# EFFECT OF ANTI-OXIDANT SUPPLEMENTATION ON POST THAW SPERM CHARACTERISTICS, CELLULAR ENZYME LEAKAGE AND LIPID PEROXIDATION IN BULL SEMEN

**M.MUTHA RAO , PAWAN KUMAR VERMA , MUZAFER AHMED BHAT, A.K.PANDEY AND R.RAINA**

*Division of Animal Reproduction, Gynaecology & Obstetrics, FVSc &AH, SKUAST-J, RSPura.-181 102, Jammu.*

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## ABSTRACT

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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, enzyme release (lactic dehydrogenase. acid phosphatase and cholinesterase) into extra cellular medium and lipid peroxidation following freezing and thawing in crossbred HF bulls. Semen was collected from three crossbred bulls (HFxT) twice a week and a total of 18 collections (six collections per bull) were utilized in this study. After initial evaluation each semen sample was split into three equal fractions and diluted in tris egg yolk extender containing either vitamin *E* 0.3mg/ml (TEY-E), BHT 2.0mM (TEY-B) or with out 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 seconds. Post-thaw semen characteristics such as progressive motilitv live sperm (%), and acrosomal integrity (%) were significantly higher and sperm abnormalities were significantly lower in samples containing Vit.E and BHT over the control. The leakage of enzymes (IU/L) such as acid phosphatase, cholinesterase and lactic dehydrogenase was significantly lower in TEY-E followed by TEY-B and highest in the TEY-C diluents. The MDA levels (umol/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 improved the post-thaw semen quality in bulls.

## INTRODUCTION

Cryopreservation coupled with artificial insemination (AI) made rapid strides in genetic improvement of livestock world over during the last millennium. However, the process of freezing and thawing of semen has deleterious effects on post-thaw semen characteristics and fertility. It has been estimated thai the sperm viabiliu is depressed by as much as 50% during freezing (Watson. 1995) partly due lo excess production of reactive oxygen species (ROS) and lipid peroxidation which leads to irreversible structural damage to sperm cell plasma membrane. Estimation of melandialdehyde (MDA) in the spermatozoa, an end product of lipid peroxidation helps in assessing the extent of cellular damage. Cells with membrane damage lose essential metabolites and enzymes. Numerous enzymes have been determined in semen of several species. These include aspartate-aminotransferase (AT-ase). lactale dehxdrogenase (I.DH). cholinesterase. acid phosphatase and alkaline phosphatase (Brown et al. 1971. Risse 1990). Following dilution of semen the ani-oxidant reserves of seminal plasma are depleted making the spermatozoa more vulnerable to cryo insults.

Corresponding author.
E mail:mutharaom@rediffmail.com,
Assistant Professor, Division of Veterinary Pharmacology & Toxicology. Professor, Division of Veterinary Pharmacology & Toxicology

Therefore, it becomes all the more essential to incorporate anti-oxidanls lo semen extenders as protective agents. Addition of natural (Vit.E) or synthetic (Butylated Hydroxyl Toluene; BUT) anti-oxidants as components of extender have yielded equivocal results in different species (Beconi et al.. 1993: Ijaz el al.. 2009). Thus the present stud}' was undertaken to assess the effect of Vit.E, a free radical chain breaking natural anti-oxidant and BHT. a synthetic analogue of Vit.E. on post-thaw semen characteristics, enzyme release into extra cellular medium and lipid peroxidation following freezing and thawing in crossbred HF bulls.

## MATERIALS AND METHODS

Three cross bred bulls (HF x 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. two ejaculates were colleeled at an interval of 15-20 minutes and the semen was transferred to a water bath at 37°C till processing. A total of 18 collections from three bulls (six collections per bull) were utilized in this study.

After evaluating the semen sample based on at least 75% initial motility. it was divided into three 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 Vit. E 0.3mg/ml (Himedia Laboratories Pvt. Ltd., Mumbai) (TEY-E) and the third fraction was diluted in TRIS egg-yolk extender containing BHT 2.0mM (Merck specialties Pvt. Ltd., Mumbai) (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 (IS-4. IMV Technologies, France). Sealed straws were equilibrated at 4°C in cold handling unit (GO-38, IMV Technologies, France) for 4h and then transferred to a bio-freezer (Digit cool-5300, IMV Technologies, France) where the temperature was brought down from 4 to -140° C in 7 minutes. The straws were then transferred to pre-cooled plastic goblets and plunged in to liquid nitrogen. After 10-15 days of preservation in liquid nitrogen, at least two straws for each extended sample of a bull in each collection were thawed at 37° C for 30 seconds and evaluated for various parameters such as progressive motility, livability (eosin-nigrosin vital stain), total sperm abnormalities (1% buffered formal saline) and acrosomal integrity (Giemsa stain). Presence of enzyme activity in the extra cellular medium was determined using standard test kits. These enzymes include acid phosphatase and cholinesterase (Reckon diagnostics Pvt.Ltd., Vadodara) and. lactic dehydrogenase (LDH) (Erba diagnostic Mannheim. Germany). Lipid peroxidation level of spermatozoa was determined in frozen-thawed semen samples by measuring the melandialdehyde (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 *(500ˣg* for 5 minutes each) and the supernatant was removed. Then the spermatozoa pellet was re-suspended in PBS (pH 7.2) to obtain a spermatozoa concentration of 20x10 /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 HC1) to 1ml of spermatozoa suspension. The mixture was kept in a boiling water bath for 45minutes. After cooling, the suspension was centrifuged at 500ˣg for ISminutes. The supernatant was separated and the absorbance was measured at 535 nm under UV spectrophotometer (U1800, Hitachi, Japan). The MDA concentration was determined by the specific absorbance coefficient ($1.56 \times 10^5$/molcirf$^3$).

$$\text{MDA produced } (\mu\text{mol/ml}) = \frac{\text{OD} \times 10^6 \times \text{total volume (3ml)}}{1.56 \times 10^5 \times \text{test volume (1ml)}} = \frac{\text{OD} \times 30}{1.56}$$

The data recorded were analyzed by using one way ANOVA as per the procedure described by 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, extra cellular enzyme leakage and MDA production is presented in Table 1. In the present study, the post thaw progressive motility, livability and acrosome integrity were observed to be significantly higher (PO.01) and sperm abnormalities were significantly lower (PO.01) in extenders containing Vit.E and BHT as compared to control diluent. However, no significant difference was observed between the TEY-E and TEY-B except for post-thaw acrosome integrity which was found to be higher in TEY-E diluent over other two extenders. Other investigators have also reported beneficial effects of Vit.E Swain *et al,* 2008 and BHT Jordi Roca *et al.,* 2004) on post thaw sperm functions. It has been documented that 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).

The mean activity of acid phosphatase (IU/L) was significantly (P<0.01) lower in the extra cellular medium of post-thaw semen supplemented with Vit.E (4.32 ± 0.59) and BHT (4.41 ± 0.61) compared to the control diluent (13.02±0.91). The leakage of enzymes lactic dehydrogenase (IU/L) and cholinesterase (IU/L) respectively \\as significant h high in the TEY-C diluent (472.19 ± 27.99 and 1093.75 ± 76.84) followed by TEY-B (301.27±10.55 and 671.71 ±13.22) and the least in the TEY-E (248.47 ± 5.5 and 378.44 ± 9,58) extenders. High enzyme activity in the extra cellular medium is an indication of sperm plasma membrane damage due to either cold shock or inappropriate concentration of extender ingredients (Singh *et at-.* 1992) and estimation of enzymes in turn reflect the efficacy of extender in maintaining the membrane integrity of spermatozoa during freezing and thawing (Prasad *et at..* 2000). In the present study supplementation of anti-oxidants. Vit.E and BHT to the extender might have provided more protection to sperm membrane there by maintaining the structural integrity and cell membrane permeabilit and. thus reduced the enzyme leakage. These results are in agreement with those of and Srivastava and Satish Kumar (2004). The results of this study further indicated the superiority of extender containing natural antioxidant (Vit.E) over its synthetic counter part (BHT) as evidenced *by* high acrosomal integrity and the least cellular enzyme leakage with TEY-E extender.

The sperm plasma membrane by virtue of its rich polyunsalurated fatly acid levels is highly susceptible to oxidalive damage. Excess free radicals attack the thiol group of cysteine residue and polyunsaturated fatty acid (PUFA) chain of phospholipids of biological membrane (Chow and Tappel. 1972). there by damaging the membrane stability (Sen *el at.,* 2006). In the present study the MDA concentration *(\i* rnol/ml) of post thaw semen was significantly (P<0.01) lower in diluents containing Vit.E (0.12±0.01) and BHT (0.19±0.02) over the control diluent (0.60±0.06). This shows that these anti-oxidant agents either decrease the free radicals production or scavenge the free radicals/reactive oxygen species generated during freezing and thawing of the semen. In contrast, Serpil *el at.* (2009) recorded the MDA levels of 1.37 i 0.21 to 2.43 ± 0.25 nmol/1 in post-thaw semen supplemented with antioxidants and these levels did not differ with that of control diluent. The values recorded in this study such as low post thaw sperm motility (44.44±1.96%), And acrosomal integrity (79,69±0.75). higher cellular enzyme leakage, more sperm MDA concentration in the diluent not provided with anti-oxidant supplementation (TEY-C) compared to those vvhh anti-oxidants (TEY-H and TEY-B). indicate that in the absence of anti-oxidant support there might ha\c been loss of sperm membrane integrity and thus increased permeability.

From the results of the present study, it may be concluded that the supplementation of anti-oxidants to the extender has beneficial effect on post-thaw sperm characteristics and membrane stability (reduced enzyme leakage) 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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