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# Impact of dietary feeding of vitamin E in buffalo bulls on fresh and frozen-thawed semen characteristics and antioxidant status

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## ABSTRACT

Vitamin E is the main chain-breaking, naturally occurring free radical scavenger that has significant biological implications on sperm. However, its role as an antioxidant on semen quality of buffalo bulls is still obscure. The present study was undertaken to investigate the effect of dietary feeding of vitamin E on fresh- and frozen-thawed semen characteristics, and antioxidant status in buffalo bull. Six apparently healthy breeding Murrah buffalo bulls were randomly selected at University bull farm for the present study. The bulls were divided into two groups, viz. control group (n = 3) and feeding group (n = 3). The bulls of feeding group were fed vitamin E @ 4000 IU/bull/day for 60 days. Accordingly, 120 ejaculates (one ejaculate/bull/session) were collected from bulls of control and feeding groups during pre-feeding, feeding and post-feeding phase of vitamin E and analyzed for semen characteristics and oxidative stress. Most beneficial effects of dietary feeding of vitamin E were observed during post-feeding phase. The percentages of total and progressive motility, viability, plasma membrane integrity, malondialdehyde (MDA) and glutathione peroxidase (GPX) in bulls fed with vitamin E were significantly higher than in their control counterparts during post-feeding phase of fresh and frozen-thawed semen. The levels of same parameters were also significantly higher as compared to that during feeding stage in fresh- and frozen-thawed semen of feeding group. It is therefore concluded that feeding vitamin E to buffalo bulls protected sperm membrane against oxidative damage and improved the fertilizing potential of spermatozoa.

**Key words**: Antioxidant status, Buffalo bulls, Cryopreservation, Semen, Seminal attributes, Vitamin E

Buffalo bulls are alleged to have poor post-thaw semen quality owing to the cryoinjuries during freezing and thawing. In India, of the 55 million breedable buffaloes, merely 15% are bred through artificial insemination due to lower freezability of semen (Kumar *et al.* 2014). During sperm cryopreservation, spermatozoa suffer molecular lesions, viz. osmotic stress, oxidative damage and apoptosis which can lead to negative results after its use in artificial insemination (Pe˜na *et al.* 2011). It is essential that biological membranes are preserved during stresses caused by physical and chemical changes that occur during the cryopreservation process (Guerra *et al.* 2004). Studies demonstrate that natural antioxidants present in seminal plasma exert a protective effect on the spermatozoon plasma membrane, preserving both metabolic activity and cellular function (O'Flaherty *et al.* 1997). However, ejaculate dilution reduces antioxidant concentrations found in seminal plasma compared to that in original volume. The number and quality of spermatozoa produced by testis is determined by interaction of two strategies, viz. pre-production (before ejaculation) optimization of semen quality through dietary supplementation of nutraceuticals and post-production (after ejaculation) nurturing of spermatozoa which involves *in vitro* treatment of semen with additives and/or proteins (Alvarez 2003, Singh *et al.* 2016). Among various nutraceuticals and additives, vitamin E is considered to be most potent, owing to beneficial effects on semen quality (Yousef *et al.* 2003). Moreover, vitamin E is the principal constituent of antioxidant defense system of spermatozoal membrane, protecting against reactive oxygen species and lipid peroxidation attack. Deficiency of vitamin E may lead to reproductive organ damage, such as degenerative spermatogonium, testicular damage and degeneration of the seminiferous tubules (Wilson *et al.* 2003). In Holstein bulls, reproductive effects of feeding vitamin E exhibited higher motility and viability in fresh ejaculates (Velasquez-Pereira *et al.* 1998). Most studies in different species have been conducted on vitamin E supplementation to cryodiluent and thereby determine its effect on post-thaw semen quality (Andrabi *et al.* 2008). However, studies on the effect of vitamin E feeding on semen quality are sparse and limited in buffalo bulls. Hence, the present study was designed to

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supplement the buffalo bull diet with vitamin E and determine its effect on sperm characteristics and antioxidant status in fresh and frozen-thawed semen.

## MATERIALS AND METHODS

*Semen collection, processing, evaluation and cryopreservation:* Six apparently healthy breeding Murrah buffalo bulls (aged 4–6 years; bodyweight 695.7±18.7 kg) were randomly selected at University bull farm for the present study. The bulls were kept under loose housing system and maintained on standard feeding schedule as per NRC recommendations and management conditions. Semen from each bull was collected through artificial vagina method (one ejaculate/bull/week). None of the bulls selected for this study was being fed vitamin E prior to start of the experiment. The bulls were randomly divided in two groups, viz. control group (3); bulls kept on standard feeding schedule and feeding group (3); bulls (600–1000 kg bodyweight) were fed vitamin E @ 4000 IU/bull/day for 60 days as per the recommendations of Velasquez-Pereira *et al.* (1998) in addition to standard feeding schedule.

A good quality fresh ejaculate (1–2 ml) of each bull in each group having prefreezing sperm motility ≥70% and mass activity >4 was collected once a week for next 20 weeks (4 weeks pre-feeding; 8 weeks during feeding and 8 weeks post-feeding). Accordingly, a total of 120 ejaculates from six bulls were collected and subjected to cryopreservation for evaluation and comparison in this experiment. The equilibrated semen was loaded into 0.25 ml plastic straws (IMV Technologies, L'Aigle, France) and cryopreserved. The straws were exposed to liquid nitrogen vapour for 12 min, plunged into liquid nitrogen ($LN_2$) and stored in $LN_2$ till further analysis. The post-thaw semen analysis was done within a week of cryopreservation.

*Collection of blood samples:* The bulls were allowed to stand in the chute for 30 min prior to blood collection to avoid any stress of restraint. Blood samples (10 ml) from each bull of control and feeding group were obtained through left jugular venipuncture in heparinized vials at weekly intervals for next 20 weeks. The plasma was harvested from blood by centrifugation at 3,000 rpm for 15 min and stored at –20°C in duplicate vials until assayed for vitamin E.

*Evaluation of sperm parameters:* The fresh and frozen-thawed sperm were evaluated for CASA-based motion traits (Biovis 2000 version 4.59), viability through Eosin-Nigrosin staining technique, plasma membrane integrity using hypo-osmotic solution (100 mosm/l), acrosome integrity and *in vitro* capacitation/acrosome reaction with chlortetracycline cysteine (CTC) stain (Wang *et al.* 1995). The number of sperm was converted to percentage.

*Oxidative stress in semen:* The assessment of oxidative stress in fresh and post-thaw semen samples was done through estimations of LPO (MDA, µmole/$10^9$ sperm); GPX (IU/$10^9$ sperm/minute) and SOD (IU/$10^9$ sperm/minute).

*Lipid peroxidation* (*LPO*)*:* Briefly, 100 µl semen was incubated with 100 µl of 150 mM Tris HCL (pH 7.1) at 37°C for 20 min. Following incubation, 0.5 ml of 10% TCA and 1.0 ml of 0.375% TBA were added and kept for 20 min in boiling water bath. Thereafter, the mixture was cooled and centrifuged for 15 min at 10,000 rpm, supernatant was taken out and absorbance was taken at 532 nm. The molar extinction coefficient for malondialdehyde (MDA) was determined according to the following formula:

$$\text{MDA } (\mu\text{mole}/10^9 \text{ sperm}) = \frac{\text{OD} \times \text{Volume of assay mixture}}{\begin{matrix}\text{Extinction}\\ \text{coefficient}\end{matrix} \times \begin{matrix}\text{Volume of}\\ \text{sample taken}\end{matrix}}$$

*Glutathione peroxidase* (*GPX*)*:* In the assay tubes, 0.1 ml of extracted semen, 0.2 ml of 8 mM GSH and 0.4 ml of 0.4 M phosphate buffer were taken. Final volume upto 2 ml was made with distilled water and reaction mixture was incubated at 37°C for 5 min. During incubation, 0.1 ml of 1.2 mM $H_2O_2$ (pre-warmed at 37°C) was added. Then, 0.5 ml of chilled TCA (10%) was added and centrifuged at 3,000 rpm for 15 min and supernatant was taken out. In the protein free filtrate, GSH was determined by mixing 0.5 ml of filtrate with 3 ml of 0.3 M $Na_2HPO_4$ and 1 ml of DTNB reagent. The absorbance was recorded at 412 nm within 5 min after the addition of DTNB reagent and calculated as:

$$\text{GPX } (\text{IU}/10^9\text{spermatozoa/minute}) = \Delta\text{T}-\Delta\text{C}$$

where, ΔT, Change in $\text{OD}_{\text{Test}}$ at 60 sec interval; ΔC = Change in $\text{OD}_{\text{Control}}$ at 60 sec interval.

*Superoxide dismutase* (*SOD*)*:* The assay mixture consisting of 0.1 ml NBT and 10 µl PMS were incubated at 25°C for 10 min. Thereafter, 10 µl of sperm extract was added and the reaction was initiated by addition of 0.1 ml of NADH. An increase in absorbance was recorded at 560 nm for 2 min at 60 sec interval. A control was also run simultaneously. The SOD activity was calculated by the following formula:

$$\text{SOD } (\text{IU}/10^9 \text{ sperm/minute}) = \frac{\Delta\text{T} \times 100}{\Delta\text{C}/2}$$

where, ΔT, Change in $\text{OD}_{\text{Test}}$ at 60 sec interval; ΔC, Change in $\text{OD}_{\text{Control}}$ at 60 sec interval.

*Vitamin E assay:* The vitamin E concentration was determined in blood plasma using a bovine vitamin E Microwell ELISA Kit (MyBioSource, Inc., San Diego, USA). The intra- and inter-assay coefficients of variations for low and high control samples were 6.9 and 5.4%, and 4.9 and 7.2%, respectively. The minimum detection limit of the assay was 0.2 µg/ml.

*Statistical analysis:* Statistical evaluations were carried out using the SAS program. Results are presented as means±SEM. The proportionality data were analyzed after angular transformation. One way analysis of variance (ANOVA) was used for comparisons of means between two groups. When the ANOVA test showed statistical

Table 1. Effect of vitamin E (Mean±SEM) feeding on CASA-based sperm motion traits in fresh and frozen-thawed semen of control (60 ejaculates) and feeding (60 ejaculates) groups

| Parameter | Fresh semen | | | | | | Frozen-thawed semen | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control group | | | Feeding group | | | Control group | | | Feeding group | | |
| | 1–4 weeks | 5–12 weeks | 13–20 weeks | PF (1–4 weeks) | DF (5–12 weeks) | POF (13–20 weeks) | 1–4 weeks | 5–12 weeks | 13–20 weeks | PF (1–4 weeks) | DF (5–12 weeks) | POF (13–20 weeks) |
| TM (%) | 75.2±4.6$^{a}$ | 77.6±2.1$^{a}$ | 76.9±2.7$^{a}$ | 75.1±4.0$^{a}$ | 79.2±3.3$^{a}$ | 85.4±1.6$^{b}$ | 36.8±2.9$^{x}$ | 37.6±1.6$^{x}$ | 37.4±3.2$^{x}$ | 37.0±1.9$^{x}$ | 36.9±1.8$^{x}$ | 44.1±2.6$^{y}$ |
| PM (%) | 48.0±3.5$^{a}$ | 46.9±1.8$^{a}$ | 45.7±2.1$^{a}$ | 47.9±2.9$^{a}$ | 50.4±3.6$^{a}$ | 57.7±2.6$^{b}$ | 24.4±2.4$^{x}$ | 23.5±1.8$^{x}$ | 24.7±2.1$^{x}$ | 23.7±2.2$^{x}$ | 25.9±2.1$^{x}$ | 32.5±3.1$^{y}$ |
| VCL (μm/s) | 88.0±2.8$^{a}$ | 88.7±4.0$^{a}$ | 90.7±2.2$^{a}$ | 89.4±3.7$^{a}$ | 85.6±2.3$^{a}$ | 99.0±3.5$^{b}$ | 62.1±4.2$^{x}$ | 64.5±3.0$^{x}$ | 62.8±3.3$^{x}$ | 60.3±4.6$^{x}$ | 63.7±3.1$^{x}$ | 66.6±2.6$^{x}$ |
| VAP (μm/s) | 60.1±3.2$^{a}$ | 62.7±3.1$^{a}$ | 64.1±2.4$^{a}$ | 62.5±2.5$^{a}$ | 61.2±1.9$^{a}$ | 64.9±2.7$^{a}$ | 46.4±3.7$^{x}$ | 42.1±3.1$^{x}$ | 44.0±3.1$^{x}$ | 43.6±4.7$^{x}$ | 41.9±3.4$^{x}$ | 47.2±2.7$^{x}$ |
| VSL (μm/s) | 53.6±1.1$^{a}$ | 56.6±3.6$^{a}$ | 54.2±2.1$^{a}$ | 53.7±2.9$^{a}$ | 57.5±2.2$^{a}$ | 63.5±2.9$^{b}$ | 33.0±3.5$^{x}$ | 36.4±2.5$^{x}$ | 36.1±2.4$^{x}$ | 33.2±2.7$^{x}$ | 35.9±1.9$^{x}$ | 44.2±2.2$^{x}$ |
| ALH (μm) | 8.47±0.64$^{a}$ | 8.79±0.73$^{a}$ | 9.50±1.09$^{a}$ | 8.60±0.47$^{a}$ | 8.36±0.46$^{a}$ | 9.64±0.80$^{a}$ | 6.72±0.48$^{x}$ | 7.08±0.60$^{x}$ | 6.57±0.44$^{x}$ | 6.84±0.49$^{x}$ | 6.97±0.35$^{x}$ | 7.27±0.33$^{x}$ |
| BCF (Hz) | 18.8±1.7$^{a}$ | 20.9±3.4$^{a}$ | 18.9±1.9$^{a}$ | 18.3±2.5$^{a}$ | 17.3±2.3$^{a}$ | 19.9±2.2$^{a}$ | 10.7±1.0$^{x}$ | 11.8±0.6$^{x}$ | 11.4±0.9$^{x}$ | 11.2±0.8$^{x}$ | 10.2±1.3$^{x}$ | 13.1±1.7$^{x}$ |
| LIN (%) | 73.7±3.7$^{a}$ | 74.9±2.6$^{a}$ | 72.0±1.1$^{a}$ | 72.2±2.2$^{a}$ | 74.3±2.7$^{a}$ | 68.6±1.6$^{b}$ | 55.5±4.5$^{x}$ | 51.4±3.9$^{x}$ | 54.4±4.3$^{x}$ | 56.2±3.3$^{x}$ | 53.7±1.3$^{x}$ | 53.1±2.7$^{x}$ |
| STR (%) | 88.2±1.8$^{a}$ | 87.7±2.7$^{a}$ | 90.4±1.9$^{a}$ | 86.7±2.5$^{a}$ | 85.6±3.4$^{a}$ | 98.8±2.2$^{b}$ | 68.4±4.8$^{x}$ | 65.5±3.4$^{x}$ | 68.9±4.1$^{x}$ | 69.1±4.2$^{x}$ | 68.6±2.7$^{x}$ | 78.6±3.2$^{y}$ |
| WOB (%) | 76.2±1.8$^{a}$ | 77.5±2.5$^{a}$ | 74.8±1.1$^{a}$ | 73.1±3.1$^{ab}$ | 71.2±1.2$^{b}$ | 67.5±2.8$^{b}$ | 55.2±5.5$^{x}$ | 57.7±3.6$^{x}$ | 58.4±3.4$^{x}$ | 54.1±4.4$^{x}$ | 55.1±2.4$^{x}$ | 52.1±3.1$^{x}$ |

Values with different superscripts ($^{a,b}$) differ significantly ($P<0.05$) in fresh semen in the same row. Values with different superscripts ($^{x,y}$) differ significantly ($P<0.05$) in frozen-thawed semen in the same row. PF, Pre-feeding; DF, During feeding; POF, Post-feeding; TM, Total motility; PM, Progressive motility; VCL, Velocity curvilinear; VAP, Velocity average path; VSL, Velocity straight line; ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency; LIN, Linearity; STR, Straightness; WOB, Wobble.

differences, the mean values of motion characteristics, viability, plasma membrane integrity, acrosomal membrane integrity, acrosome reaction and oxidative stress were compared using Duncan's multiple range test (DMRT) and Tukey-Kramer multiple comparison test. Kruskal-Wallis test was used to compare plasma alpha tocopherol values in bulls of control and vitamin E fed groups. For all analyses, a confidence level of $P<0.05$ was considered to be significant.

## RESULTS AND DISCUSSION

*Vitamin E levels in blood plasma of buffalo bulls:* The pattern of vitamin E release and average pre-feeding vitamin E concentration in bulls of feeding (1.66±0.34 μg/ml) and control groups (1.71±0.37 μg/ml) was similar and did not appear to have any effect on semen quality during this period in either group. In feeding group, vitamin E release in response to dietary supplementation was considered to have started from the first rise in vitamin E concentration of more than double the pre-feeding value and its duration was considered up to the last similar value during the course of study. During feeding period, the maximum concentration of vitamin E was significantly higher ($P< 0.05$) in bulls of feeding group (7.72±0.48 μg/ml) than in control group (1.86±0.43 μg/ml) but exhibited maximum effect on semen quality during post-feeding period of feeding group. The mean maximum plasma vitamin E levels were similar to that in dairy bulls (6.9 μg/ml) fed similar (4000 IU/bull/day) concentrations of vitamin E for ten weeks (Velasquez-Pereira *et al.* 1998). During post-feeding period, the levels of vitamin E in feeding group returned to pre-feeding concentrations (2.30±0.49 μg/ml) and corresponded to values of control group (1.78±0.41 μg/ml). The levels of vitamin E in control group, however, remained similar throughout the study period.

*Effect of dietary vitamin E on CASA-based sperm motion and kinetic traits in fresh and frozen-thawed semen of buffalo bulls:* The sperm motility and kinetics of fresh and frozen-thawed semen in two groups (control and feeding) of bulls are shown in Table 1. The percentage of total and progressive motility in bulls fed with vitamin E was significantly higher ($P<0.05$) than in their control counterparts during post-feeding phase in fresh and frozen-thawed semen (Table 1). Similarly, the percentage of total and progressively motile sperm during post-feeding as compared to that during feeding stage of feeding group was significantly ($P<0.05$) higher in fresh and frozen-thawed semen. There were no differences in total and progressive motility between the two groups during pre-feeding and feeding period. These observations were in accordance with the findings of Velasquez-Pereira *et al.* (1998) that feeding vitamin E @ 4000 IU/bull/day for 10 weeks enhanced ($P>0.05$) the percentage of motility (71.0±6.1 in fed bulls vs 63.7±6.9 in control bulls) in Holstein bulls. Electron microscopy of bull sperm revealed that vitamin E protects and reduces gaps in mitochondrial helix of midpiece of spermatids during late spermatogenesis that may be

Table 2. Effect of vitamin E (Mean±SEM) feeding on semen characteristics in fresh and frozen-thawed semen of control (60 ejaculates) and feeding (60 ejaculates) groups

| Parameter | Fresh semen | | | | | | Frozen-thawed semen | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control group | | | Feeding group | | | Control group | | | Feeding group | | |
| | 1–4 weeks | 5–12 weeks | 13–20 weeks | PF (1–4 weeks) | DF (5–12 weeks) | POF (13–20 weeks) | 1–4 weeks | 5–12 weeks | 13–20 weeks | PF (1–4 weeks) | DF (5–12 weeks) | POF (13–20 weeks) |
| Viability (%) | 74.2±3.3$^{a}$ | 76.1±2.1$^{a}$ | 76.3±2.1$^{a}$ | 75.3±2.1$^{a}$ | 78.1±1.1$^{a}$ | 84.4±2.2$^{b}$ | 57.2±1.7$^{x}$ | 57.6±2.1$^{x}$ | 58.5±2.1$^{x}$ | 57.1±2.6$^{x}$ | 59.2±2.2$^{x}$ | 65.6±2.3$^{y}$ |
| PMI (%) | 69.2±2.7$^{a}$ | 67.3±2.7$^{a}$ | 68.3±3.5$^{a}$ | 68.3±2.1$^{a}$ | 72.3±2.3$^{a}$ | 77.3±1.6$^{b}$ | 29.2±1.3$^{x}$ | 30.9±2.1$^{x}$ | 30.1±1.1$^{x}$ | 29.3±1.1$^{x}$ | 32.3±2.6$^{x}$ | 39.0±1.8$^{y}$ |
| AI (%) | 84.2±2.1$^{a}$ | 83.5±2.3$^{a}$ | 85.0±2.1$^{a}$ | 83.5±3.0$^{a}$ | 82.7±1.5$^{a}$ | 84.5±2.0$^{a}$ | 59.6±1.4$^{x}$ | 60.3±2.6$^{x}$ | 59.8±2.5$^{x}$ | 58.5±3.5$^{x}$ | 59.7±2.8$^{x}$ | 66.1±2.6$^{y}$ |
| AR (%) 0 h | 7.4±0.9 | 7.9±1.2 | 7.8±1.0 | 7.8±0.9 | 7.0±1.4 | 7.6±1.3 | 6.6±1.6 | 5.8±1.2 | 6.1±0.9 | 5.9±1.5 | 6.7±0.9 | 6.2±0.8 |
| AR (%) 6 h | 54.5±2.0$^{a}$ | 54.4±2.6$^{a}$ | 56.3±1.4$^{a}$ | 55.3±2.2$^{a}$ | 54.0±1.4$^{a}$ | 56.3±2.2$^{a}$ | 34.2±2.8$^{x}$ | 35.4±1.7$^{x}$ | 36.9±2.1$^{x}$ | 35.4±2.3$^{x}$ | 34.8±1.7$^{x}$ | 35.1±1.6$^{x}$ |

Values with different superscripts ($^{a,b}$) differ significantly (P<0.05) in fresh semen in the same row. Values with different superscripts ($^{x,y}$) differ significantly (P<0.05) in frozen-thawed semen in the same row. PF, Pre-feeding; DF, During feeding; POF, Post-feeding; PMI, Plasma membrane integrity; AI, Acrosome integrity; AR, Acrosome reaction.

responsible for improved motility (Luo *et al.* 2004).

Most sperm kinematics observed in feeding group revealed higher values in both fresh and frozen-thawed semen than in control ones during post-feeding phase (Table 1) which may be due to beneficial effect of vitamin E related to its biological function that prevents LPO of sperm membranes (Sarlós *et al.* 2002). It was also observed in rams that vitamin E (trolox) favourably affects sperm kinematic parameters (STR, VAP, VCL and VSL) in supplemented group as compared to control group (Silva *et al.* 2013). Sperm cells with these elevated fertility indicative parameters may be better able to migrate through cervical mucus. However, higher LIN in samples of control group than in feeding group may be indicative of lower *in vivo* fertility rates. Verstegen *et al.* (2002) stated that increase in LIN values may be related to reduce fertilization capacity. Dietary feeding of α-tocopherol helped in preserving the ability to generate oxidative energy, thereby improving sperm motility and kinematics, an essential requirement for oocyte fertilization (Satorre *et al.* 2007).

*Effect of dietary vitamin E on sperm characteristics in fresh and frozen-thawed semen of buffalo bulls:* Changes in mean percentages of sperm viability, plasma membrane integrity, acrosome integrity and acrosome reaction after feeding vitamin E to three bulls compared to control (unfed, n = 3) bulls in fresh and frozen-thawed semen are shown in Table 2. In the present study, sperm viability was significantly (P<0.05) higher during post-feeding phase of feeding group as compared to their control counterparts in fresh and frozen-thawed semen (Table 2). The percentage of live sperm was also significantly (P< 0.05) higher during post-feeding than during feeding period of feeding group. These findings were in agreement with the observations of Jeong *et al.* (2009) that percentage of viable sperm was remarkably higher in fresh (96.3±1.0 vs 75.8±1.1%) and frozen-thawed (54.9±1.4 vs 36.3±0.8%) semen samples of boars supplemented with α-tocopherol than in control ones. They further reported that vitamin E regulates phospholipase $A_2$ activity that plays a pivotal role in stabilization of viable sperm by interaction with phospholipids.

Observations on 120 ejaculates from six bulls (three control and three feeding) revealed a significant difference (P<0.05) between the two groups for plasma membrane integrity during post-feeding phase in fresh and frozen-thawed semen (Table 2). Likewise, percentage of sperm with intact membrane during post-feeding stage of feeding group was significantly higher (P<0.05) as compared to that during feeding stage. Eventually, there were no significant differences in plasma membrane integrity during pre-feeding and feeding periods of both groups. Similar studies (Velasquez-Pereira *et al.* 1998) also showed that dietary supplementation of vitamin E increased the percentage of sperm with intact plasma membrane in vitamin E fed bulls (68.3±6.7%) than in control bulls (55.1±6.0%). Feeding vitamin E may cause a direct stimulation of steroidogenic enzymes, alteration of cAMP second-messenger function and/or interference with cell membrane properties that could result in maintaining the conformation of cell membrane (Chase *et al.* 1990).

The acrosomal status of sperm is an important indicator of their ability for fertilization. There were no significant differences (P>0.05) in acrosome integrity of fresh semen during pre-feeding, feeding and post-feeding phases among two groups (Table 2). However, percentage of intact acrosomes in frozen-thawed semen during feeding period in feeding group was significantly higher (P<0.05) than in control group. The acrosome integrity in frozen-thawed semen of feeding group during post-feeding stage was also significantly higher (P<0.05) as compared to that during feeding stage within the same group. The assessment of inducibility of acrosome reaction remained similar (P>0.05) in bulls of both groups immediately after swim-up at 0 h and after 6 h of incubation in fresh and frozen-thawed semen (Table 2). Chase *et al.* (1990) demonstrated that percentage of intact acrosome in bulls fed vitamin E was similar to those in unfed bulls showing the inability of spermatids to respond to vitamin E in influencing the acrosome during spermatogenesis. Therefore, even if animal was fed with optimum levels of vitamin E, its activity could not promote an improvement in acrosome integrity in fresh semen. Alternatively, an increase in proportion of intact acrosomes in frozen-thawed semen indicated that endogenous vitamin E activity was effective to counteract the ROS action on phospholipids of acrosome membrane generated during cryopreservation. Despite its positive effect on acrosome membrane integrity, feeding of vitamin E did not persuade capacitation-like changes in fresh and frozen-thawed semen. These findings were in accordance with the observations of Satorre *et al.* (2007) in boars that addition of α-tocopherol to freezing extender could not induce acrosome reaction in frozen-thawed spermatozoa.

*Effect of dietary vitamin E on antioxidative status in fresh and frozen-thawed semen of buffalo bulls:* The mean levels of malondialdehyde (MDA), glutathione peroxidase (GPX) and superoxide dismutase (SOD) in fresh and frozen-thawed ejaculates of bulls of control and feeding groups are presented in Table 3. The production of MDA was significantly lower (P<0.05) in feeding group than in control group during post-feeding period, and within group during feeding period in fresh and frozen-thawed semen demonstrating a reduction in generation of ROS through vitamin E feeding to bulls (P<0.05; Table 3). Incidentally, no differences in MDA levels were noticed during pre-feeding and feeding phases of both groups. A significant difference (P<0.01) in MDA profiles was seen between fresh and frozen-thawed semen samples of respective groups. Similar studies (Zeitoun and Al-Damegh 2015) also showed that dietary feeding of vitamin E to ram reduces MDA in fed rams (2.13±0.19 µM per $10^9$ sperm) as compared to unfed rams (15.13±0.45 µM per $10^9$ sperm). The lesser amount of MDA in bulls fed vitamin E than in control group could be attributed to the scavenging action of vitamin E on free radicals, thereby, preventing the damaging process

Table 3. Effect of vitamin E (Mean±SEM) feeding on antioxidative status in fresh and frozen-thawed semen of control (60 ejaculates) and feeding (60 ejaculates) groups

| Parameter | Fresh semen | | | | | | Frozen-thawed semen | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control group | | | Feeding group | | | Control group | | | Feeding group | | |
| | 1–4 weeks | 5–12 weeks | 13–20 weeks | PF (1–4 weeks) | DF (5–12 weeks) | POF (13–20 weeks) | 1–4 weeks | 5–12 weeks | 13–20 weeks | PF (1–4 weeks) | DF (5–12 weeks) | POF (13–20 weeks) |
| MDA (µmole/$10^9$ spermatozoa) | 52.5±5.2$^{a}$ | 50.8±3.2$^{a}$ | 50.5±4.2$^{a}$ | 53.0±3.0$^{a}$ | 51.6±3.8$^{a}$ | 41.0±4.2$^{b}$ | 248.8±29.6$^{x}$ | 251.9±23.6$^{x}$ | 243.8±32.4$^{x}$ | 256.1±32.2$^{x}$ | 238.2±19.2$^{x}$ | 171.3±23.1$^{y}$ |
| GPX (IU/$10^9$ spermatozoa/min) | 0.82±0.26$^{a}$ | 0.85±0.13$^{a}$ | 0.79±0.09$^{a}$ | 0.81±0.07$^{a}$ | 0.82±0.11$^{a}$ | 1.17±0.10$^{b}$ | 0.20±0.06$^{\#}$ | 0.20±0.05$^{\#}$ | 0.25±0.03$^{\#}$ | 0.20±0.04$^{\#}$ | 0.19±0.05$^{\#}$ | 0.61±0.10$^{*}$ |
| SOD (IU/$10^9$ spermatozoa/min) | 553.6±41.0$^{a}$ | 542.3±48.6$^{a}$ | 560.1±54.7$^{a}$ | 560.0±37.1$^{a}$ | 556.8±39.5$^{a}$ | 583.4±51.6$^{a}$ | 281.5±29.7$^{x}$ | 273.6±19.5$^{x}$ | 277.0±35.2$^{x}$ | 281.7±34.3$^{x}$ | 292.5±26.9$^{x}$ | 328.8±35.9$^{x}$ |

Values with different superscripts ($^{a,b}$) differ significantly (P<0.05) in fresh semen in the same row. Values with different superscripts ($^{x,y}$) differ significantly (P<0.05) in frozen-thawed semen in the same row. Values with different superscripts ($^{\#,*}$) differ significantly (P<0.01) in frozen-thawed semen in the same row. PF, Pre-feeding; DF, During feeding; POF, Post-feeding; MDA, Malondialdehyde; GPX, Glutathione peroxidase; SOD, Superoxide dismutase.

from propagating through plasma membrane. Bansal and Bilaspuri (2009) observed that higher MDA in frozen-thawed than in fresh sperm could be due to the fact that frozen-thawed spermatozoa were more easily peroxidized than fresh spermatozoa.

The GPX activity in fresh semen of feeding group during post-feeding period was significantly higher ($P<0.05$) as compared to control group (Table 3). The level of significance was even more pronounced ($P<0.01$) in frozen-thawed semen samples of respective groups during same period. Within feeding group, the GPX profile was significantly higher ($P<0.05$) during post-feeding than during feeding stage in fresh and frozen-thawed semen. The SOD activity was non-significantly higher ($P>0.05$) in fresh and frozen-thawed semen of feeding group as compared to their control counterparts during post-feeding phase (Table 3). The effect of cryopreservation on SOD values was clearly ($P<0.05$) observed in fresh and frozen-thawed semen of two groups. GPX and SOD are key enzymes in natural defense against free radicals (Menvielle-Bourg 2005). Our findings were in accordance with the results of Zeitoun and Al-Damegh (2015) who clearly demonstrated the protective effect of vitamin E enrichment on GPX and SOD levels in ram semen. Further, greater positive effect during sperm storage is also associated with pretranslational mechanisms including GPX and SOD gene expression and cytosolic mRNA stabilization (Christensen and Burgener 1992).

Overall, in feeding group, the fresh and frozen-thawed semen attributes and oxidative stress exhibited favourable values during post-feeding phase (60 days) than during pre-feeding and feeding phase. Senger (2005) demonstrated that nutritional effects of feeding on sperm quantity and quality show carryover effect, since mature sperm is produced over a 61-day period before ejaculation. Incidentally, few sperm characteristics also showed slightly encouraging values during feeding period which could possibly be due to species variation and individual differences, if any (Holt 2000).

Thus, it can be concluded that vitamin E feeding @ 4000 IU/bull/day for 60 days provides higher integrity and protection to plasma membrane from destabilization as well as better kinematics and reduced oxidative stress for buffalo sperm.

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