Original Research

Changes in Ultrastructure of Crossbred Bovine Spermatozoa in Butylated Hydroxytoluene Supplemented Semen

Mridula Sharma* and Anoop Singh

Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, G.B Pant University of Agriculture and Technology, Pantnagar-263145, Uttarakhand, INDIA

***Corresponding author:** sharmavetmridula@yahoo.co.in

Rec. Date:	Dec 13, 2017 10:05
Accept Date:	Feb 22, 2019 13:35
DOI	10.5455/ijlr.20171213100530

Abstract

The present study was planned to observe the effect of Butylated hydroxytoluene (BHT) on ultra-structure changes in bovine crossbred spermatozoa. The sperm plasma membrane is the primary site of damage induced by cryopreservation. There are many harmful conditions such as cold shock, osmotic stress, ice crystal formation or oxidative damage etc. which cause sperm cryo-injury and loss of sperm viability and fertility during cryopreservation. At the time of cryopreservation there were considerable ultra-structure changes to the acrosomes and middle piece studied by transmission electron microscopy. Semen from two crossbred bulls were collected and extended with egg yolk tris dilutor along with addition of different concentrations of BHT i.e. 0.0mM (Control), 0.5mM (T_1) and 1.0mM (T_2), respectively. The effect of BHT on ultrastructural alterations was revealed by Transmission electron microscopy during post dilution (Stage I), post equilibration (Stage II) and post thaw (Stage III), respectively. Per cent difference in ultastructural defects between three stages (After dilution, after equilibration and after thawing) was observed. There was significantly ($p<0.05$) lowest number of ultrastructural defects between three stages in group T_2 compared to T_1 and control groups, respectively. The study indicated that 1.0mM BHT increases the cryosurvival of sperm.

Key words: BHT, Crossbred Bull, Electron Microscopy, Semen, Ultrastructure

How to cite: Singh, A., & Sharma, M. (2019). Changes in Ultrastructure of Crossbred Bovine Spermatozoa in Butylated Hydroxytoluene Supplemented Semen. International Journal of Livestock Research, 9(5), 64-69. doi: 10.5455/ijlr.20171213100530

Introduction

Cryopreservation is detrimental to sperm function and fertility even if we use the most up to date technique. Sperm viability and fertility is decreased by about 50% after cryopreservation (Lessard *et al.*, 2000; Luque and Bao, 2006). There are many harmful conditions such as cold shock, osmotic stress, ice crystal formation or oxidative damage etc. which cause sperm cryoinjury and loss of sperm viability and fertility (Amirat *et*

al., 2004; Li *et al.*, 2005; Asadpour *et al.*, 2012; Bucack *et al.*, 2010). The most pronounced damage was observed in the plasma membrane (Khalil *et al.*, 2018). The characteristic feature of biological cell membrane is the asymmetrical arrangement of phospholipids bilayer with cholesterol, complex carbohydrate and protein. Sperm cells consist of a high amount of polyunsaturated fatty acids (PUFA) which makes the membranes more susceptible to oxidative damage by ROS. At the time of cryopreservation there were considerable ultrastructural changes to the acrosomes and middle piece studied by transmission electron microscopy (TEM) (Lopez Armengol *et al.*, 2012; Kumar, 2012; Khan *et al.*, 2015). Changes in acrosome include breakage of the plasma and outer acrosomal membranes and efflux of the acrosomal contents. Changes in middle piece include breakage of the plasma membrane, irregular mitochondrial helix (Kumar, 2012). Improved understanding the causes of the cryoinjury is important to improve the efficiency of semen cryopreservation (Watson, 1995). In the past years, a series of functional assays have been developed to determine the structural morphology and functional integrity of the plasma membrane and sperm acrosomal membrane (Tartaglionea and Ritta, 2004). Ultrastucture damage is always accompanied by biochemical changes or even loss of their vital contents (Salamon and Maxwell, 1995).

Butylated hydroxytoluene (BHT) also known as butylhydroxytoluene, an artificial antioxidant is a lipophilic (fat-soluble) organic compound and has an excellent antioxidant capacity (Merino *et al.*, 2015). It is chemically a derivative of phenol hence used as antioxidant. It behaves as a synthetic analogue of vitamin E. It was primarily acting as terminating agent that suppresses autoxidation, which is a process where unsaturated (usually) organic compounds are targeted by atmospheric oxygen. BHT stops this autocatalytic reaction by converting peroxy radicals to hydroperoxides (Patel *et al.*, 2015). Lipid solubility is a unique property of BHT due to which it functions as an antioxidant within and outside the sperm membrane.

Thus, the present study was planned to observe the protective effect of Butylated hydroxytoluene (BHT) on ultrastructure of crossbred bovine spermatozoa by electron microscopy.

Material and Methods

Two crossbred bulls (1/2 HF x 1/2 J) of aged 4-6 years weighing 450-500 Kg, reared at Semen Production Centre, Department of Veterinary Gynaecology & Obstetrics, College of Veterinary and Animal Sciences, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand-263145 were selected for the study. The bulls were kept under identical feeding and managemental conditions. 2-3 ejaculates/week were collected by artificial vaginal method. The fresh semen samples collected from two crossbred bulls were evaluated for volume, pH, mass motility and sperm concentration. For uniformity, ejaculate with mass activity of 3.0 + (0-5.0 scale) and a progressive motility of 70% or more were selected for study.

 DOI 10.5455/ijlr.20171213100530

Dissolution of 275.44 mg BHT (SRL, India) in 25 ml of ethanol was done for making a BHT concentration of 0.05 mole/ml. Then this solution was added in the two glass test tubes @ 0.05 and 0.1ml and kept them for few minutes at 37^0C in an incubator so that ethanol containing BHT was then evaporated resulting in sticking of BHT in the inner wall of the test tubes. The semen was extended with glycerolated egg yolk tris (EYT) extender as per need. To each of this test tubes 5 ml of extended semen was added making a concentration of 0.5 mM BHT (T_1) and 1.0 mM BHT (T_2). The extended semen along with BHT was then kept at 37^0C for 5 min to allow uptake of BHT by spermatozoa. Simultaneously, the same amount of extended semen was kept in another test tube (without BHT) and considered as control.

Electron microscopy was performed at three stages i.e., after dilution (Stage I), pre freeze (Stage II) and post thaw (Stage III) to study the effect of BHT on structural properties of spermatozoa. 1ml of semen from each group and stage was taken into a 5 ml eppendrof tube and immediately fixed by addition of 0.5 ml of Karnovsky's fixative (2.5% gluteraldehyde and 2% parafarmaldehyde in PBS). After fixation, samples were kept for 4 hours at 4^0C and subsequently washed two times with PBS by gentle centrifugation (170g x 10 min each) at 4^0C. A second fixation was performed with 1% osmium tetraoxide solution (OsO_4) in 0.1 M PBS for 1h at 4^0C and then washed again in PBS twice for 10 min. each. The samples were dehydrated in a graded ethanol series (50%, 70%, 80%, 95% and 100% ethanol) for 10 min. in each solution. Clearing of samples was done by toluene for 30 min. For block preparation, spermatozoa were infiltrated with epoxy resin overnight and embedded in fresh epoxy resin and placed into an oven at 60^0C for 12 h. The blocks were then sectioned using an Ultra Cut (UCT Leica Ultra-microtome) at 60-80 nm width with a glass knife and mounted onto copper grids and stained with drops of 2% uranyl acetate, followed by lead citrate for 1-2 min. Sperms were examined with a FEI Tecnai G220 S- Twin (Holland) transmission electron microscope (80 kv) at Sophisticated Advanced Instrument Facility (SAIF) AIIMS, New Delhi, India.

Results

Per cent difference in ultra-structural defects (dilated plasma membrane, dilated plasma membrane and outer acrosomal membrane and broken plasma membrane and acrosomal membrane) between three stages (After dilution, after equilibration and after thawing) is presented in Table 1. Plasma membrane defects are depicted in Fig. 1 and 2. There was significantly ($p<0.05$) lower number of dilated plasma membrane of sperms from dilution to post equilibrium and also from dilution to post freezing (post thaw) in group T_2 compared to T_1 and control groups, respectively. Likewise, dilated plasma membrane and outer acrosomal membrane were also significantly ($P<0.05$) lower in between three stages in 1 mM added BHT group compared to others. Moreover, broken plasma membrane and outer acrosomal membrane were also significantly ($P<0.05$) higher in control and 0.5mM added BHT between three stages compared to T2 Group.

 DOI 10.5455/ijlr.20171213100530

Table 1: Difference of ultrastructural defects (%) between three stages i.e. stage I, stage II and stage III

Group	Sperm head								
	Dilated plasma membrane			Dilated plasma membrane and outer acrosomal membrane			Broken plasma membrane and outer acrosomal membrane		
	AD-AE	AE-AF	AD-AF	AD-AE	AE-AF	AD-AF	AD-AE	AE-AF	AD-AF
T_1	17.37^{b} ±0.16	18.70^{b} ±0.31	36.07^{b} ±0.94	2.08^{b} ±0.04	2.65^{a} ±0.13	4.73^{b} ±0.18	3.72^{a} ±0.09	0.42^{c} ±0.01	3.30^{b} ±0.13
T_2	10.81^{c} ±0.38	23.11^{a} ±0.47	33.92^{c} ±0.94	1.96^{c} ±0.08	1.30^{b} ±0.04	3.26^{c} ±0.09	1.92^{c} ±0.09	0.78^{b} ±0.03	2.70^{c} ±0.04
Control	18.69^{a} ±0.29	22.78^{a} ±0.49	41.47^{a} ±0.81	6.58^{a} ±0.04	0.20^{c} ±0.0001	6.38^{a} ±0.13	2.93^{b} ±0.08	2.17^{a} ±0.08	5.11^{a} ±0.09

Control, Treatment 1 and treatment 2 contain 0, 0.5 & 1.0 mM of BHT respectively; AD= after dilution, AE= after equilibration; AF= after freezing (post thaw); AD-AE= difference between dilution to equilibration stage; AE-AF= difference between equilibration to post freezing (post thaw); AD-AF= difference between dilution to post freezing (post thaw)

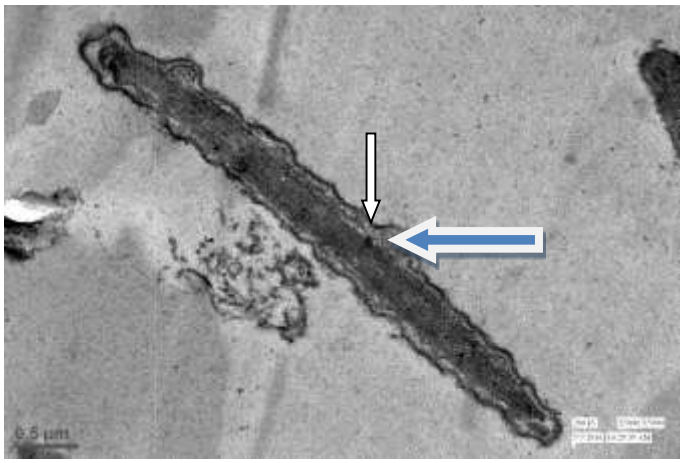

Fig. 1: Transmission electron microphotograph of longitudinal section of head showing dilated plasma membrane (white arrow) and acrosome membrane (blue arrow) of bovine bull spermatozoa X 25000

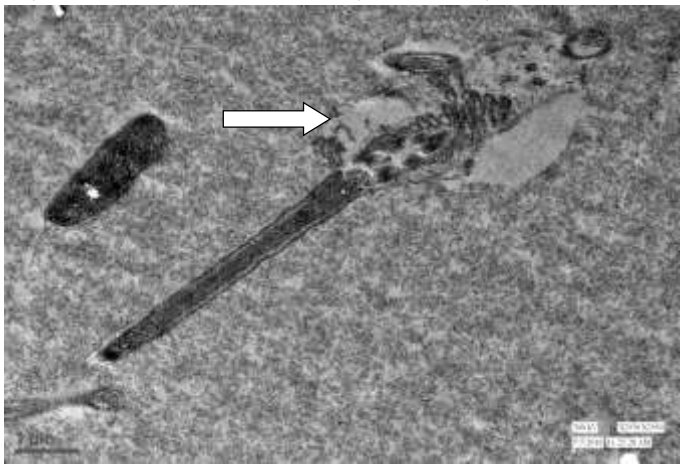

Fig. 2: Transmission electron microphotograph of longitudinal section of head showing broken plasma membrane and acrosome membrane (white arrow) of bovine bull spermatozoa X 56250

Discussion

In our study, heads with dilated plasma membrane was mainly observed. There were no detectable changes observed in mitochondria and axonemal complex of midpiece in longitudinal and transverse section of

DOI 10.5455/ijlr.20171213100530

sperm, respectively, after equilibration in all groups. In BHT added semen the plasma membrane abnormalities were not increased from dilution to equilibration and post thaw indicated the cryoprotective role of BHT. Similarly, ultrastructure of spermatozoa in CLC added semen after equilibration was assessed (Kumar, 2012) and significantly ($p<0.05$) lower number of dilated plasma membrane (15.46%) were observed than control (33.77%) group. Our observations also confirm that the most negative effect of cold shock and freezing was on the acrosome and plasma membrane of spermatozoa (Lopez Armengol *et al.,* 2012; Kumar, 2012; Khan *et al.,* 2015). The damaging effects of freeze-thawing were more pronounced in control group compared to BHT treated groups. Although there is no report in the literature related to ultrastructural changes after BHT addition in dilutor. But in buffalo semen, addition of 2mg CLC (Cholesterol loaded cyclodextrin) resulted significantly ($p<0.05$) lower number of dilated plasma membrane and outer acrosomal membrane (2.90%) than control (12.5%) at post thaw stage (Kumar, 2012). The minimum alterations in sperm ultrastructure in BHT treated groups were observed as BHT stabilized the membrane, reduced membrane permeability and resistant to osmotic pressure change which occur during the freeze-thawing process (Hammerstedt *et al.,* 1990). The BHT treated groups preserved the integrity of plasma and acrosomal membrane more efficiently than control.

Conclusion

Beneficial effects of BHT addition on semen preservation was confirmed Ultrastructural findings of spermatozoa supported the protective action of BHT as it reduced the damage of membrane and organelles of bovine spermatozoa.

Acknowledgments

The authors are highly thankful to Dean, College of Veterinary and Animal Sciences and Vice Chancellor of G.B Pant University of Agriculture and Technology, Pantnagar-263145, Uttarakhand, India for providing necessary facilities and financial support for carrying out the MVSc. research work of first author.

References

1. Amirat, L., Tainturier, D., Jeanneau, L., Thorin, C., Gerard, O., Courtens, J.L. and Anton, M. 2004. Bull semen in vitro fertility after cryopreservation using egg yolk LDL: a comparison with optidyl, a commercial egg yolk extender. Therio. 61: 895–907.
2. Asadpour, R., Jafari, R. and Tayefi-Nasrabadi, H. 2012. The effect ofantioxidant supplementation in semen extenders on semen quality and lipid peroxidation of chilled bull spermatozoa. *Iran Journal of Veterinary Research,* 13: (3), 246–249.
3. Bucak, M.N., Sariozkan, S. and Tuncer, P.B. 2010. The effect of antioxidants on post-thawed Angora goat (*Capra hircus ancryrensis*) sperm parameters, lipid peroxidation and antioxidant activities. *Small Rumin. Res*. 89 (1): 24–30.

DOI 10.5455/ijlr.20171213100530

4. Hammerstedt, R.H., Graham, J.K. and Nolan, J.P. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. *Journal of Andrology*. 11: 73–88.
5. Khalil W.A., Hairary A. E., Zeidan, A. E. B., Mahmood, A. E., Elsaeed, O.M. 2018. Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultra-structural insights. *International Journal of Veterinary Science and Medicine*, 6*:* 549-556.
6. Khan, M.H., Nath, K.C., Naskar, S., Deka, B.C. and Kumae, C. 2015. Electron microscopic studies of porcine sperm: changes during freezing and post-thawing. *Indian Journal of Animal Sciences*. 85.
7. Kumar, A. 2012. Studies on effect of cholesterol loaded cyclodextrin on freezibility and *in vitro* fertility of buffalo spermatozoa. Thesis, Ph.D. Deemed University, IVRI, Izatnagar, U.P, India.
8. Lessard, C., Parent, P., Leclerc, P., Bailey, J.L. and Sullivan, R. 2000. Cryopreservation alters the levels of the bull sperm surface protein P25b. *Journal of Andrology*. 21: 700-707.
9. Li, Y.H., Cai, K.J., Su, L., Gaun, M., He, X.C., Wang, H., Kovacs, A. and Ji, W.Z. 2005. Cryopreservation of cynomolgus monkey (*Macaca fascicularis*) spermatozoa in a chemically defined extender. *Asian Journal of Andrology*. 7: 139-144.
10. Lopez-Armengol, M.F., Jurado, S.B., Pelufo, V. and Eisen, E.G., 2012. A quantitative ultramorphological approach for systematic assessment of sperm head regions: An example in rams. *Cryobiology*, 64; 223-234.
11. Luque, M.C.A. and Bao, S.N. 2006. Structural and ultrastructural characterization of zebu (*Bos indicus*) spermatozoa. *Biocell*. 30:01.
12. Merino, O., Aguaguina, W.E., Esponda, P., Risopatrón, J., Isachenko, E., Isachenko, V. and Scnchez, R. 2015. Protective effect of butylated hydroxytoluene on sperm function in human spermatozoa cryopreserved vitrification technique. *Andrologia*. 47 (2): 186-93.
13. Patel, A., Saxena, A., Swain, D.K., Yadav, D., Yadav, S.S. and Kumar, A. 2015. Effect of supplementation of butylated hydroxytoluene on post-thaw sperm viability, motility and membrane integrity of Hariana bulls. *Veterinary World. 8* (6): 808-812.
14. Salamon, S. and Maxwell, W.M.C. 1995. Frozen storage of ram semen. Cause of low fertility after cervical insemination and methods of improvement. *Animal Reproduction Science*, 38:1-36.
15. Tartaglionea, C.M. and Ritta, M.N. 2004. Prognostic value of spermatological parameters as predictors of in vitro fertility of frozen-thawed bull semen. *Theriogenology*. 62: 1245-1252.
16. Watson, P.F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post thawing function. Reprod. Fertil. Dev. 7: 871–891.

DOI 10.5455/ijlr.20171213100530