Pakistan J. Zool., vol. 43(2), pp. 273-277, 2011.

Storage of Nili-Ravi Buffalo (*Bubalus bubalis*) Semen in Skim Milk Extender Supplemented with Ascorbic Acid and α-Tocopherol

Shamim Akhter,[1]* Bushra Allah Rakha[1], Muhammad Sajjad Ansari[1], Syed Murtaza Hassan Andrabi[3] and Nemat Ullah[2]

[1]*Animal Physiology Laboratory, Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi*

[2]*Faculty of Veterinary and Animal Sciences, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi*

[3]*Animal Reproduction Programme, Animal Sciences Institute, National Agriculture Research Center, Islamabad*

Abstract.- We investigated the effect of ascorbic acid and α-tocopherol supplementation in skim milk extender on the preservability of buffalo bull spermatozoa stored at 5°C. For this purpose, semen samples were collected from Nili-Ravi buffalo (*Bubalus bubalis*) bulls (n = 3) and diluted at 37°C with skim milk extender containing ascorbic acid (0.5mM) or α-tocopherol (1.0mM) or without any supplement (Control). The sperm concentration in the extender was adjusted at 10 x 10^6 motile spermatozoa per ml. Diluted semen was cooled from 37°C to 5°C in 2 hours and stored at 5°C for five days. Semen quality assays for sperm motility, plasma membrane integrity, normal apical ridge and abnormalities were performed at 1st, 3rd and 5th day of storage. Percentage of sperm motility was lower ($P < 0.05$) in extender containing α-tocopherol as compared to ascorbic acid and control at 1st and 3rd day of storage. However, it did not differ at 5th day of storage. There was no difference ($P > 0.05$) in percentage of plasma membrane integrity and normal apical ridge of buffalo bull spermatozoa for five days of storage. Moreover, sperm abnormalities (head, mid piece and tail) remained similar ($P > 0.05$) in all experimental extenders for five days to storage. It is concluded that ascorbic acid and α-tocopherol addition in skim milk did not improve the semen quality of Nili-Ravi buffalo bull spermatozoa stored at 5°C for five days.

Key words: Buffalo bull semen, antioxidant, glutathione, liquid storage.

INTRODUCTION

Sperm cells have a high content of unsaturated fatty acids in their membranes and lack a significant cytoplasmic component containing antioxidants (Andrabi *et al*., 2009). Therefore, it makes spermatozoa more susceptible to lipid peroxidation by reactive oxygen species molecules (ROS; Storey, 1997). During freezing and storage of spermatozoa, exposure to oxygen and light radiation accelerate the production of ROS molecules and lipid peroxidation of sperm plasma membrane (Andrabi *et al*., 2008).

It is well established that chilling of buffalo semen resulted in deceased semen quality which is highly associated with decreased antioxidant activity and higher ROS production (El-Sissy *et al*., 2007; Kumaresan *et al*., 2005, 2006). Moreover, buffalo bull spermatozoa are more susceptible to oxidative damage as compared to cattle bull spermatozoa (Nair *et al*., 2006; Kumaresan *et al*., 2005, 2006). It is believed that this difference is due to higher contents of polyunsaturated phospholipids present in plasma membrane of buffalo bull spermatozoa (Sansone *et al*., 2000). Freezing process accelerate the production of ROS molecules which may decrease the viability of buffalo bull spermatozoa during storage (Kumaresan *et al*., 2005; 2006; Garg *et al*., 2008). Therefore, supplementation of antioxidants in semen extender is required to decrease the ROS-mediated damages to buffalo spermatozoa.

Ascorbic acid and α-tocopherol are naturally occurring antioxidants in buffalo semen, to protect the spermatozoa from oxidative damage (Sansone *et al*., 2000). However, the indigenous antioxidant system to protect the spermatozoa integrity from ROS during freezing is insufficient (Baumber *et al*., 2005; Sreejith *et al*., 2006; Nichi *et al*., 2006). It was observed that supplementation of ascorbic acid and α-tocopherol in semen extender improved the quality of cryopreserved Nili-Ravi buffalo semen

* Corresponding author: sashraf1993@gmail.com
0030-9923/2011/0002-0273 $ 8.00/0

(Andrabi *et al*., 2008). We hypothesized that supplementation of ascorbic acid and α-tocopherol in skim milk extender may improve the semen quality of Nili-Ravi buffalo bull spermatozoa stored at 5°C.

Therefore, present experiment was designed to investigate the effect of ascorbic acid and α-tocopherol supplementation in skim milk extender on the semen quality (motility, plasma membrane integrity and morphology) of Nili-Ravi buffalo bull spermatozoa stored at 5°C.

MATERIALS AND METHODS

Skim milk (SKIMZ®; CANDIA) 10% (w/v) was used as stock extender for preparation of experimental extenders. Experimental extenders were prepared by adding ascorbic acid 0.5mM and α-tocopherol acetate 1.0mM in extenders, extender without supplement served as control. Antibiotics (Streptomycin sulphate @ 1 mg/ml and Benzyl Penicillin @ 1000 IU/ml) were added in each extender at room temperature.

Two consecutive ejaculates were collected from three Nili-Ravi buffalo bulls maintained at Semen Production Unit, Qadirabad, Sahiwal, Pakistan with artificial vagina for three weeks (replicates). Colleted semen was immediately transferred to the laboratory for initial evaluation. Sperm motility was assessed using phase contrast microscope at 37 °C and 400X. Sperm concentration was determined by Neubauer haemocytometer. Qualifying semen ejaculates were pooled from three bulls having motility > 60% and split into three aliquots for further processing. Three aliquots were diluted at 37°C with skim milk extender containing ascorbic acid (0.5mM) or α-tocopherol acetate (1.0mM) or without any supplement (control). The diluted semen was cooled from 37°C to 5°C in 2 hours and stored at 5°C for five days.

Plasma membrane integrity of buffalo bull spermatozoa was assessed by hypo-osmotic swelling assay (HOS). The HOS solution contained sodium citrate 0.735g and fructose 1.351g dissolved in 100ml distilled water. A semen sample (50µl) was mixed with 500µl pre-warmed (37°C) HOS solution and incubated at 37°C for 30-40 min. After incubation, a drop of semen sample was evaluated with phase contrast microscope at 400X. One hundred spermatozoa were observed and the percentage of cells with curled tails (intact plasma membrane) was recorded as HOS positive (Akhter *et al*., 2008). Sperm morphology (acrosome, head, mid piece and tail) was assessed by fixing 500 µl of semen samples with 50µl of 1% formal citrate. A drop of semen sample was studied using phase contrast microscope at 1000X. One hundred spermatozoa were studied to determine the percentage of intact acrosomes, head, mid piece and tail abnormalities (Andrabi *et al*., 2008).

The data are presented as means ± (SD). Effects of treatments on different semen quality parameters were analyzed by using Analysis of Variance (ANOVA). When the F–ratio was found significant ($P<0.05$), LSD test was used, to compare the treatment means (MINITAB® Release 12.22, 1998).

RESULTS

Motility of buffalo bull spermatozoa

The data on motility of buffalo bull spermatozoa in three experimental extenders are presented in Figure 1. Percentage of motility was observed lower ($P < 0.05$) in extender containing α-tocopherol as compared to ascorbic acid and control at 1st and 3rd day of storage at 5°C, respectively. However, sperm motility in all experimental extenders did not differ ($P>0.05$) at 5th day of storage at 5°C.

Plasma membrane integrity of buffalo bull spermatozoa

The data on the plasma membrane integrity of buffalo bull spermatozoa in three experimental extenders are presented in Figure 2. Percentage of buffalo bull spermatozoa with intact plasma membrane remained similar ($P > 0.05$) in extender containing ascorbic acid, α-tocopherol and control at 1st, 3rd and 5th days of storage at 5°C.

Normal apical ridge of buffalo bull spermatozoa

The data on normal apical ridge of buffalo bull spermatozoa in three experimental extenders are presented in Figure 3. Percentage of buffalo bull

spermatozoa with intact acrosomes did not differ (P>0.05) in extender containing ascorbic acid, α-tocopherol and control at 1st, 3rd and 5th days of storage at 5°C.

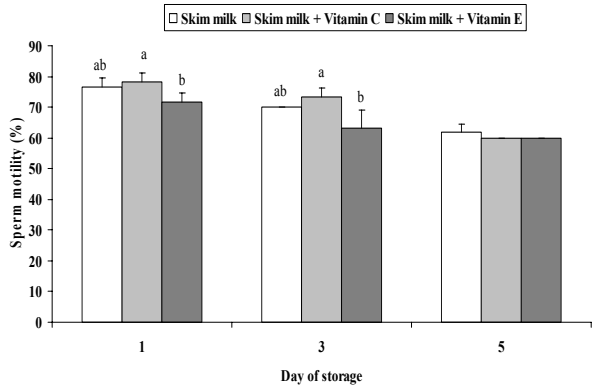

Fig. 1. Effect of ascorbic acid (Vitamin C 0.5mM) and α-tocopherol (Vitamin E; 1.0mM) supplementation in skim milk extender on the motility (Mean ± SD, n=3) of buffalo bull spermatozoa at 1st, 3rd, and 5th day of storage at 5 °C. Bars with different letters differ ($P < 0.05$) at a given day.

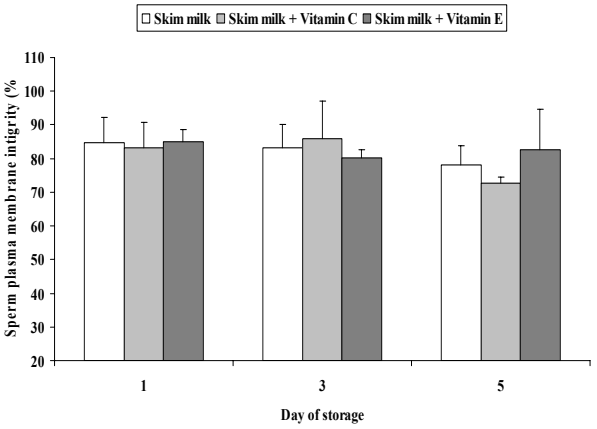

Fig. 2. Effect of ascorbic acid (Vitamin C; 0.5mM) and α-tocopherol (Vitamin E; 1.0mM) supplementation in skim milk extender on plasma membrane integrity (Mean ± SD: n=3) of buffalo bull spermatozoa at 1st, 3rd, and 5th day of storage at 5°C.

Sperm abnormalities of buffalo bull spermatozoa

The data on the abnormalities (head, mid piece and tail) of buffalo bull spermatozoa in three experimental extenders are given in Table I. Percentage of abnormalities (head, mid piece and tail) of buffalo bull spermatozoa remained similar (P > 0.05) in all three experimental extender for five days of storage at 5°C.

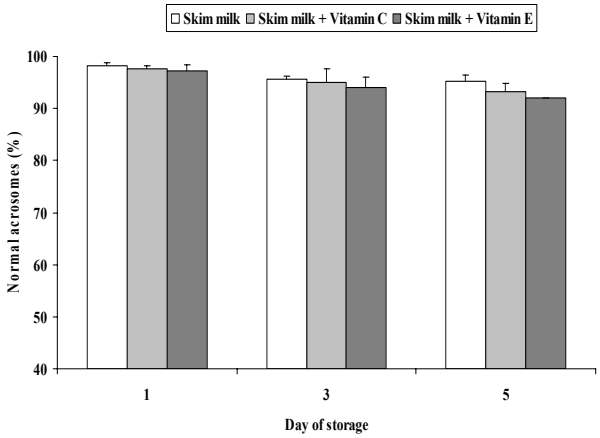

Fig. 3. Effect of ascorbic acid (Vitamin C 0.5mM) and α-tocopherol (Vitamin E; 1.0mM) supplementation in skim milk extender on the acrosomal integrity (Mean±SD, n=3) of buffalo bull spermatozoa at 1st, 3rd, and 5th day of storage at 5 °C.

Table I.- Effect of ascorbic acid (Vitamin C; 0.5mM) and α-tocopherol (Vitamin E; 1.0mM) supplementation in skim milk extender on abnormalities (Mean ± SD: n=3; head, mid piece and tail) of buffalo bull spermatozoa at 1st, 3rd, and 5th day of storage at 5°C.

	Day of storage	Extender		
		Skim milk	Skim milk + vitamin C	Skim milk + vitamin E
Head	1	1.00±0.0	1.00±1.0	0.67±0.6
	3	2.33±2.3	1.67±0.6	3.00±2.0
	5	1.00±0.0	2.00±0.0	1.67±0.6
Mid piece	1	0.33±0.6	1.00±0.0	0.67±0.6
	3	1.00±1.0	1.00±0.0	0.33±0.6
	5	1.00±0.0	1.33±1.2	4.33±1.2
Tail	1	2.33±1.2	2.67±1.2	3.33±0.6
	3	3.67±1.5	2.33±0.6	4.33±1.5
	5	4.67±1.2	0.67±1.2	5.67±1.5

Means within rows did not differ (P>0.05).

DISCUSSION

Freezing process accelerated the ROS production and reduced anti-oxidative activity which resulted in decreased motility, plasma membrane integrity and intact acrosomes of buffalo bull spermatozoa (El-Sissy *et al*., 2007). In our study, motility of liquid preserved buffalo bull spermatozoa did not differ in extender containing ascorbic acid and α-tocopherol as compared to control. Contrary to our findings, higher sperm motility of Murrah buffalo bull spermatozoa was observed in tris-egg yolk and milk egg yolk extender (Raina *et al*., 2002). Similarly, in a recent study (Andrabi *et al*., 2008) on Nili-Ravi buffalo semen higher post thaw sperm motility, plasma membrane integrity and normal apical ridge was reported after the addition of ascorbic acid and α-tocopherol in tris-citric acid extender. It is pertinent to mention that supplementation of vitamin C and E in milk based extenders failed to improve the preservability of bovine semen (Beconi *et al.,* 1993; Foote *et al*., 2002). Similarly, catalase supplementation in milk extender at 5°C in egg yolk extender found non beneficial for bovine spermatozoa (Foote, 1962). It is believed that supplementation of antioxidants in milk based extender did not improve semen quality because of a naturally occurring antioxidant casein in milk which alter the requirement of extra antioxidant supplementation (Foote *et al*., 2002). Reactive oxygen species molecules at physiological levels are essential for the spermatozoa, and it was observed that higher concentration of Vitamin C and E may result in impairment of semen quality (Andrabi *et al*., 2008) and decreases the success of fertility of bull semen *in vitro* (Dalvit *et al*., 1998).

Assessment of sperm abnormalities is one of the commonest methods to assess the viability of buffalo bull spermatozoa (Sajjad *et al*., 2007). In our study, sperm head, mid piece and tail abnormalities did not differ in all the three experimental extenders. It is noteworthy to mention that semen processing does not increase the proportion of buffalo bull spermatozoa with head, mid piece and tail abnormalities (Akhter *et al*., 2008)

It is concluded that ascorbic acid and α-tocopherol addition in skim milk did not improve the sperm motility, plasma membrane integrity and morphology of Nili-Ravi buffalo bull spermatozoa stored at 5°C for five days.

REFERENCES

AKHTER, S., ANSARI, M. S., ANDRABI, S. M. H., ULLAH, N. AND QAYYUM, M., 2008. Effect of antibiotics in extender on bacterial control and spermatozoal quality of cooled buffalo (*Bubalus bubalis*) bull semen. *Reprod. Domest. Anim.*, **43**: 272-278.

ANDRABI, S.M.H., 2009. Factors affecting the quality of cryopreserved buffalo (*bubalus bubalis*) bull spermatozoa. *Reprod. Domest. Anim.*, **44**: 552-569.

ANDRABI, S. M. H., ANSARI, M. S., ULLAH, N. AND AFZAL, M., 2008. Effect of non-enzymatic antioxidants in extender on post-thaw quality of buffalo *(bubalus bubalis)* bull spermatozoa. *Pakistan Vet. J.*, **28**: 159-162.

BAUMBER, J., BALL, B.A. AND LINFOR, J.J., 2005. Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. *Am. J. Vet. Res*., **66**: 772-779.

BECONI, M.T., FRANCIA, C.R., MORA, N.G. AND AFFRANCHINO, M.A., 1993. Effect of natural antioxidants on frozen bovine semen preservation. *Theriogenology*, **40**: 841-851.

DALVIT, G.C., CETICA, P.D. AND BECONI M.T., 1998. Effect of α-tocopherol and ascorbic acid on bovine *in vitro* fertilization. *Theriogenology*, **49**: 619-627.

EL-SISSY, G.A., EL-NATTAT, W.S. AND EL-SHESHTAWY, R.I., 2007. Buffalo semen quality, antioxidants and peroxidation during chilling and cryopreservation. *J. Vet. Res*., **11**: 55-61.

FOOTE, R. H., 1962. Survival of bull sperm in milk and yolk extenders with added catalase. *J. Dairy Sci.*, **45**: 907-910.

FOOTE, R.H., BROCKETT, C.C. AND KAPROTH, M.T., 2002. Motility and fertility of bull sperm in whole milk extenders containing antioxidants. *Anim. Reprod. Sci.*, **71**: 13-23.

GARG, A., KUMARESAN, A. AND ANSARI, M.R., 2008. Effect of hydrogen peroxide on fresh and cryopreserved buffalo sperm functions during incubation at 37^{o}C *in vitro*. *Reprod. Domest. Anim.*, **10**: 1439-1445.

KUMARESAN, A., ANSARI, M.R. AND GARG, A., 2005. Modulation of post thaw sperm functions with oviductal proteins in buffaloes. *Anim. Reprod. Sci*., **90**: 73-84.

KUMARESAN, A., ANSARI, M. R., GARG, A. AND KATARIA, M., 2006. Effect of oviductal proteins on sperm functions and lipid peroxidation levels during cryopreservation in buffaloes. *Anim. Reprod. Sci*., **93**: 264-257.

NAIR, S.J., BRAR, A.S., AHUJA, C.S., SANGHA, S.P. AND

CHAUDHARY, K.C., 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Anim. Reprod. Sci*., **96**: 21-29.

NICHI, M., BOLS, P.E.J., ZUGE, R.M., BARNABE, V.H., GOOVAERTS, I.G.F., BARNABE, R.C. AND CORTADA, C.N.M., 2006. Seasonal variation in semen quality in *Bos indicus* and *Bos taurus* bulls raised under tropical conditions. *Theriogenology*, **66**: 822-828.

RAINA, V.S., GUPTA, A.K. AND SINGH, K., 2002. Effect of antioxidant fortification on preservability of buffalo semen. *Asian-Aust. J. Anim. Sci*., **15**: 16-18.

SAJJAD, M., ALI, S., ULLAH, N., ANWAR, M., AKHTER, S. AND ANDRABI, S.M.H., 2007. Blood serum testosterone level and its relationship with scrotal circumference and semen characteristics in Nili-Ravi buffalo bulls. *Pakistan Vet. J*., **27**: 63-66.

SANSONE, G., NASTRI, M.J.F. AND FABBROCINI, A., 2000. Storage of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci*., **62**: 55-76.

SREEJITH, J.N., BRAR, A.S., AHUJA, C.S., SANGHA, S.P.S. AND CHAUDHARY, K.C., 2006. A comparative study on lipid peroxidation, activities of antioxidant enzymes and viability of cattle and buffalo bull spermatozoa during storage at refrigeration temperature. *Anim. Reprod. Sci.*, **96**: 21-29.

STOREY, B.T., 1997. Biochemistry of the induction and preservation of lipod peroxidative damage in human spermatozoa. *Mol. Hum. Reprod*., **3**: 203-214.

(Received 5 April 2010, revised 28 April 2010)