility of caudal epididymal spermatozoa (not longer than 1hr) upon dilution was reported by Cascieri et al (1976), and Acott and Carr (1984), Igboeli and Foote (1968) reported that caudal epididymal spermatozoa were motile after dilution in tris diluent and could be stored for up to 60 hrs in tris- buffered diluent at 5^{o}C and maintained spermatozoa motility of 41 percent.

The results of this study compares quite closely with that of Igboeli and Foote (1968) showing that tris is a better diluent and maintained a higher percentage of motile caudal epididymal spermatozoa. Our study was able to store the caudal epididymal spermatozoa for a longer period of 96 hrs and the spermatozoa were still motile at 24.2±2.2 percent when stored in tris diluent.

The data presented in the present study shows that the motility of caudal epididymal spermatozoa decreases with time during storage. This was remarkable at 39^{o}C, than 25^{o}C and 4^{o}C. As the caudal epididymal spermatozoa are mature, it is likely that the effect of temperature on metabolism and motility would be the same as for the ejaculated spermatozoa.

From the results of this study it can be concluded that under the same conditions, tris can sustain a higher number of progressively motile spermatozoa than egg yolk citrate or skim milk diluents, and 4^{o}C was better in prolonging the viability of spermatozoa than 25^{o}C and 39^{o}C. Caudal epididymal spermatozoa could possibly be stored for artificial insemination or for *in vitro* fertilization and more research is recommended to test their viability after storage in tris diluent.

References

Acott, T. S. and Carr, D. W. (1984). Inhibition of bovine spermatozoa by caudal epididymal fluid. II. Interaction of pH and quiescence factor. *Biol Reprod.,* **30**: pp 926–935.

Akhter, S., Sajjad, M., Andrabi, S. M. H., Ullah, N, and Qayyum, M. (2007). Effect of antibiotics in extender on fertility of liquid buffalo bull semen Pakistan. *Vet. J.,* **27**: pp 13-16.

Amann, R. P. (1987). Function of the epididymis in bulls and rams. J *Reprod Fert (Suppl),* **34**: pp 115–131.

Amann, R. P. and Griel, L. G. Jr. (1974). Fertility of bovine spermatozoa from the rete, cauda epididymides and ejaculated semen. *J Dairy Sci.,* **57**: pp 212–219

Bedford, J. M. and Hoskins, D. D. (1990). The Mammalian Spermatozoon: Morphology, Biochemistry and Physiology. In Marshall's Physiology of Reproduction (ed GE Lamming) Churchill, Livingstone. U.K **Vol 2** pp 379–567

Carlos-Reyes, M., Mathieu, B., Robert, S and Marc-Andre' S. (2002). Characterisation and identification of epididymal factors that protect ejaculated bovine sperm during in vitro storage. *Biol. of Reprod*., **66**: pp 159-166.

Carr, D. W. and Acott, T. S. (1984). Inhibition of bovine spermatozoa by caudal epididymal fluid. I. Study of sperm motility quiescence factor. *Biol Reprod,* **30:** pp 913–925.

Cascieri , M., Amann, R. P. and Hammerstedt, R. H. (1976). Adenine nucleotide changes in the initiation of bull sperm motility. *J Biol Chem.,* **251**: pp787–793.

Cooper, T. G. (1986). The epididymis, sperm maturation and fertilisation. Springer Verlag Berlin pp 118–162

Daader, A. H., Zeidan, A. S. B. (2008). Motility and acrosomal integrity of frozen rabbit spermatozoa as affected by different extenders, cryoprotectants and packaging methods. 9th world Congress, Verona –Italy.

Davis. I. S., Bratton, R. W. and Foote, R. H. (1963a). Livability of bovine spermatozoa at 5°C in tris-buffered and citrate-buffered yolk-glycerol extenders. *J Dairy Sci.,* **46**: pp 57–60.

Davis, I. S., Bratton, R. W. and Foote, R. H. (1963b). Livability of bovine spermatozoa at 5, –25 and –85°C in tris-buffered and citrate-buffered yolk-glycerol extenders. *J Dairy Sci***., 46**: pp 333–336.

De Jarnette, J. M., Marshall, C. E., Lenz, R. W., Monk, D. R., Ayars, W. H. and Sattler, C. G. (2004). Sustaining the fertility of artificially inseminated dairy cattle :The role of the artificial insemination industry. J. Dairy Sci., **87** (E.suppl.), E93-E104.

Dong, Q, Rodenberg, S. E., Huang, C. and Vandevoor,T. C. A. (2008). Cryopreservation of Rhesus monkey (Macaca muitatta) epididymal spermatozoa before and after refrigerated storage. *J. Androl.* **29**: pp 283–92.

Dott, H. M. and foster, G. C. A. (1972). A technique for studying the morphology of mammalian spermatozoa which is eosinophilic in a differential live/dead stain. *J Reprod Fert.,* **29**: pp 443–445.

Foote, R. H. (1970). Fertility of bull semen at high extension rate in Tris-buffered extender. *J Dairy Sci.,* **53**: pp 1475–1477.

Foote, R. H. (2002). The history of artificial insemination: Selected notes and notables. *J.Anim. Sci*. Bibliography and History series 1-10.

Graffer, T. and Field, A. R. (1988). Studies on the Tris diluter for the bull semen. *11th Cong Anim Reprod Artif Insem.,* pp 248–250.

Hammerstedt, R. H. and Park, J. E. (1987). Changes in sperm surface associated with epididymal transit. *J Reprod Fert (Suppl)* **34:** pp 133–147.

Haycock, J. L. (1951). A staining technique for the study of temperature shock in the semen. *Nature* **167**: pp 323–324.

Igboeli, G. and Foote, R. H. (1968). Maturation changes in bull epididymal spermatozoa. *J Dairy Sci*., **51**: pp 1703–1705.

Julie, A. C., Scott, M., Kelly, F., Justin, T. H., Lisa, D. and Mei, L. F. (2004). A comparison of electroinjaculation and epididyma sperm collection technique in stallions. *Canadian Vet. J.,* **45:** pp 35-41.

Karabinus, D. S., Evenson, D. P. and Kaproth, M. T. (1991). Effects of egg yolk-citrate and milk extenders on chromatin structure and viability of cryopreserved bull sperm. *J Dairy Sci.,* **74:** pp 3836–3848.

Mann,T. and Lutwak-Mann, C. (1981). *Male reproductive function and semen*. Springer-Verlag New York.

Mathews, N., Bester, N. and Schwalbach, L. M. J. (2003). A comparison of ram semen collected by artificial vagina and electro-ejaculation *SA-Anim Sci .,* **4:** pp 28-30.

Robaire, B. and Hermo, L.(1988). Efferent duct, epididymis and vas deferent: Structure, function and their regulation. In *The Physiology of Reproduction* (eds E Knobil and JD Neill) Raven Press **Vol 1** pp 999–1080.

Tomlinson, M., Turner, J., Powell, G. and Sakkas, D. (2001). One–step disposable chambers for sperm concentration and motility assessment: how do they compare with the world health organization's recommended methods. *Human Reprod*. **16**: pp 121-124.

Uttam, D., Chandra, S. M., Manik, L. H. Raju, D. (2009). Development of a new method to preserve caprine cauda epididymal spermatozoa in-situ at -10°C with electrolyte free medium. *J Assist Reprod Genet.,* **26**: pp 467–473.

VYT, P., Mase, D., Dejonckeere, E. Castryck, F and Soom Van, A. (2004). Comparative study on five different commercial extenders for boar semen. *Reprod. In Dom. Anim*. **39**: pp 8-12.

White, I. G. and Voglmayr, J. K. (1986). ATP-induced reactivation of ram testicular, cauda epididymal and ejaculated spermatozoa extracted with Triton X–100. *Biol Reprod.,* **34**: pp 183–193.

Wildeus, S. A. J. (1983). Some effects of development, seasonal and heat stress on reproductive function of *Bos Indicus* cross bulls. Ph.D. Thesis, James Cook University, pp 199–220.

Zimmerman, K. J., Crabo, B. G., Moore, R., Weiseberg, S., Deibel, F. C. and Graham, E. F. (1979). Movements of sodium and potassium into epididymal boar spermatozoa. *Biol Reprod,.* **21**: pp 173–179.