



Evaluation of linolenic acid supplementation in extender for freezability and fertility of Murrah buffalo (*Bubalus bubalis*) bull semen

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Received: 20 July 2018; Accepted: 27 August 2018

ABSTRACT

Linolenic acid is integral component of cell membrane that has the ability to protect the structural and functional integrity of buffalo spermatozoa during freeze-thawing. Therefore, present study was designed to evaluate supplementation of linolenic acid (0, 2.5, 5, 7.5 and 10.0 ng/ml) in extender on freezability and *in vivo* fertility of buffalo bull spermatozoa. Semen from healthy breeding Murrah buffalo bulls (4) was collected using artificial vagina (one ejaculate/bull/session; n=24). Qualified semen ejaculates (1–2 ml volume; >70% motility; ≥4 mass activity; 1.0 billion/ml concentration) were diluted with Tris-citric acid extender containing 0.0 (control), 2.5, 5.0, 7.5 and 10.0 ng/ml linolenic acid at 37°C and cryopreserved following established protocol. Sperm progressive motility, viability and plasma membrane integrity were recorded higher in extender containing 5.0 ng/ml of linolenic acid compared to control and other concentrations. Sperm acrosome and DNA integrity exhibited no difference in all experimental extenders with linolenic acid compared to control. Total 60 artificial inseminations were performed with the best evolved extender having linolenic acid (5.0 ng/ml) and control (30 inseminations each). *In vivo* fertility rates of buffalo semen were recorded higher with extender containing linolenic acid (5.0 ng/ml; 46.7%) compared to control (36.7%). In conclusion, supplementing 5.0 ng/ml linolenic acid in extender improved the post-thaw quality and *in vivo* fertility of cryopreserved Murrah buffalo bull semen.

Key words: Cryopreservation, Extender, Fertility, Linolenic acid, Murrah, Oxidative stress, Semen

In India, of 55 million breedable buffaloes, merely 15% are bred through artificial insemination due to lower freezability of semen (Kumar *et al.* 2014). A significant extent of cryodamage is being caused during freeze-thawing process owing to damage of plasma membrane integrity and had been positively correlated with fertility (Andrabi 2009). Moreover, during cryopreservation sperm membrane is more prone to lipid peroxidation by free radicals such as O₂⁻ and H₂O₂ which leads to structural damage of sperm membrane (Kadirvel *et al.* 2009). The number and quality of spermatozoa is determined pre-production through optimization of semen quality by dietary supplementation of nutraceuticals and post-production by *in vitro* treatment of semen with additives (Memon *et al.* 2012). Dietary supplementation of fatty acids had previously been reported to improve semen quality in different species (Gholami *et al.* 2010). Fatty acids have also been added directly to the semen extender with variable effects in different species. Linolenic acid, a major long chain omega-3 fatty acid, is

the main antioxidant of biological membranes. Linolenic acid present in the plasma membrane provide energy, modulate the structure and composition of lipid rafts, regulate plasma membrane proteins, maintain and normalize plasma membrane function, sustain sperm viability and fertility during chilling and freezing (Shevchenko and Simons 2010). Preliminary studies had shown successful use of linolenic acid in cryopreservation of bovine semen (Abavisani *et al.* 2013). Likewise, alpha linolenic acid supplemented into BioXcell® extender improved the quality of cooled and frozen-thawed bull spermatozoa (Kaka *et al.* 2015). However, studies in buffalo bull semen are sparse and limited. Keeping in view above facts, the present work was planned to evaluate the effect of linolenic acid supplementation in extender on freezability and *in vivo* fertility of buffalo bull spermatozoa.

MATERIALS AND METHODS

Semen collection, evaluation, processing and cryopreservation: Twenty four semen ejaculates from four healthy breeding Murrah buffalo bulls (one ejaculate/bull/session; bulls aged between 4–6 years and weighing 689.3±31.1 kg) maintained under identical feeding and management systems at University bull farm were collected using artificial vagina at different interval. Each ejaculate was divided into five equal fractions and diluted to final

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concentration of 100×10^6 sperm/ml using Tris-egg yolk semen extender supplemented with different doses of linolenic acid (Sigma-Aldrich, St. Louis, USA), viz. 0.0 (control), 2.5, 5.0, 7.5 and 10.0 ng/ml to optimize the most effective linolenic acid concentration for its cryoprotective effect. As fatty acids are insoluble in water, ethanol (0.05%) was added as a solvent. Ejaculates having prefreezing sperm motility >70% and mass activity ≥ 4 subjectively assessed under phase contrast microscope equipped with a warm stage (37°C) at 400× magnification were used throughout the study. Briefly, the extended semen of each ejaculate in five fractions was equilibrated at 4°C for 3–4 h in a cold cabinet. 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 8 min, plunged into liquid nitrogen (LN₂) and stored in LN₂ till further analysis. The post-thaw semen analysis was done within a week of cryopreservation. The thawing of cryopreserved semen was done in water bath at 37°C for 30 sec.

Evaluation of post-thaw sperm functional assays: The frozen-thawed sperm were evaluated for CASA-based motion traits, viability, plasma membrane integrity, acrosome integrity, *in vitro* capacitation/acrosome reaction and DNA integrity. 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 (37°C for 30 sec), 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.

Sperm viability: The live sperm count was determined through Eosin-Nigrosin staining technique. Briefly, 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:

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

Plasma membrane integrity: Functional membrane integrity of sperm was evaluated by hypo-osmotic swelling test using hypo-osmotic solution (HOS, 100 mosm/l). Firstly, 300 mosm/l HOS was prepared by mixing 7.35 g Trisodium citrate and 13.51 g D-fructose in double distilled water (DDW) and volume was made up to 1000 ml. Thereafter, 1.0 ml of 300 mosm/l HOS was mixed with 0.5 ml DDW to obtain 100 mosm/l concentration. Briefly, 100 µl of frozen-thawed semen was mixed with 1.0 ml of hypo-osmotic solution and incubated at 37°C for 1 h.

Simultaneously, 100 µl of semen was incubated in 1.0 ml of phosphate buffer saline (PBS) under similar conditions. Incubated semen (10 µl) 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 (100×) 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: Acrosomal integrity of spermatozoa was assessed using Giemsa stain. Briefly, a smear (10 µl) of washed semen was prepared on a clean glass slide, air dried and fixed in methanol for 30 min. After drying, the smear was stained in Giemsa working solution (stock Giemsa stain 3 ml, 0.1 M phosphate buffer 2 ml, pH 7.4 and DDW 35 ml) for 4 h. The slides removed from the stain were rinsed quickly in DDW, air dried and examined under oil immersion (1000×) of the bright field microscope. At least 200 spermatozoa with intact acrosome and damaged acrosome (partially or completely) from each slide were counted in different fields. The percent acrosome integrity was calculated as:

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

Acrosomal status: 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 NaHCO₃, Na₂HPO₄, 10 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O, 1.3 mM sodium pyruvate, 7.6 mM sodium lactate and 20 mM HEPES) by centrifuging at 1000 rpm for 5 min (Yanagimachi 1994). The sperm suspension was then re-suspended in the energy rich TALP medium (0.5 ml), at 37°C for 4 h. After 4 h, a 10 µl sperm suspension was removed from the aliquot, smear was prepared, stained with Giemsa stain as mentioned for acrosome integrity and assessed for acrosome reaction using Giemsa stain. Evaluation of smear was carried under bright field microscope (1000×). About 200 spermatozoa showing swelling, vesciculation and shedding of acrosome were counted in different fields.

DNA integrity: Sperm DNA integrity was evaluated using Acridine Orange (AO) as per the method described by Lui and Baker (1992). About 200 spermatozoa were evaluated under an epifluorescent microscope (400×; model CX31RTSF, Olympus Corporation, Tokyo, Japan) at 480 nm. The heads of the sperm cells with normal DNA integrity (double stranded) emitted green fluorescence, whereas those with denatured or single stranded DNA had orange, yellow and/or red fluorescence/streak. The slides were evaluated within 1 h after staining.

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,

$\mu\text{mole}/10^9$ sperm), superoxide dismutase (IU/ 10^9 sperm/min) and glutathione peroxidase (IU/ 10^9 sperm/min).

Lipid peroxidation (LPO): Briefly, 100 μl of sperm suspension 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 in boiling water bath for 20 min. Thereafter, the mixture was cooled and centrifuged at 10,000 rpm for 15 min, supernatant was taken out and absorbance was taken at 532 nm. The molar extinction coefficient for MDA was determined according to the following formula:

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

Superoxide dismutase: 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:

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

where ΔT , Change in OD test at 60 sec interval; ΔC , Change in OD Control at 60 sec interval.

Glutathione peroxidase (GPX): In the assay tubes, 0.1 ml of sperm extract, 0.2 ml of 8 mM GSH and 0.4 ml of 0.4 M phosphate buffer saline were taken. Final volume was made upto 2 ml 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:

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

where ΔT , Change in OD Test at 60 sec interval; ΔC , Change in OD Control at 60 sec interval.

In vivo fertility rate: Two different experimental extenders were prepared having linolenic acid 0.0 ng/ml (control) and 5.0 ng/ml (optimal concentration for all semen quality parameters studied). For artificial insemination, semen straws were thawed at 37°C for 30 sec. Total 60 inseminations were performed under field conditions (control, 30; extender supplemented with 5.0 ng/ml linolenic acid, 30). All the buffaloes (60) enrolled for fixed time insemination program were healthy, multiparous (2nd to 5th parity), free from physical problems, vaginal discharge and maintained under standard feeding and management systems. Prior to start of breeding program, the clinical assessment of genitalia was done ultrasonographically twice at ten days interval using a B-mode linear array trans-rectal

transducer with 5/7.5 MHz interchangeable frequency (AGROSCAN, ECM, France) to visualize a cyclic CL. The buffaloes were synchronized using doublesynch protocol (PGF2_α -GnRH- PGF2_α -GnRH on day -2, 0, 7 and 9, respectively) followed by FTAI at 16 and 40 h after last GnRH injection, respectively. To confirm ovulation, all the buffaloes were submitted to ultrasound examination on the two days of FTAI i.e. disappearance of follicle(s) ≥ 10.0 mm in two consecutive evaluations. The pregnancy diagnosis was performed on day 45 post-insemination and confirmed on day 60 using ultrasonography. The first service pregnancy rate (FSPR) was recorded on non-return basis and calculated according to the formula:

$$\text{FSPR (\%)} = \frac{\text{Number of buffaloes pregnant after first insemination}}{\text{Total number of first services}}$$

Statistical analysis: Statistical evaluations were carried out using the SAS program. Results are presented as means $\pm$ SEM. The proportionality data were analyzed after angular transformation. Effect of various concentrations of linolenic acid supplementation in extender on post thaw quality of cryopreserved buffalo sperm were statistically analyzed using analysis of variance (ANOVA) in a randomized complete block design and a confidence level of $P < 0.05$ was considered to be significant. When F-ratio was found significant ($P < 0.05$), least significant difference test was used to compare the treatment means. The data on *in vivo* fertility rates were analyzed using Chi-square test.

RESULTS AND DISCUSSION

Effect of linolenic acid supplementation to extender on sperm motion traits in post-thaw semen of buffalo bulls: The total motility was nonsignificantly higher ($P > 0.05$) at 2.5 and 5.0 ng/ml linolenic acid as compared to other concentrations. The percentage of sperm progressive motility significantly improved ($P < 0.05$) in extender containing 5.0 ng/ml of linolenic acid than in 2.5, 7.5, 10.0 ng/ml and control. The results of present study were consistent with previous observations of Kaka *et al.* (2015) where addition of α -linolenic acid to commercially available soya lecithin-based semen extender (BioXcell®) at 5.0 ng/ml exhibited positive impact on sperm motility. Similar studies (Ejaz *et al.* 2017) in buffalo bulls in Tris-citric acid extender also revealed a higher ($P < 0.05$) percentage of total motility in linolenic acid supplemented group at 5.0 ng/ml (53.0 ± 2.64) in comparison to control (47.66 ± 4.16), 10.0 ng/ml (48.33 ± 7.23) and 20.0 ng/ml (50.0 ± 4.0). Eventually, greater number of motile spermatozoa present in samples frozen with linolenic acid would increase the fertilizing potential of post-thaw spermatozoa.

Addition of linolenic acid at 5.0 ng/ml showed significantly higher ($P < 0.05$) values of VAP, VCL and STR when compared to control and other concentrations (Table 1). A substantial proportion of VSL and ALH were also higher ($P > 0.05$) in linolenic supplemented samples than in control ones. However, no difference was observed for BCF. Alternatively, LIN and WOB were higher ($P < 0.05$)

Table 1. CASA-based sperm motion traits (Mean±SEM) in frozen-thawed buffalo bull semen cryopreserved in extender supplemented with different concentrations of linolenic acid (24 ejaculates)

Parameter	Linolenic acid (ng/ml)				
	0.0	2.5	5.0	7.5	10.0
TM (%)	52.2±2.9	57.8±3.0	56.8±3.4	51.5±2.8	50.2±2.4
PM (%)	35.8±2.6 ^{ab}	37.8±1.9 ^b	43.1±3.0 ^c	33.1±1.7 ^a	32.8±2.0 ^a
VSL (µm/s)	81.1±2.4 ^a	92.3±3.3 ^b	90.3±1.8 ^b	85.3±2.5 ^a	86.3±2.2 ^{ab}
VAP (µm/s)	92.4±3.2 ^a	101.5±3.5 ^b	109.4±3.7 ^c	96.8±2.6 ^{ab}	93.5±2.8 ^a
VCL (µm/s)	132.5±3.5 ^a	153.7±2.9 ^b	165.5±3.3 ^c	141.3±2.1 ^d	144.9±2.7 ^d
ALH (µm)	6.6±1.0	7.1±0.5	7.8±0.6	7.0±0.7	6.8±0.9
BCF (Hz)	37.7±2.8	38.4±3.7	37.2±1.9	39.7±2.4	38.8±2.1
LIN (%)	65.5±3.0 ^a	59.2±1.6 ^b	51.9±2.0 ^c	57.3±2.5 ^b	61.9±1.7 ^{ab}
STR (%)	85.2±2.5 ^a	79.3±2.3 ^b	91.0±1.4 ^c	84.9±2.1 ^a	83.0±3.0 ^{ab}
WOB (%)	79.1±2.4 ^a	72.7±3.6 ^b	72.0±1.8 ^b	71.1±2.2 ^b	76.2±3.3 ^{ab}

Mean values with different superscripts within the row differ significantly ($P<0.05$). TM, Total motility; PM, Progressive motility; VSL, Velocity straight line; VAP, Velocity average path; VCL, Velocity curvilinear; ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency; LIN, Linearity; STR, Straightness; WOB, Wobble.

in control than in linolenic 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 and other concentrations as compared to greater values in 5.0 ng/ml linolenic acid group (Kaka *et al.* 2015). Estienne *et al.* (2008) also reported improved sperm kinetic percentages following dietary linolenic acid in boar cryopreserved sperm. Lipid peroxidation (LPO) causes failure in metabolic exchange mechanisms and in extreme conditions, cellular death, causing changes in kinematic parameters and lowering overall motility (Shannon and Curson 1972).

Effect of linolenic acid supplementation to extender on sperm function assays in cryopreserved semen of buffalo bulls: For optimum post-thaw fertility, the plasma membrane of spermatozoa should remain viable and intact. Supplementation of linolenic acid at 5.0 ng/ml to Tris-egg yolk extender significantly improved ($P<0.05$) the proportion of viable (live) sperm cells than in their counterparts (Table 2). These findings were in agreement with the observations of Towhidi and Parks (2012) that PUFA mainly linolenic acid are main constituent of sperm membrane. They further reported that linolenic acid at 5.0 ng/ml concentration is important for lipid bilayer to control ionic exchange, help sperm during development, acquire viability and regulate membrane physiology. At higher

(>10.0 ng/ml) levels, linolenic acid is believed to increase membrane fluidity, decrease resistance to cold shock and make sperm susceptible to oxidation of lipids (Kandelousi *et al.* 2013). In current study, higher proportion of live sperm in extender supplemented with 5.0 ng/ml linolenic acid than in their counterparts could have resulted from lesser membrane damage and reduced ATP depletion from H_2O_2 due to action of ROS during cryopreservation.

Plasma membrane integrity was recorded higher ($P<0.05$) in all experimental extenders containing 2.5, 5.0, 7.5 and 10.0 ng/ml linolenic acid as compared to control (Table 2). Amongst different concentrations, highest percentage of membrane integrity was noticed in 5 ng/ml linolenic acid group. Concentrations higher or lower than 5 ng/ml resulted in lower membrane integrity after freezing. Similar findings by Nasiri *et al.* (2012) in bulls also showed that increasing linolenic acid concentration from 0 to 5.0 ng/ml helped in improving plasma membrane integrity. Moreover, inclusion of linolenic acid in extender might have replaced and/or replenished antecedently lost phospholipids during freezing and thawing stages, thereby increased the osmotic tolerance limits, reduced ice crystal formation and improved cryostability of sperm (Glazar *et al.* 2009). Therefore, addition of linolenic acid at 5.0 ng/ml to semen freezing extender seemed to exert a protective effect on sperm plasma membrane, preserving both metabolic activity

Table 2. Sperm function tests (Mean±SEM) in frozen-thawed buffalo bull semen cryopreserved in extender supplemented with different concentrations of linolenic acid (24 ejaculates)

Parameter	Linolenic acid (ng/ml)				
	0.0	2.5	5.0	7.5	10.0
Viability (%)	58.9±3.2 ^a	61.7±1.8 ^a	67.5±3.6 ^b	60.2±2.0 ^a	61.0±2.7 ^a
PMI (%)	61.9±2.9 ^a	67.4±2.0 ^b	73.2±3.2 ^c	65.2±3.0 ^{ab}	64.6±2.6 ^{ab}
Acrosome integrity (%)	72.2±2.6	71.8±1.9	72.6±2.9	70.5±2.2	69.5±3.3
DNA integrity (%)	90.3±1.5	91.3±1.9	90.0±2.1	89.1±2.6	88.2±3.1
Acrosome reaction (%) 4 h	36.3±1.8	37.2±2.4	38.7±3.1	35.9±2.5	36.7±3.4

Mean values with different superscripts within the row differ significantly ($P<0.05$). PMI, Plasma membrane integrity.

and cellular function after freeze-thaw process.

Optimum fertility depends upon acrosome being structurally and functionally intact which is a prerequisite for capacitation, normal acrosome reaction and successful fertilization (Ramio-Lluch *et al.* 2011). In current study, percent acrosome integrity appeared to be less affected since similar values with no significant difference ($P < 0.05$) was noticed across all treatments (Table 2). Previous studies (Lee *et al.* 2016) in boar stated that addition of linolenic acid (3.0 ng/ml) to extender did not enhance ($P > 0.05$) acrosomal integrity in frozen-thawed spermatozoa (59.5% in alpha linolenic acid supplemented vs 58.3% in control). In present study, addition of linolenic acid to semen extender revealed no change in the proportion of sperm with intact acrosomes among all treatments as well as in control.

The capacitation / *in vitro* acrosome reaction of mammalian spermatozoa is a prerequisite for successful fertilization (Feliipe-Perez *et al.* 2008). In present study, percent acrosome reaction in linolenic acid supplemented semen was similar to control after 4 h of incubation (Table 2). In boar cryopreserved semen, Lee *et al.* (2016) noticed decrease ($P < 0.05$) proportion of acrosome-reacted sperm in alpha linolenic acid supplemented group (24%) as compared to their control counterparts (30%). On the other hand, induction of acrosome reaction (34 vs 22.5%) was significantly increased ($P < 0.05$) in n-3 fatty acid supplemented diluent than in control at 4 h of incubation in same species (Hossain *et al.* 2007). Parrish *et al.* (1988) reported that individual bull differences, interference of seminal proteins as well as length of time needed for capacitation might be the possible factors causing disparity in the proportion of reacted acrosome.

Spermatozoal DNA plays an important role in fertilization process and optimum fertility depends on structurally and functionally intact DNA (Evenson and Wixon 2006). Staining of sperm smears with acridine orange revealed that percentage of intact DNA was similar ($P > 0.05$) in control and all the experimental extenders (Table 2, Fig. 1). These results were in consonance with the observations of Ejaz *et al.* (2017) in buffalo bulls who demonstrated no difference in the percentage of chromatin integrity in control (96.7 ± 0.91) and alpha linolenic acid supplemented semen samples at 5.0 (97.86 ± 0.56), 10.0 (97.83 ± 1.09) and 20.0 ng/ml (97.4 ± 1.05). Though, the measurement of intact DNA is one of the sensitive parameters for assessing the semen quality (Agarwal and Said 2003), however, difference could

be appreciated in the percentage of sperm DNA integrity between different concentrations of linoleic acid and control in the current study.

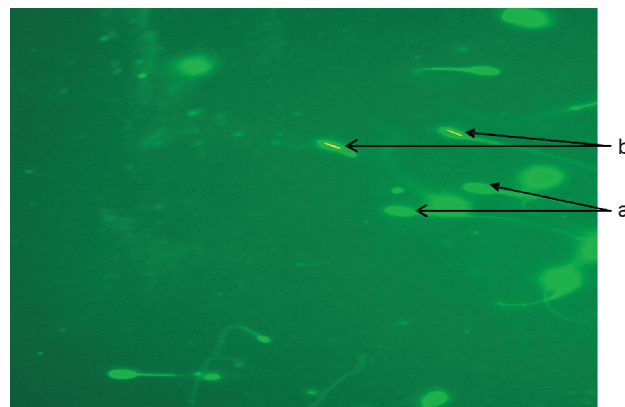


Fig. 1. Acridine orange staining of frozen-thawed spermatozoa in buffalo bulls. (a) Spermatozoa with green fluorescence on nucleus indicated the presence of intact DNA, (b) Spermatozoa with yellowish-orange fluorescence/streak on nucleus indicated the presence of damaged DNA (400 $\times$).

Effect of linolenic acid supplementation to extender on oxidative stress in post-thaw semen of buffalo bulls: Amongst different concentrations, the MDA production was significantly lower ($P < 0.05$) in the extender supplemented with 5.0 ng/ml and 2.5 ng/ml linolenic acid than in those supplemented with 7.5 ng/ml and 10.0 ng/ml linolenic acid as well as in control (Table 3). Interestingly, the MDA production was lower in all linolenic acid supplemented extenders as compared to control. These findings were in accordance with the observations of Kaka *et al.* (2015) who recorded lower MDA concentrations in alpha linolenic acid supplemented semen at 2.5 and 5.0 ng/ml. They further reported that higher MDA levels in frozen semen at concentrations > 5.0 ng/ml could be attributed to observed higher recovery of alpha linolenic acid absorbed with subsequent increased in LPO reaction and MDA production that probably may cause oxidative stress to sperm cells.

A considerably higher ($P > 0.05$) level of superoxide dismutase (SOD) was seen in semen cryopreserved in extender at 5.0 and 7.5 ng/ml linolenic acid concentrations than in control and other experimental concentrations (Table 3). SOD is one of the key enzymes involved in detoxification of ROS in mammalian spermatozoa (Menvielle-Bourg 2005). Previously, Gürlér *et al.* (2015)

Table 3. Level of oxidative stress and antioxidant enzymes (Mean $\pm$ SEM) in frozen-thawed buffalo bull semen cryopreserved in extender supplemented with different concentrations of linolenic acid (24 ejaculates)

Parameter	Linolenic acid (ng/ml)				
	0.0	2.5	5.0	7.5	10.0
MDA (μ mole/ 10^9 spermatozoa)	288.9 $\pm$ 42.3 ^a	177.9 $\pm$ 23.7 ^b	173.1 $\pm$ 37.5 ^b	253.4 $\pm$ 28.8 ^a	275.1 $\pm$ 32.2 ^a
Superoxide dismutase (IU/ 10^9 spermatozoa/min)	275.9 $\pm$ 25.5	283.4 $\pm$ 26.9	339.1 $\pm$ 33.8	330.6 $\pm$ 37.9	268.5 $\pm$ 42.7
Glutathione peroxidase (IU/ 10^9 spermatozoa/min)	0.26 $\pm$ 0.04	0.31 $\pm$ 0.07	0.27 $\pm$ 0.08	0.29 $\pm$ 0.05	0.21 $\pm$ 0.09

Mean values with different superscripts within the row differ significantly ($P < 0.05$). MDA, Malondialdehyde.

Table 4. Field fertility of buffalo semen cryopreserved in extender supplemented with linolenic acid (5.0 ng/ml)

Extender supplementation	Number of animals inseminated	Number of animals conceived	Number of animals ovulated	Ovulation rate (%)	FSPR (%)		Chi square value	P-value
					Ovulated animals	All animals		
Control	30	11	26	86.7	42.3	36.7	0.247	0.183 ^{NS}
Linolenic acid (5.0 ng/ml)	30	14	27	90.0	51.9	46.7		

NS, Non-significant.

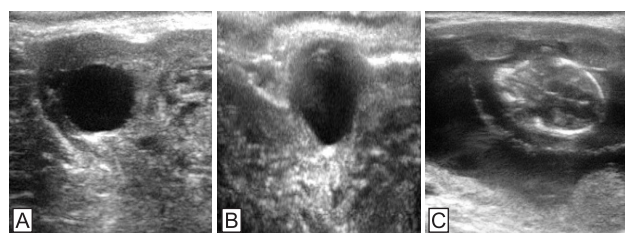


Fig. 2. Ultrasonographic images of dominant follicle at 1st AI (A), reduction in size of dominant follicle at 2nd AI (B) and fetus at day 60 of pregnancy (C).

had also shown a non-significant increase ($P>0.05$) in intracellular SOD activity in frozen-thawed semen of bulls before (10016 ± 1084 U/ml) and after (10769 ± 1504 U/ml) feeding alpha linolenic acid. In present study, addition of linolenic acid to extender helped in maintaining ($P>0.05$) SOD levels in frozen-thawed semen when compared to control but due to less concentration in the cytoplasm it was unable to neutralize the peroxidation in sperm during freezing (Bucak *et al.* 2007).

The glutathione peroxidase (GPX) activity exhibited non-significant difference ($P>0.05$) between all the extenders containing linolenic acid and control (Table 3). These observations were in consonance with the findings of Gürlér *et al.* (2015) who recorded nearly similar ($P>0.05$) GPX activity in frozen-thawed semen of bulls before (66.4 ± 16.9 nmol NADPH oxidized/min/mg of protein) and after (64.4 ± 19.0 nmol NADPH oxidized/min/mg of protein) feeding linolenic acid.

Effect of linolenic acid supplementation in extender on in vivo fertility of cryopreserved buffalo sperm: Although non-significant, the *in vivo* first service pregnancy rate (FSPR) of cryopreserved buffalo bull semen was considerably improved ($P>0.05$) with extender supplemented with 5.0 ng/ml linolenic acid (46.7%) compared to control (36.7%). In ovulated buffaloes, FSPR were 51.9% and 42.3%, respectively ($P<0.05$) for the pooled semen cryopreserved in extender supplemented with 5.0 ng/ml linolenic acid and control (Fig. 2). Determination of fertility rate after artificial insemination could help to predict the quality of cryopreserved semen accurately (Mirmahmoudi and Prakash 2012). Previously, Abd El-Razek *et al.* (2009) recorded that fish oil supplementation to extender improved the *in vivo* fertility rate up to 53.5% following cervical artificial insemination in sheep. In present study, linolenic acid supplementation in extender might have stabilized membranes, countered peroxidation

and decreased calcium uptake that in turn resulted in efficient transport of frozen-thawed spermatozoa and their increased survival in the female reproductive tract leading to better fertility rates than control.

It is concluded that addition of 5.0 ng/ml linolenic acid extender improves semen quality and *in vivo* fertility rate. Furthermore, it is recommended to adopt in routine semen cryopreservation protocols for artificial insemination program in buffaloes to improve fertility rates under field conditions.

ACKNOWLEDGEMENTS

The authors are grateful to the Indian Council of Agricultural Research for providing required funding under All India Research Coordinated Project (AICRP) for conducting this study.

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