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Effect of Vitamin E Supplementation during Buffalo Semen Cryopreservation on Sperm Characteristics and Oxidative Stress

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ABSTRACT

The present study was undertaken to determine the effect of vitamin E supplementation to Tris-egg yolk diluent on post-thaw semen quality and oxidative parameters of buffalo bulls. Semen samples (eighteen ejaculates) were diluted in Tris-yolk egg medium without antioxidant (control group) and with vitamin E in different concentrations (0.5, 1.0, 1.5 and 2.0 mg/ml) and cryopreserved. After thawing, samples were subjected to selective semen parameters. Based on post-thaw selected sperm parameters, the concentration of 1.0 mg/ml vitamin E seemed to be most effective. Therefore, thirty ejaculates from same bulls were further collected, divided into two fractions [control and vitamin E supplemented (1.0 mg/ml)] and evaluated for semen characteristics and oxidative stress in post-thaw semen. The results revealed that progressive motility (29.0 ± 2.2 vs 24.1 ± 1.7%), sperm viability (63.2 ± 2.5 vs 56.7 ± 1.9%) and plasma membrane integrity (36.1 ± 2.2 vs 28.9 ± 1.4%) were significantly higher ($P<0.05$) in extender supplemented with vitamin E than in control. Regarding sperm kinematics, addition of vitamin E to extender significantly increased ($P<0.05$) VCL, VSL and STR when compared to control. Lipid peroxidation, as manifested by MDA production was significantly lower ($P<0.05$) in vitamin E supplemented than in control semen (187.0 ± 19.8 vs 245.5 ± 25.9 MDA μmole/10^9 spermatozoa). It is, therefore, concluded that addition of vitamin E to extender prior to cryopreservation of buffalo bull semen protected sperm membrane against oxidative damage and improved the fertilizing potential of spermatozoa.

Keywords: Buffalo bull semen, Cryopreservation, Oxidative stress, Seminal attributes, Vitamin E

Artificial insemination of buffalo is favored for dissemination of superior germplasm and impregnation of multiple females to increase milk production. At the same time, 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 percent are bred through AI due to lower freezability of semen (Kumar *et al.,* 2014). During sperm cryopreservation spermatozoa suffer molecular lesions during freezing and thawing procedures viz. osmotic stress, oxidative damage and apoptosis which can lead to negative results after its use in artificial insemination (Pena *et al.,* 2011). They further reported that the damage caused by freezing and thawing procedures may be derived from action of reactive oxygen species responsible for depletion of sperm adenosine triphosphate, with consequent decreases in motility, ruptured spermatozoon membrane as well as lipid peroxidation. 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. Thus, addition of these substances and/or proteins to freezing extender may be an alternative to preserve sperm parameters after freeze-thaw process (Singh *et al.,* 2016; Soares and Guerra, 2009). Among various nutraceuticals and additives, vitamin E addition at freezing of semen is considered to be most potent, owing to beneficial effects on semen quality

(Keskes-Ammar *et al.,* 2003). Vitamin E is the principal constituent of antioxidant defense system of spermatozoal membrane, protecting against reactive oxygen species and lipid peroxidation attack (Yousef *et al.,* 2003). Addition of vitamin E (1.0 mg/ml) to cryodiluent showed post-thaw (0 h) increase in sperm motility and membrane integrity (Andrabi *et al.,* 2008). However, studies on the effect of vitamin E supplementation on post-thaw semen quality are sparse and limited in buffalo bulls. Hence, the present study was designed to supplement the freezing media with vitamin E and determine its effect on sperm characteristics and oxidative stress in frozen-thawed semen of buffalo bulls.

MATERIALS AND METHODS

Semen collection, evaluation, processing and cryopreservation

Eighteen semen ejaculates from six breeding Murrah buffalo bulls (one ejaculate/bull/session; bulls aged between 4-6 years) maintained under identical feeding and management systems at University bull farm were collected using artificial vagina at different interval, divided into five equal fractions each time and diluted to final concentration of 80 × 10^6 sperm/ml using Tris-egg yolk semen extender supplemented with different concentrations of vitamin E viz. 0 (control), 0.5, 1.0, 1.5 and 2.0 mg/ml to optimize the most effective vitamin E concentration for its cryoprotective effect. Ejaculates having prefreezing sperm motility ≥70% and mass activity >4 subjectively assessed under phase contrast microscope equipped with a warm stage (37ºC) at 400x magnification were used throughout the study. The equilibrated semen was loaded into 0.25 ml plastic straws (IMV Technologies, L'Aigle, France) and cryopreserved till further analysis. The post-thaw semen evaluation on selected parameters was done within a week of cryopreservation.

Following optimization of vitamin E concentration, the study was conducted on 30 ejaculates (one ejaculate/bull/session) obtained from same selected buffalo bulls. Each ejaculate was divided in two equal fractions (control and vitamin E supplemented), cryopreserved and evaluated and compared for sperm characteristics and oxidative stress.

Evaluation of sperm after freeze-thawing

The frozen-thawed sperm were evaluated for CASA-based motion traits, viability, plasma membrane integrity, acrosome integrity and *in vitro* capacitation/acrosome reaction. The number of sperm were converted to percentage

Assessment of sperm motility and kinematics

A previously validated computer assisted semen analysis (CASA; Biovis 2000 version 4.59) was used to evaluate sperm motion traits. Immediately after thawing in water bath (37ºC for 30s), 10 µl semen was mounted on a pre-warmed CASA slide (depth 10 µm). For each semen sample, five aleatory fields with at least 150 sperm per field were considered. The CASA software settings for recording sperm motility and kinetics are shown in table 1.

Table 1: Software settings of the CASA machine during semen evaluation in the current study

Parameters	Settings
Optic calibration	
Magnification	Objective 10 X Phase
	Image Pixels – 1.48 pixels/unit
Camera frequency (FPS)	160
Frame rate (FPS)	60
Frames acquired (FPS)	60
Detection of motility and velocity parameters	
Non progressive limit (µm/sec)	0-10
Maximum velocity for tracking (µm/sec)	150
Minimum VCL (µm/sec)	> 25
Minimum VAP (µm/sec)	> 10
Minimum VSL (µm/sec)	> 1
Minimum track Length (% of frames)	51
Shape and size	
Area (µm)	1-9999
Aspects	0-99999
Axis major (µm)	5-16
Axis minor (µm)	3-10
Compactness	0-50
Stage configuration	
Chamber depth (micron)	10
Chamber area (mm^2)	100 × 0.01

The following motion characteristics were recorded in frozen-thawed semen samples: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), velocity curvilinear (VCL, µm/s), average lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %) and wobble (WOB, %) of the spermatozoa.

Sperm viability

The live sperm count was determined through Eosin-Nigrosin staining technique. In brief, a 10 µl aliquot of semen was thoroughly mixed with 10 µl of stain at 37°C and a thin smear was prepared on a clean and grease free glass slide from the semen stain mixture. The slides were observed under oil immersion at 100× of light microscope. About 200 live (white head), partial dead (light pink head) and dead (dark pink head) spermatozoa were counted in different fields. The percent viability was calculated by the formula:

$$\text{Sperm viability (\%)} = \frac{\text{No. of live sperm}}{\text{Total sperm}} \times 100$$

Plasma membrane integrity

Functional membrane integrity of sperm was evaluated by hypo-osmotic swelling test using hypo-osmotic solution (100 mosm/L). Briefly, 100 µl of frozen-thawed semen was mixed with 1.0 ml of hypo-osmotic solution and incubated at 37ºC for 1 h. Simultaneously, 100 µl of semen was incubated in 1.0 ml of PBS under similar conditions. A 10 µl of incubated semen both from hypo-osmotic solution as well as from PBS was placed on separate glass slides and covered with a cover slip. The semen was examined under bright field microscope (100x) for curled tail spermatozoa. About 200 coiled and uncoiled spermatozoa were counted separately in PBS and hypo-osmotic solution in different fields. The number of curled tail spermatozoa in PBS was deducted from that in hypo-osmotic solution and the resultant figure was taken as the HOS-reactive sperm.

Acrosome integrity

Acrosome integrity of spermatozoa was assessed by chlortetracycline cysteine stain (CTC) as per the method of Wang *et al.* (1995). Sperm suspension (25 µl) was mixed with 25 µl of 750 µM CTC solution in 20 mM Tris HCl, 130 mM NaCl and 5 mM cysteine and incubated at 37ºC for 30 seconds. Thereafter, 100 µl of 12.5% paraformaldehyde and one drop of 0.22 M DABCO were added. Then a drop of sperm suspension was placed on a glass slide, covered with cover slip and observed under fluorescence microscope (Olympus) at 400x using blue filter at 480 nm. Two hundred spermatozoa from each slide were counted in different fields. A green fluorescence detected over the whole region of sperm head was designated as an intact acrosome, a green fluorescence in sperm head except in the post-acrosomal region as cryo-capacitated sperm and no fluorescence in head except for a bright band in the equatorial segment as a cryo-acrosome reacted sperm. The percent acrosome integrity was calculated as:

$$\text{Acrosome integrity (\%)} = \frac{\text{No. of sperm with intact acrosome}}{\text{Total sperm}} \times 100$$

Acrosomal status

Semen from frozen-thawed semen (ten straws per ejaculate per bull) were taken separately in 15 ml graduated tube and washed twice with the basic TALP medium (2 ml; 92.9 mM NaCl, 4 mM KCl, 25.9 mM $NaHCO_3$, Na_2HPO_4, 10 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, 1.3 mM sodium pyruvate, 7.6 mM sodium lactate and 20 mM HEPES) by centrifuging at 1000 rpm for 5 minutes (Yanagimachi, 1994). The sperm suspension was then re-suspended in the energy rich TALP medium (0.5 ml), motile spermatozoa separated by swim up, transferred to eppendorf tubes and placed in a dry water bath at 37°C for 6 h. Immediately after swim-up and at the end of 6 h, capacitation/acrosome reaction was assessed in the same way as mentioned in acrosome integrity by counting 200 spermatozoa in CTC stained smear under fluorescence microscope at 400x (Olympus). A green fluorescence detected over the whole region of sperm head was designated as an intact acrosome, a green fluorescence in sperm head except in the post-acrosomal region as capacitated sperm and no fluorescence in head except for a bright band in the equatorial segment as an acrosome reacted sperm.

Oxidative stress status in frozen-thawed semen

The assessment of oxidative stress in post-thaw semen

samples was done through estimations of lipid peroxidation (MDA, µmole/10^9 sperm), glutathione peroxidase (IU/10^9 sperm/minute) and superoxide dismutase (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 minutes. Following incubation, 0.5 ml of 10% TCA and 1.0 ml of 0.375% TBA were added and kept for 20 minutes in boiling water bath. Thereafter, the mixture was cooled and centrifuged for 15 minutes at 10000 rpm, supernatant was taken out and absorbance was taken at 532 nm. The molar extinction coefficient for MDA was determined according to the following formula:

$$\text{MDA, µmole/}10^9\text{ sperm} = \frac{\text{O.D.} \times \text{Volume of assay mixture}}{\text{Extinction coefficient} \times \text{Volume of sample taken}}$$

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 minutes. 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 3000 rpm for 15 minutes 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 minutes after the addition of DTNB reagent and calculated as:

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

ΔT - Change in OD $_{Test}$ at 60 second interval

ΔC - Change in OD $_{Control}$ at 60 second interval

Superoxide dismutase (SOD)

The assay mixture consisting of 0.1 ml NBT and 10 µl PMS were incubated at 25°C for 10 minutes. 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 minutes at 60 seconds interval. A control was also run simultaneously. The SOD activity was calculated by the following formula:

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

ΔT - Change in OD $_{Test}$ at 60 second interval

ΔC - Change in OD $_{Control}$ at 60 second interval

Statistical analysis

Statistical evaluations were carried out using the SAS program. Results are presented as means ± S.E.M. The proportionality data were analyzed after angular transformation. One way Analysis of variance (ANOVA) was used for comparisons of means. When the ANOVA test showed statistical 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 independent Sample 't'-test, and a confidence level of $P<0.05$ was considered to be significant.

Ethical approval

The study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA; F. No. 25/33/2016-CPCSEA).

RESULTS AND DISCUSSION

Optimization of vitamin E concentration in semen freezing media for buffalo bull

Different concentrations of vitamin E viz. 0 (control), 0.5, 1.0, 1.5 and 2.0 mg/ml were standardized and carried out to determine the most effective dose for its cryoprotective effect on motility, viability, plasma membrane and acrosome integrity (Table 2). The beneficial cryoprotective effects of vitamin E were seen in all concentrations of vitamin E viz. 0.5, 1.0, 1.5 and 2.0 mg/ml as evident from motility, viability, plasma membrane and acrosome integrity in frozen-thawed semen that were either equal to

Table 2: Effect of different concentrations of vitamin E on sperm parameters during frozen-thaw phase of buffalo bull semen (n = 18 ejaculates)

Parameters	Concentration of vitamin E (mg/ml)				
	0.0	**0.5**	**1.0**	**1.5**	**2.0**
Motility (%)	36.7 ± 2.5	37.5 ± 1.4	40.0 ± 2.5	38.9 ± 2.1	37.3 ± 1.4
Viability (%)	56.5 ± 2.1^{a}	61.3 ± 2.2^{bc}	64.6 ± 3.0^{c}	58.7 ± 2.0^{ab}	56.6 ± 1.4^{a}
Plasma membrane integrity (%)	28.7 ± 2.1^{a}	30.6 ± 2.1^{a}	35.5 ± 2.7^{b}	32.2 ± 2.7^{ab}	32.0 ± 2.2^{ab}
Acrosome integrity (%)	60.0 ± 2.6	60.4 ± 3.2	62.3 ± 2.0	62.3 ± 1.7	61.7 ± 3.3

Values with different superscripts (^{a,b,c}) differ significantly (P<0.05) in the same row.

or more than that in control. Amongst different doses of vitamin E, the concentration of 1.0 mg/ml was found to be most effective. The percentage of sperm viability and plasma membrane integrity were significantly higher (P < 0.05) in frozen-thawed semen supplemented with 1 mg/ml vitamin E as compared to control. The sperm motility was non-significantly higher (P>0.05) in supplemented than in control. Alternatively, supplementation of vitamin E to extender did not affect the acrosome integrity in frozen-thawed semen. Previous studies (Rao *et al.,* 2017) in cattle bulls have shown that supplementation of 1.0 mg/ml vitamin E to extender improved the quality of semen and prevented cryodamage subsequently. At higher or lower concentrations vitamin E may act as an oxidation stimulator rather than an antioxidant (Breininger *et al.,* 2005). However, studies indicate a species specific variation in most effective concentration of vitamin E supplemented in freezing extender viz. 120 µM in ram (Silva *et al.,* 2013), 200 µg/ml in boar (Breininger *et al.,* 2005) and 1.0 mg/ml in cattle bull and buffalo bull (Raina *et al.,* 2002; Rao *et al.,* 2017) to prevent oxidative damage and improve semen quality of cryopreserved semen. Based on post-thaw sperm motility, viability, plasma membrane and acrosome integrity, the concentration of 1.0 mg/ml vitamin E seemed to be most effective which was further evaluated on thirty ejaculates of six selected buffalo bulls.

Effect of vitamin E supplementation to Tris-egg yolk diluent on CASA-based sperm motion traits in frozen-thawed semen of buffalo bulls

The effect of vitamin E supplementation (1.0 mg/ml) on CASA-based post-thaw sperm motility and velocity parameters are presented in table 3. Vitamin E supplementation exhibited beneficial effect for most sperm motion traits following cryopreservation. The percentage of progressive motility in vitamin E supplemented samples was significantly higher (P<0.05) as compared to their control counterparts. The total motility was also non-significantly higher (P>0.05) in supplemented than in control samples (Table 3).

Table 3: Effect of vitamin E (1.0 mg/ml) supplementation on post-thaw CASA-based sperm motion traits (Mean ± SEM; n = 30 ejaculates)

Parameter	Control	Vitamin E supplemented
Total motility (%)	37.6 ± 2.4	40.5 ± 2.0
Progressive motility (%)	24.1 ± 1.7^{a}	29.0 ± 2.2^{b}
VCL (µm/s)	61.7 ± 2.4^{a}	72.8 ± 4.8^{b}
VAP (µm/s)	46.0 ± 2.4	49.8 ± 4.4
VSL (µm/s)	33.3 ± 2.6^{a}	45.4 ± 4.6^{b}
ALH (µm)	6.53 ± 0.45	6.80 ± 0.50
BCF (Hz)	11.7 ± 0.4	13.0 ± 1.1
LIN (%)	57.3 ± 2.9	55.7 ± 2.3
STR (%)	63.6 ± 3.8^{a}	83.7 ± 2.4^{b}
WOB (%)	55.6 ± 2.4	51.7 ± 1.8

Values with different superscripts (^{a,b}) differ significantly (P<0.05) in the same row.

Sperm motility is commonly believed to be one of the most important characteristics for evaluating the fertility potential of ejaculated spermatozoa (Singh *et al.,* 2017). The results of present study are consistent with previous observations of Rao *et al.* (2017) where supplementation of α-tocopherol (vitamin E) to freezing extender at 1.0 mg/ml prevented oxidative damage and improved sperm motility. Similar studies (Silva *et al.,* 2013) in ram revealed that addition of vitamin E in extender was effective at preserving progressive sperm motility. Eventually, greater

number of motile spermatozoa present in samples frozen with vitamin E would increase the fertilizing potential of post-thaw spermatozoa.

Regarding sperm kinematics, addition of vitamin E to extender significantly increased (P<0.05) VCL, VSL and STR when compared to control (Table 3). A substantial proportion of VAP was also higher (P>0.05) in vitamin E supplemented than in control. However, no difference was observed for ALH and BCF, although they were marginally higher (P>0.05) in extender supplemented with vitamin E. Alternatively, WOB and LIN were higher (P>0.05) in control than in vitamin E supplemented samples. Higher reactive oxygen species (ROS) production increased the number of damaged cells and caused negative effects on sperm kinematic parameters observed in control group as compared to higher values in vitamin E (120 mM) supplemented group (Rover Jr *et al.,* 2001). Increased STR percentages following vitamin E analogue addition in extender were also reported by Maia *et al.* (2009) in ram cryopreserved sperm. Lipid peroxidation (LPO) causes failure in metabolic exchange mechanisms and in extreme conditions, cellular death. Once dead, a spermatozoon releases enzymes that exert toxic effects on living sperm, causing changes in kinematic parameters and lowering overall motility (Benzie, 1996).

Effect of vitamin E supplementation to Tris-egg yolk diluent on sperm characteristics in frozen-thawed semen of buffalo bulls

The results of sperm characteristics after the freezing–thawing process are presented in fig. 1. For optimum post-thaw fertility, the plasma membrane of spermatozoa should remain viable and intact. In the present study, post-thaw sperm viability was significantly higher (P<0.05) in the extender supplemented with vitamin E than in their control counterparts (Fig. 1A). These findings are in agreement with the observations of Silva *et al.* (2013) that vitamin E helps in maintaining the viability of spermatozoa with consequent increase in livability. In the current study, higher proportion of live sperm in vitamin E supplemented than in control samples could have resulted from lesser membrane damage and reduced ATP depletion from H_2O_2 due to action of ROS during cryopreservation.

The addition of vitamin E to extender significantly increased (P<0.05) the percentage of plasma membrane integrity in post-thaw semen as compared to control (Fig. 1B). These findings are in accordance with the findings of Bhakat *et al.* (2011) that after-freeze plasma membrane integrity was more in vitamin E supplemented group as compared to non-supplemented group. Alpha-tocopherol break covalent links that ROS form between fatty acid side chains in membrane lipids and exert a protective effect on sperm plasma membrane, preserving both metabolic activity and cellular function after freeze-thaw process (Soares and Guerra, 2009).

Acrosome integrity is one of the main parameters in evaluation of sperm viability and membrane functionality since only sperm with intact acrosome are able to undergo acrosome reaction, penetrate the zona pellucida and fuse with oocyte. In the current study, no significant differences in acrosome integrity following vitamin E supplementation were observed (Fig. 1C). Previous studies (Beheshti *et al.,* 2011) in buffalo bull reported that addition of vitamin E (1 mg/ml) to extender did not enhance acrosomal integrity of frozen-thawed spermatozoa. Similarly, no significant differences (P>0.05) for acrosome integrity (56.67 ± 6.15% in vitamin E supplemented vs 56.67 ± 6.15% in control) were found among groups in ram (Silva *et al.,* 2013). In the present study, addition of vitamin E to semen extender did not maintain an increased proportion of sperm with intact acrosomes (Petrunkina *et al.,* 2005). The discrepancy between motility, viability, plasma membrane and acrosome integrity suggested that cryopreservation may affect different intracellular and/or membrane components required for cell functionality (Breininger *et al.,* 2005).

Like acrosome integrity, the acrosome reaction in vitamin E supplemented semen was also similar to its counterparts (Fig. 1D). This indicates that vitamin E has no role in membrane destabilization and permeability, necessary for capacitation and acrosome reaction of spermatozoa. Similar findings by Satorre *et al.* (2007) revealed non-significant differences in acrosome-reacted spermatozoa of α-tocopherol supplemented (41%) and control groups (39%) in cryopreserved porcine sperm.

Effect of vitamin E supplementation to Tris-egg yolk diluent on oxidative stress in frozen-thawed semen of buffalo bulls

The effect of vitamin E on oxidative stress in extender

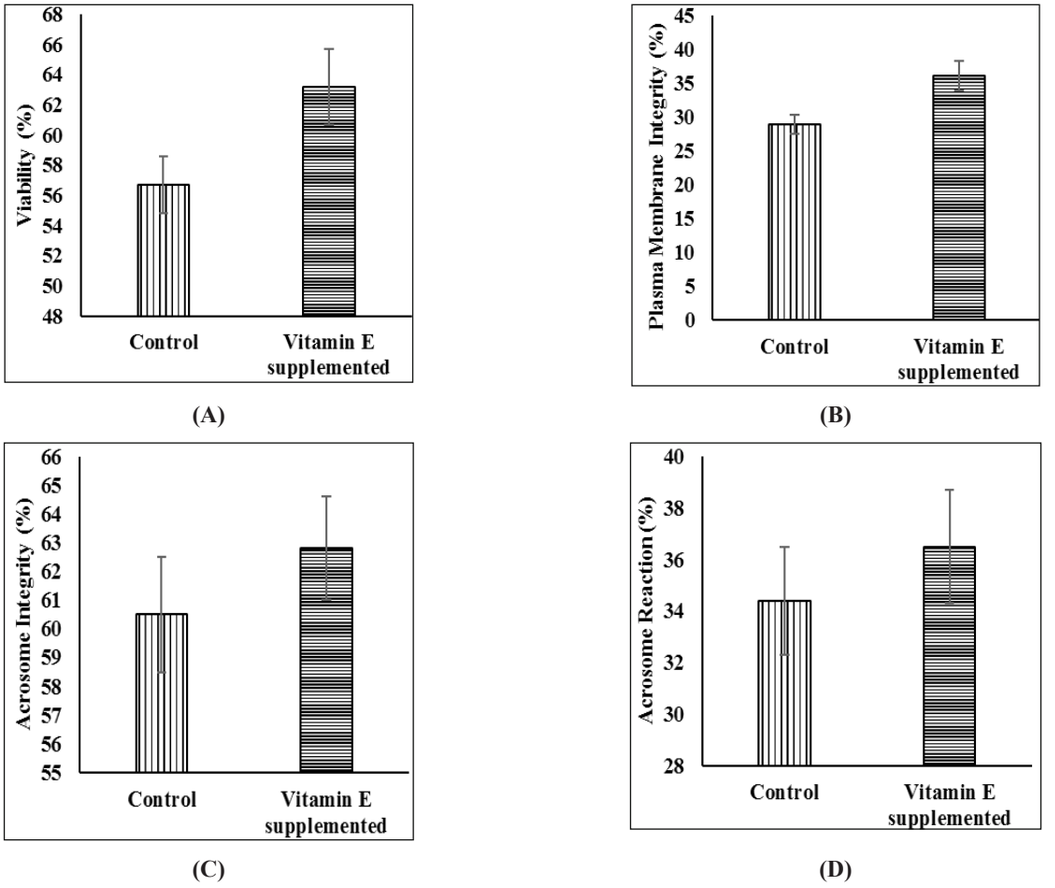

Fig. 1: Effect of vitamin E (1.0 mg/ml) supplementation on post-thaw semen parameters (n = 30 ejaculates; Mean ± SEM). Values with different letter (a,b) differ significantly (P<0.05)

is shown in Table 4. The malondialdhyde (MDA) production was significantly lower (P<0.05) in vitamin E supplemented semen than in control semen. These findings are in agreement with the observations of Singh *et al.* (1989) that addition of 2.5 µM vitamin E to buffalo sperm causes a drop (P<0.05) in MDA production from 35.2 to 29.3%. Bansal and Bilaspuri (2009) also reported reduced LPO levels in vitamin E supplemented cattle bull semen than in their counterparts. The lesser amount of LPO produced in freezing media supplemented with vitamin E than in control ones could have resulted by inhibiting the propagation of peroxidative chain reaction and forming a relatively stable complex called tocopheroxyl radical since vitamin E functions as a chain-breaking antioxidant (Sinclair, 2000).

The glutathione peroxidase (GPX) activity was considerably higher (P>0.05) in vitamin E supplemented semen compared to control (Table 4). These observations are in consonance with the findings of Zeitoun and Al-Damegh (2015) who recorded higher (P<0.05) GPX activity in extender supplemented with vitamin E (5 IU/ml) both in seminal plasma as well as in spermatozoa as compared to respective controls in ram. GPX in sperm is considered to be the main enzyme that removes peroxides and protects the cell against damage caused by free radicals and products of LPO (Griveau *et al.,* 1995). Maia *et al.* (2010) found that generation of H_2O_2 was less in semen cryopreserved in extender containing trolox (vitamin E analogue) than in control due to occurrence of competition for substrate to oxidation mediated by peroxidase.

Like GPX, the concentration of superoxide dismutase (SOD) was also greater (P>0.05) in semen cryopreserved in extender supplemented with vitamin E than in control (Table 4). Mammalian spermatozoa are highly susceptible to oxidative damage and natural antioxidative enzymes in seminal plasma and spermatozoa are frontline defense mechanisms against the oxidative damage (Bansal and Bilaspuri, 2011). SOD is one of the key enzymes involved in detoxification of ROS in mammalian spermatozoa (Menvielle-Bourg, 2005). Previous studies (Kadirvel *et al.,* 2014) have shown a significant (P<0.01) reduction in intracellular SOD activity after freeze-thawing compared to that in fresh spermatozoa. In the present study, addition of vitamin E to extender helped in maintaining (P>0.05) SOD levels in frozen-thawed semen when compared to control.

Table 4: Effect of vitamin E (1.0 mg/ml) supplementation on oxidative stress and antioxidant enzymes (Mean ± SEM) in post-thaw semen (n = 30 ejaculates)

Parameter	Control	Vitamin E supplemented
Lipid Peroxidation (MDA µmole/10^9 spermatozoa)	245.5 ± 25.9[a]	187.0 ± 19.8[b]
Glutathione Peroxidase ($IU/10^9$ spermatozoa/ minute)	0.22 ± 0.06	0.33 ± 0.08
Superoxide Dismutase ($IU/10^9$ spermatozoa/ minute)	272.6 ± 31.1	324.4 ± 29.1

Values with different superscripts ([a,b]) differ significantly (P<0.05) in the same row.

CONCLUSION

Thus, it can be concluded that vitamin E addition to Tris-egg yolk extender at 1.0 mg/ml concentration provides higher integrity and protection to plasma membrane from destabilization as well as better kinematics and reduced oxidative stress for buffalo sperm post-cryopreservation.

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