

## Effect of Butylated Hydroxytoluene Supplementation in Extender on Motility, Plasmalemma and Viability of Sahiwal Bull Spermatozoa

Muhammad Sajjad Ansari\*, Bushra Allah Rakha and Shamim Akhter

Animal Physiology Laboratory, Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi-46300, Pakistan

**Abstract.** This experiment was designed to evaluate the effect of butylated hydroxytoluene supplementation in extender on motility, plasmalemma and viability of Sahiwal bull spermatozoa. Semen was collected from three Sahiwal bulls of similar age group with artificial vagina for three weeks (replicates). Qualifying semen ejaculates were split into five aliquots and cryopreserved in *tris*-citric extender having either 0.0 (control), 0.5, 1.0, 2.0 or 3.0mM butylated hydroxytoluene. After thawing at 37°C, semen was evaluated for sperm motility, plasma membrane integrity and viability. Sperm plasma membrane integrity was assessed using supravital hypo-osmotic swelling test, while viability was assessed with dual staining procedure using Trypan blue and Giemsa stain. Sperm motility, plasma membrane integrity and viability were higher in extender containing 0.5mM butylated hydroxytoluene compared to the control and extenders containing 1.0, 2.0 and 3.0mM butylated hydroxytoluene. It is concluded that supplementation of 0.5mM butylated hydroxytoluene in *tris*-citric acid extender improved the motility, plasma membrane integrity and viability of Sahiwal bull spermatozoa.

**Key words:** Sahiwal bull spermatozoa; cryopreservation; butylated hydroxytoluene; sperm viability

### INTRODUCTION

Physiological levels of reactive oxygen species (ROS) play essential roles in sperm capacitation, acrosomal reaction and fertilization process (Bailey *et al.*, 2000). However, higher production of ROS due to oxidative stress (Baumber *et al.*, 2005) during freezing and thawing of bull semen is responsible for the deterioration of semen quality (Bilodeau *et al.*, 2000). The damage due to increased ROS can occur in sperm bio-membrane system due to presence of high content of polyunsaturated fatty acids (Lenzi *et al.*, 2002; Kankofer *et al.*, 2005). Increased ROS affect motility, plasma membrane integrity, viability and acrosomal integrity (Aitken *et al.*, 1998; Vishwanath and Shannon, 1997; Akiyama, 1999; Bilodeau *et al.*, 2001; Chatterjee *et al.*, 2001; Lenzi *et al.*, 2002) which ultimately reduce the fertilization potential of the spermatozoa.

Although antioxidants naturally present in bovine semen protect the spermatozoa against oxidative stress but these are insufficient for

protecting the spermatozoal integrity during freeze-thaw process (Baumber *et al.*, 2005; Sreejith *et al.*, 2006; Nichi *et al.*, 2006). It has also been observed that levels of naturally occurring antioxidants in semen decrease during freeze-thaw cycle (Beconi *et al.*, 1993; Bilodeau *et al.*, 2000). Therefore, supplementation of suitable antioxidants in semen extender is recommended to reduce the oxidative stress and to maintain the quality of cryopreserved semen. Butylated hydroxytoluene supplementation was tested in semen extender and found effective to improve the semen quality after cryopreservation of Holstein-Frisian bull semen (Shoae and Zamiri, 2008). However, information on its use in semen extender for Sahiwal bull semen is lacking.

It has been well recognized that there is a remarkable difference in antioxidant defense system and production of ROS of taurine and zebu bull semen (Nichi *et al.*, 2006). Therefore, it is required to test and identify the suitable dose of butylated hydroxytoluene in Sahiwal bull semen extender to improve quality of Sahiwal bull spermatozoa during freeze-thaw process. This experiment was designed to evaluate the effect of butylated hydroxytoluene supplementation in extender on motility, plasmalemma and viability of Sahiwal bull spermatozoa.

\* Corresponding author: m.sajjad.ansari@gmail.com  
0030-9923/2011/0002-0311 \$ 8.00/0  
Copyright 2011 Zoological Society of Pakistan.

## MATERIALS AND METHODS

The stock extender contained *tris*-(hydroxymethyl)-aminomethane (3.03%; w/v), citric acid (1.56%; w/v), fructose (0.2%; w/v), glycerol (7%; v/v), egg yolk (20%; v/v), streptomycin sulphate (1mg/ml), procaine penicillin (300iu/ml), and benzyl penicillin (100iu/ml). Five experimental extenders were prepared by adding 0.0 (Control), 0.5, 1.0, 2.0 or 3.0mM butylated hydroxytoluene.

Semen ejaculates from three Sahiwal bulls were used in this study. From each bull two consecutive ejaculates were collected in graduated plastic tubes using artificial vagina (42°C) at weekly intervals for a period of three weeks. Each ejaculate was immediately transferred to the laboratory for initial evaluation for volume, motility and sperm concentration. Qualifying semen ejaculates from three bulls were split into five aliquots and diluted in five different experimental extenders at a concentration of  $50 \times 10^6$  motile spermatozoa per ml. Diluted semen was cooled to 4°C over a 2 h period and equilibrated for 4h at 4°C. It was then filled in 0.5 ml French straws (IMV, France) with suction pump at 4°C in a cold cabinet and kept in liquid nitrogen vapours for 10 min. Straws were then plunged and stored in liquid nitrogen (-196°C). After 24h, semen straws were thawed in a water bath at 37°C for 30 seconds and assessed for sperm motility, plasma membrane integrity and viability.

Sperm progressive motility was assessed using a phase contrast microscope at 200X by placing semen sample on a pre-warmed (37°C) glass slide and covered with a cover slip. Sperm plasma membrane integrity was assessed by supravital hypo-osmotic swelling (HOS) test as described by Jeyendran *et al.* (1984). After the incubation period, a 5µl aliquot of the suspension was placed on a warm slide and a 5µl droplet of Eosin (0.5% w/v in sodium citrate 2.92%) was mixed for 10 seconds. A cover slip was placed on the mixture and evaluated with phase contrast microscope at 400x. A total of 100 spermatozoa were observed in at least five different fields. Unstained heads and tails but swollen tails indicated intact, biochemically active sperm membranes, while pink heads and tails but unswollen tails indicated disrupted, inactive sperm membranes. Sperm viability was determined by dual

staining procedure (Kovacs and Foote, 1992). The supravital stain Trypan-blue was used to distinguish live and dead spermatozoa while Giemsa stain was used to evaluate the integrity of the acrosome membrane. Briefly, equal sized drops of Trypan-blue and semen were placed on a slide and mixed quickly. Smears were air-dried and slides were fixed with formaldehyde-neutral red for 5 min. After rinsing with running distilled water, Giemsa stain (7.5%) was applied for 4 hours. The slides were rinsed, air-dried and mounted with Balsam of Canada. Trypan-blue penetrated non-viable spermatozoa with disrupted membrane, which appeared stained in blue, while alive spermatozoa appeared unstained. Giemsa accumulated in spermatozoa with an intact acrosome, staining the acrosome region in purple. One hundred spermatozoa were evaluated in at least five different fields in each smear by a phase contrast microscope at 1000x.

MSTAT-C Ver.1.42 was used to analyze the data and the values for post thaw sperm motility, plasma membrane integrity and viability of Sahiwal bull spermatozoa were reported as Mean  $\pm$ SD. Analysis of variance (ANOVA) in completely randomized design was used to analyze the data and when appropriate, Post-hoc comparisons for different treatments were performed with LSD. A level of 5% ( $P < 0.05$ ) was used to determine statistical significance.

## RESULTS AND DISCUSSION

Figure 1 shows the effect of butylated hydroxytoluene (BHT) supplementation in extender on post thaw motility, sperm plasma membrane integrity and viability of Sahiwal bull spermatozoa. All the three parameters showed higher ( $P < 0.05$ ) values in extender containing 0.5mM BHT. The percentage of motility was  $58.3 \pm 3.0$  in 0.5 mM as compared to the values in extenders containing 0 ( $41.7 \pm 2.9$ ), 1.0 ( $46.7 \pm 2.9$ ), 2.0 ( $43.3 \pm 3.0$ ) and 3.0mM ( $41.7 \pm 2.9$ ) BHT.

The percentage of sperm with intact plasma membrane was  $57.0 \pm 2.6$  in 0.5 mM BHT as compared to the values in extenders containing 0 ( $39.3 \pm 3.2$ ), 1.0 ( $46.0 \pm 2.6$ ), 2.0 ( $41.3 \pm 3.1$ ) and 3.0mM ( $40.3 \pm 3.2$ ) BHT.

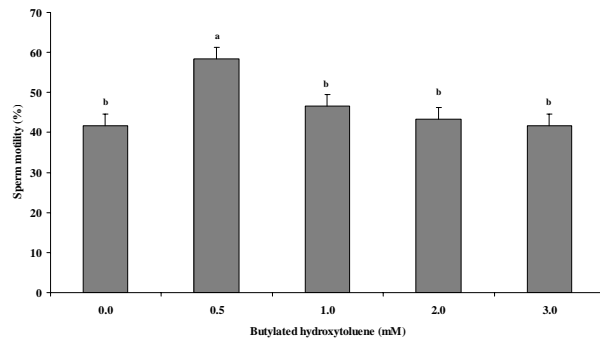
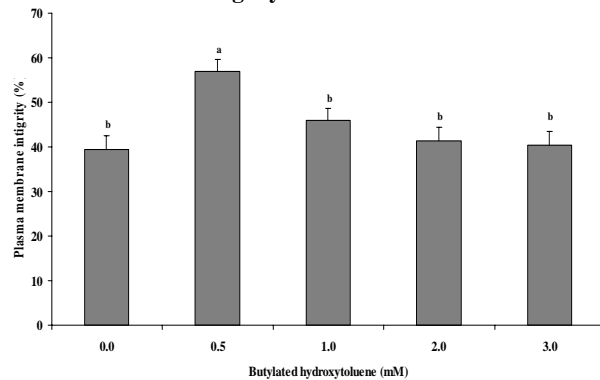
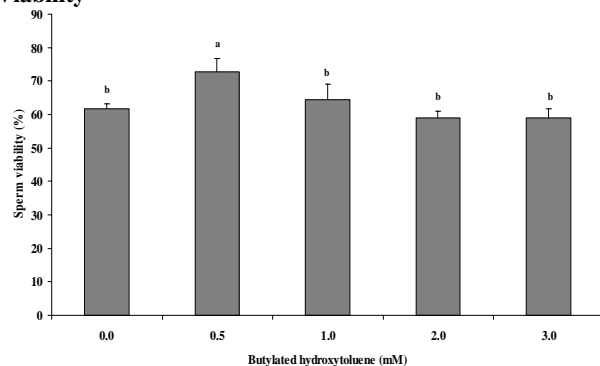
**Motility****Plasma membrane integrity****Viability**

Fig. 1. Effect of supplementation of different concentrations of butylated hydroxytoluene in extender on motility, plasma membrane integrity and viability of Sahiwal bull spermatozoa. Bars with different letters show significant ( $P < 0.05$ ) differences.

The percentage of live sperm with intact acrosomes was  $72.7 \pm 4.2$  in 0.5mM BHT as compared to values in extenders containing 0 ( $61.7 \pm 1.5$ ), 1.0 ( $64.3 \pm 4.7$ ), 2.0 ( $59.0 \pm 2.0$ ) and 3.0mM ( $59.0 \pm 2.6$ ) BHT.

In a similar study, 1.0mM BHT improved post thaw motility of Holstein-Frisian bull spermatozoa in sodium citrate extender. It is believed that species and type of extender affect the sperm protection by BHT (Ball *et al.*, 2001; Roca *et al.*, 2004). It is pertinent to mention that taurine bull semen has higher level of ROS as compared to zebu bull semen (Nichi *et al.*, 2006). It is suggested that increase in sperm motility after BHT supplementation is due to a decrease in oxidative stress and ROS production (Alvarez and Storey, 1983).

It is well documented that sperm viability and integrity of the sperm plasma membrane have critical role in the process of fertilization. Therefore, assessment of sperm viability and plasma membrane integrity is of particular importance due to its interaction with surrounding medium for metabolic exchange. In addition, capacitation, acrosome reaction and the oocyte penetration require a biochemically active sperm plasma membrane (Jeyendran *et al.*, 1984). In this study sperm viability and plasma membrane integrity was higher in extender containing 0.5mM BHT. Our results are in agreement with the study on Holstein-Friesian bull semen (Shoae and Zamiri, 2008), where percentage of sperm with intact plasma membrane was higher in sodium citrate extender containing 0.5mM BHT.

It is concluded that supplementation of 0.5mM BHT in *tris*-citric acid extender improved the motility, plasma membrane integrity and viability of Sahiwal bull spermatozoa. However, further investigations are required to explore its effects on the fertility in artificial insemination programmes.

**REFERENCES**

- AITKEN, R.J., GORDON, E., HARKISS, D., TWIGG, J.P., MILNE, P., JENNING, Z. AND IRVINE, D.S., 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol. Reprod.*, **59**: 1037-1046.
- AKIYAMA, M., 1999. *In vivo* scavenging effect of ethylcysteine on reactive oxygen species in human semen. *Nippon Hinyokika Gakkai Zasshi*, **90**: 421-428.
- ALVAREZ, J.G. AND STOREY, B.T., 1983. Role of superoxide dismutase in protecting rabbit spermatozoa from  $O_2$  toxicity due to lipid peroxidation. *Biol. Reprod.*, **28**: 183-197.
- BAILEY, J.L., BILODEAU, J.F. AND CORMIER, N., 2000.

- Semen cryopreservation in domestic animals: A damaging and capacitating phenomena. *J. Androl.*, **22**: 508-515.
- BALL, B.A., MEDINA, V., GRAVANCE, C.G. AND BUMBER, J., 2001. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. *Theriogenology*, **56**, 577-589.
- 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., FRANZIA, C.R., MORA, N.G. AND AFFRANCHINO, M.A., 1993. Effect of natural antioxidants on frozen bovine semen preservation. *Theriogenology*, **40**: 841-851.
- BILODEAU, J.F., BLANCHETTE, S., GAGNON, C. AND SIRAD, M.A., 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol. Reprod. Develop.*, **55**: 282-288.
- BILODEAU, J.F., BLANCHETTE, S., GAGNON C. AND SIRAD, M.A., 2001. Thiols prevent H<sub>2</sub>O<sub>2</sub> -mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology*, **56**: 275-286.
- CHATTERJEE, S., LAMIRANDE, E.D. AND GAGNON, C., 2001. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: Protection of oxidized glutathione. *Mol. Reprod. Develop.*, **60**: 498-506.
- JEYENDRAN, R.S., VAN DER VEN, H.H., PEREZ-PELAEZ, M., CRABO, B.G. AND ZANEVELD, L.J.D., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.*, **70**: 219-228.
- KANKOFER, M., KOLM, G. AND AURICH, J., 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5°C. *Theriogenology*, **63**: 1354-1365.
- KOVACS, A. AND FOOTE, R.H., 1992. Viability and Acrosome staining of bull, boar and rabbit spermatozoa. *Biotech. Histochemistry*, **67**: 119-124.
- LENZI, A., GANDINI, L., LOMABARDO, F., PICARDO, M., MARESCA, V. AND PANFILI, E., 2002. Polyunsaturated fatty acids of germ cell membranes, glutathione and glutathione-dependant enzyme-PHGPx from basic to clinic. *Contraception*, **65**: 301-304.
- 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.
- ROCA, J., GILT, M.A., HERNANDEZ, M., PARRILIA, I., VAZQUEZ, J.M. AND MARTINEZ, E.M., 2004. Survival and fertility of boar spermatozoa after freeze-thawing in extender supplemented with butylated hydroxytoluene. *J. Androl.*, **25**: 397-405.
- SHOAE, A. AND ZAMIRI, M.G., 2008. Effect of butylated hydroxytoluene on bull spermatozoa frozen in egg yolk-citrate extender. *Anim. Reprod. Sci.*, **104**: 414-418.
- 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.
- VISHWANATH, R. AND SHANNON, P., 1997. Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. *Reprod. Fertil. Dev.*, **9**: 321-332.

(Received 12 July 2010, revised 18 August 2010)