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Effect of anti-oxidant supplementation on post-thaw sperm characteristics, membrane integrity, migration capability and lipid peroxidation in bull semen

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ABSTRACT

A study was conducted to assess the influence of natural (vitamin E) and synthetic (butylated hydroxyl toluene; BHT) anti-oxidants on post-thaw semen characteristics, migration ability and functional integrity and lipid peroxidation following freezing and thawing in crossbred HF bulls. Semen was collected from 3 crossbred bulls (HF×T) twice a week and 18 collections (6 collections per bull) were utilized in this study. After initial evaluation each semen sample was split into 3 equal fractions and diluted in tris egg yolk extender containing either vitamin E @0.3mg/ml (TEY-E), BHT @2.0mM/ml (TEY-B) or without any additive (TEY-C) which acted as control. Filling and sealing of individual diluted semen samples in PVA straws, equilibration at 4°C for 4h, freezing in programmable freezer and plunging of straws into liquid nitrogen were followed as per standard procedure. Thawing of semen was performed at 37°C for 30 sec. Post-thaw semen characteristics such as progressive motility, live sperm percent and acrosomal integrity were significantly higher and sperm abnormalities were significantly lower in samples containing vitamin E and BHT over the control. The sperm penetration distance (mm/20min) was highest in TEY-E (50.19±1.36) followed by TEY-B (40.94±1.54) and the least in TEY-C (36.83±1.49) diluents. Similarly the HOS positive spermatozoa (%) were significantly higher in vit E (49.21±0.97) and BHT (48.12±0.89) added extenders over the control (37.86±1.39).The MDA levels (µmol/ml) increased significantly in non-antioxidant added samples (0.60±0.05) over vit E (0.11±0.01) and BHT (0.19±0.02) containing samples following thawing. The results revealed that supplementation of vit E and BHT to tris egg yolk extender improve the post-thaw semen quality in bulls.

Key words: BHT, Lipid peroxidation, Post-thaw semen quality, Tris, Vitamin E

Cryopreservation coupled with artificial insemination (AI) made rapid strides in genetic improvement of livestock world over during the last millennium. The sperm viability is depressed by as much as 50% during freezing (Watson 1995). Estimation of melanaldehyde (MDA) in the spermatozoa, an end product of lipid peroxidation helps in assessing the extent of cellular damage. The final outcome of oxidative stress is the reduced success rate following AI (Chatterjee and Gagnon 2001). Further more, due to dilution of semen the anti-oxidant reserves of seminal plasma are depressed making the spermatozoa more vulnerable to cryo insults. Therefore, it becomes all the more essential to incorporate anti-oxidants to semen extenders as protective agents. Assessing the structural and functional integrity of sperm membrane is of paramount importance in knowing the fertility potential of a spermatozoa. Hypo-osmotic swelling test (HOST) is based on functional integrity of sperm cell membrane (Jayendran *et al.* 1984). Addition of natural (vit E) or synthetic (butylated hydroxyl toluene, BHT) anti-oxidants as components of extender have yielded equivocal results in different species (Beconi *et al*. 1993, Ijaz *et al*. 2009). Thus the present study was undertaken to assess the beneficial effects of vit E, a free radical chain breaking natural anti-oxidant and butylated hydroxyl toluene, a synthetic analogue of vit E, on post-thaw semen characteristics, membrane integrity, migration capability of sperm in cervical mucus and lipid peroxidation following freezing and thawing in crossbred HF bulls.

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MATERIALS AND METHODS

Three crossbred bulls (HF ×Tharparkar) aged between 2 and 9 years maintained at frozen semen bull station, Hakkal, Jammu were selected for this study. Semen was collected twice a week between 7.00 AM and 8.00 AM with artificial vagina following standard protocol. On each collection day, 2 ejaculates were collected at an interval of 15–20 min

and the semen was transferred to a water bath at 37°C till processing. Semen collections (18) from 3 bulls (6 collections/bull) were utilized in this study.

After evaluating the semen sample based on at least 75% initial motility, it was divided into 3 equal fractions; one fraction was diluted in TRIS egg-yolk extender which acted as control (TEY-C), second fraction was diluted in TRIS egg-yolk extender containing anti-oxidant vit E @0.3mg/ml (TEY-E) and the third fraction was diluted in TRIS egg-yolk extender containing anti-oxidant butylated hydroxyl toluene @2.0mM/ml (TEY-B). The semen was diluted in such a way that it contained 80 million spermatozoa/ml, post dilution. The diluted semen was filled in 0.25ml poly vinyl chloride straws in an automatic filling and sealing machine. Sealed straws were equilibrated in cold handling unit for 4h and transferred to a bio-freezer where the temperature was brought down from 4 to –140° C in 7 min. The straws were transferred to pre-cooled plastic goblets and plunged into liquid nitrogen. After 10–15 days of preservation in liquid nitrogen, at least 2 straws for each extended sample of a bull in each collection were thawed at 37° C for 30 sec and evaluated for various parameters such as progressive motility, livability (eosin-nigrosin vital stain), sperm abnormality (1% buffered formal saline), acrosomal integrity (Giemsa stain), hypo-osmotic swelling test and cervical mucus penetration test. Lipid peroxidation level of spermatozoa was determined in frozen–thawed semen samples by measuring the malanaldehyde (MDA) production, using thiobarbituric acid (TBA) as described by Kumaresan *et al.* (2006) with slight modifications. One semen straw per bull per collection for each extended sample was thawed, washed twice in Tris buffer by centrifugation (500×*g* for 5 min each) and the supernatant was removed. The spermatozoa pellet was re-suspended in PBS (pH 7.2) to obtain a spermatozoa concentration of 20×10^6/ml. Lipid peroxide levels were measured in spermatozoa after the addition of 2ml of thiobarbituric acid trichloroacetic acid (TBATCA) reagent (15%, w/v TCA; 0.375%, w/v TBA and 0.25N HCl) to 1ml of spermatozoa suspension. The mixture was kept in a boiling water bath for 45min. After cooling, the suspension was centrifuged at 500×*g* for 15 min. The supernatant was separated and the absorbance was measured at 535 nm under UV spectrophotometer. The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5 molcm^{-3}).

$$\text{MDA produced} = (\mu\text{mol/ml}) \frac{\text{OD} \times 106 \times \text{total volume (3ml)}}{1.56 \times 105 \times \text{test volume (1ml)}} = \frac{\text{OD} \times 30}{1.56}$$

The data recorded were analyzed by using one-way ANOVA as per Snedecor and Cochran (1989).

RESULTS AND DISCUSSION

The effect of addition of vit E and BHT to tris egg yolk extender on post-thaw semen parameters, membrane integrity, migration capability in cervical mucus and MDA production are presented in Table 1. In the present study, the post-thaw progressive motility, livability and acrosome integrity were significantly higher (P<0.01) and sperm abnormalities significantly lower (P<0.01) in extenders containing vit E and BHT compared to control diluent. However, no significant differences could be observed between the TEY-E and TEY-B except for post-thaw acrosome integrity which was higher in TEY-E diluent over other 2 extenders. Other investigators have also reported beneficial effects of vitamin E (Beconi *et al.* 1993, Swain *et al.* 2008, Andrabi *et al.* 2008) and BHT (Killian *et al.* 1989, Graham and Hammerstedt, 1992, Arangasamy *et al.* 2002 and Jordi Roca *et al.* 2004) on post-thaw sperm functions. BHT modifies the properties of lipid bilayer and membrane of sperm cell and serves as a scavenger of oxygen-free radicals associated with diluent and sperm (Killian *et al.* 1989). Similarly, the beneficial effects of vit E can be attributed to its protective action on the sperm cell membrane against ROS and lipid peroxidation (Surai *et al.* 1998).

Table 1. Sperm characteristics, membrane integrity, migration ability and, MDA production in frozen-thawed bovine semen supplemented with Vitamin E and BHT

Attribute	TEY-C	TEY-E	TEY-B
Post-thaw sperm characteristics			
Progressive motility (%)	44.44 ±1.96[a]	56.39 ±1.54[b]	55.55 ±1.51[b]
Live sperm count (%)	51.91 ±1.56[a]	61.08 ±1.01[b]	58.19 ±0.93[b]
Sperm abnormalities (%)	18.00 ±0.49[a]	15.14 ±0.46[b]	15.69 ±0.49[b]
Acrosome integrity (%)	79.69 ±0.75[a]	84.72 ±0.48[b]	82.86 ±0.69[c]
Functional integrity and migration capability			
HOS positive sperms (%)	37.86 ±1.39[a]	49.21 ±0.97[b]	48.12 ±0.89[b]
SPD (mm/20 min.)	36.83 ±1.49[a]	50.19 ±1.36[b]	40.94 ±1.54[c]
MDA production (μ mol./ml)	0.60 ±0.06[a]	0.12 ±0.01[b]	0.19 ±0.02[b]

Means bearing different superscripts with in a row differ significantly (P<0.01).

In the present study, the average SPD values (mm/20min) were 36.83±1.49, 50.19±1.36 and 40.94±1.54 in TEY-C, TEY-E and TEY-B diluents, respectively. Sushant and Kumar (2006) and Kumar (2007) reported similar SPD values in crossbred bulls. On the contrary, much lower values (16–21mm/20min) of SPD were reported by Kumar *et al.* (2001) and Kumar *et al.* (2003). The major spermatozoal characteristics that determine the capacity of spermatozoa to penetrate either gel or mucus are post-thaw motility, concentration, per cent abnormal spermatozoa and acrosome

integrity (Galli *et al*. 1991 and Bals Pratsch *et al*. 1988). In this study, since the sperm concentration is constant in all the diluents, the variation in SPD between extenders might be due to differences in the post-thaw motility, abnormal sperms and acrosome integrity. Of all the parameters acrosome integrity was found to have more significant contribution to SPD (Kumar *et al*. 2001). In line with this observation, the highest acrosome integrity (%) obtained in post-thaw semen of TEY-E extender (84.72±0.48) might explain the superiority of this extender in respect of SPD (50.19±1.36 mm) over the other 2 extenders. Similarly the superiority of TEY-E and TEY-B over TEY-C diluent in respect of all post-thaw semen characteristics might substantiate the higher SPD values in the experimental diluents compared to control diluent.

In our study, significantly higher % of HOS positive sperm was observed in the presence of additives (49.21±0.97 in TEY-E and 48.12±0.89 in TEY-B) compared to control diluent TEY-C (37.86±1.39). This may be substantiated by the fact that these non-enzymatic antioxidants (vit E and BHT) reduce the damage caused by free radicals and also act as potential scavengers of such radicals. Frozen–thawed spermatozoa produce reactive oxygen species (Alvarez and Storey 1992) and excessive formation of reactive oxygen species during cryopreservation has been associated with decrease in quality of thawed spermatozoa (Stradaioli *et al*. 2007). The HOS responsive sperm (%) observed in this study is much higher than earlier reports (Kumar *et al.* 2003, Rana and Dhami 2003) and the observed difference may be attributed to variation in the breed of bulls (Prasad *et al*. 1999), season (Kale *et al.* 2000), post-thaw motility, livability and intact acrosome (Prasad *et al.* 1999) and individual fertility levels (Jayendran *et al.* 1984).

The sperm plasma membrane by virtue of its rich polyunsaturated fatty acid levels is highly susceptible to oxidative damage which may interfere with fertilizing ability. The lipid peroxides, assayed in terms of malanaldehyde (MDA) concentration cause membrane damage and reduced motility. In the present study the MDA concentration (µmol/ml) of post-thaw semen was significantly lower in diluents containing vit E (0.12±0.01) and BHT (0.19±0.02) over the control diluent (0.60±0.06). In contrast, Serpil *et al.* (2009) recorded the MDA levels of 1.37±0.21 to 2.43±0.25 µmol/l in post-thaw semen supplemented with antioxidants and these levels did not differ with that of control diluent.

It may be concluded that the supplementation of anti-oxidants to the extender has beneficial effect on post-thaw sperm characteristics and membrane integrity and sperm migration ability, and the production of MDA was significantly inhibited. Detailed information on field based fertility studies in a more controlled manner would throw more light on the influence of antioxidant additives in preserving the potential sperm characteristics and fertilizing ability following cryopreservation.

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REFERENCES

Alvarez J G and Storey B T. 1992. Evidence for increased lipid peroxidative damage and loss of superoxide desmutage activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *Journal of Andrology* **13**: 232–41.

Andrabi S M H, Ansari M S, Ullah N and Afzal M. 2008. Effect of non-enzymatic antioxidant in extender on post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. *Pakisthan Veterinary Journal* **28**: 159–62.

Arangasamy A, Singh L P, Ansari M R, Gupta O P and Bisht G S. 2003. Effect of protaglandin F2α, oxytocin and butylated hydroxyl toluene on enzymatic activities of crossbred (HF × Hariana) bull semen. *Indian Journal of Animal Reproduction* **24**: 54–55.

Bals Pratsch M, Knuth U A and Nieschlag E. 1988. Does sperm penetration in bovine mucus provide additional information to the classical spermiogram and motility characteristics. *Zentralhl Gynakol* **110**: 224–31.

Beconi M T, Affranchino M A, Schang L M and Beorlegni N B. 1993. Effect of natural antioxidants on frozen bovine spermatozoa preservation. *Theriogenology* **40**: 841–51.

Chatterjee S and Gagnon. 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. *Molecular Reproduction Development* **59**: 451–58.

Galli A, Basetti W, Baldizzi D, Martignoni M, Bornaghi V and Miffi M. 1991. Frozen bovine semen quality and bovine cervical mucus penetration test. *Theriogenology* **35**: 837–44.

Graham J K and Hammerstedt R H. 1992. Differential effect of BHT analogues on bull sperm subjected to cold induced membrane stress. *Cryobiology* **29**: 106–17.

Ijaz A, Hussain A, Alewem M, Yousaf M S and Rehman H. 2009. Butylated hydroxytoluene inclusion in semen extender improves the post-thawed semen quality of Nili-Ravi buffalo (*Bubalus bubalis*). *Theriogenology* **71***:* 1326–29.

Jayendran R S, Vandervent H H, Pereze-Peleae Z M, Crobo B G and Zenevald L J D. 1984. Development of assay to access the functional integrity of human sperm membrane and its relation to other semen characteristics. *Journal of Reproduction Fertilility* **70**: 219–25.

Jordi Roca, Maria A, Gil, Marta Hernandez, Inma Parilla, Vazquez J M and Martinez E A. 2004. Survival and fertility of boar spermatozoa after freez-thawing in extender supplemented with butylated hydroxytoluene. *Journal of Andrology* **25**: 397–405.

Kale M M, Manik R S and Tomer O S. 2000. *In-vitro* assessment of crossbred buck fertility. *Indian Journal of Animal Sciences* **70(1)**: 25–29.

Killian G, Honadel T, McNutt T, Henault M, Wegner C and Dunlap D. 1989. Evaluation of BHT as cryoprotective added to whole or skim milk diluent for bull semen. *Journal of Dairy Science* **72**: 1291–95.

Kumar Ajeet, Jagir Sigh, Nanda A S, Matharoo J S and Prahlad. 2003. Assessment of osmotic resistance and sperm penetration in polyacrylamide gel after sephadex filtration of post-thaw bull semen. *Indian Journal of Animal Sciences* **73**: 854–57.

Kumar Anil R, Devanathan T G, Pattabiraman S R and Edwin M J. 2001. Correlation between spermatozoa characteristics and sperm penetration distance in PAG and bovine cervical mucus. *Theriogenology* **55**: 685–91.

Kumar Satish. 2007. Effect of certain additives in the diluter on post-thaw recovery rate of bovine semen. *Indian Journal of Veterinary Research* **1**: 29–42.

Kumaresan A, Ansari M R and Garg. A. 2006. Modulation of post-thaw sperm functions with oviductal proteins in buffaloes. *Animal Reproduction Science* **90** : 73–84.

Prasad J K, Kumar S, Mohan G, Agarwal S K and Shankar U. 1999. Simple modified method for cervical mucus penetration test for quality assessment of bull semen. *Indian Journal of Animal Science*s **69**: 103–05.

Rana C M and Dhami A J. 2003. Acrosomal integrity and HOS response at initial, post-thaw and post-refrigeration stages in sephadex filtered Gir (*Bos indicus*) and Jafarabadi (*Bubalus bubalis*) bulls' semen. *Indian Journal of Animal Reproduction* **24**: 113–20.

Serpil Sariözkan, Pürhan Barbaros Tuncer, Mustafa Numan Bucak and Pinar Alkim Ulutas. 2009. Influence of various antioxidants on microscopic-oxidative stress indicators and fertilizing ability of frozen-thawed bull semen. *Acta Veternaria Brno* **78** : 463–69.

Snedecor G W and Cochran W G. 1976. *Statistical Methods*. 6th edn. Iowa State University Press, Ames.

Stradaioli G, Noro T, Sylla L and Monaci M. 2007. Decrease in Glutathione content in bovine sperm after cryopreservation: comparison between two extenders. *Animal Reproduction Science* **67**: 1249–55.

Surai P, Kostjuk I, Wishart G, Macpherson A, Speake B, Noble R, Ionov I and Kutz E. 1998. Effect of vit. E and selenium supplementation of cockeret diets on glutathione peroxidase activity and lipid peroxidation succeptibility in sperm,testes and liver. *Biology of Trace Element Re*search **64**: 119–32.

Sushant S and Kumar S. 2006. Effect of certain additives on the freezability of crossbred bull semen. *Indian Journal of Animal Reproduction* **27**: 1–5.

Swain D K and Kundu A K. 2009. Effect of antioxidants on membrane integrity of cryopreserved bull spem, *Indian Veterinary Journal* **86**: 1076–77.

Watson P F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction Fertility Development* **7**: 871–91.