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α-Linolenic acid supplementation in BioXcell® extender can improve the quality of post-cooling and frozen-thawed bovine sperm

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ABSTRACT

The present study was conducted to determine the effects of supplementing α-linolenic acid (ALA) into BioXcell® extender on post-cooling, post-thawed bovine spermatozoa and post thawed fatty acid composition. Twenty-four semen samples were collected from three bulls using an electro-ejaculator. Fresh semen samples were evaluated for general motility using computer assisted semen analyzer (CASA) whereas morphology and viability with eosin–nigrosin stain. Semen samples extended into BioXcell® were divided into five groups to which 0, 3, 5, 10 and 15 ng/ml of ALA were added, respectively. The treated samples were incubated at 37 °C for 15 min for ALA uptake by sperm cells before being cooled for 2 h at 5 °C. After evaluation, the cooled samples were packed into 0.25 ml straws and frozen in liquid nitrogen for 24 h before thawing and evaluation for semen quality. Evaluation of cooled and frozen-thawed semen showed that the percentages of all the sperm parameters improved with 5 ng/ml ALA supplement. ALA was higher in all treated groups than control groups than control group. In conclusion, 5 ng/ml ALA supplemented into BioXcell® extender improved the cooled and frozen-thawed quality of bull spermatozoa.

1. Introduction

Semen cryopreservation is a well-developed technique commonly used worldwide; it prolongs the life of spermatozoa by decreasing metabolism and toxin production (Bailey et al., 2003; Curry, 2000; Hammerstedt et al., 1990). During cryopreservation, development of ice crystals changed the plasma membrane structure and functions such as redistribution of membrane bound phospholipids and proteins, membrane permeability and ion exchange (Lessard et al., 2000; Amann and Graham, 1993), resulting in decreased in viability, fertility (Wongtawan et al., 2006) and ultimately, death of sperm (Bailey et al., 2003; Royere et al., 1996).

Cryopreservation damages sperm structures and is detrimental to post-thawed sperm characteristics, including motility and plasma membrane integrity (Lessard et al., 2000; Yoshida, 2000; Royere et al., 1996). The n-3 fatty acids (ALA) present in the plasma membrane provide energy and regulate plasma membrane proteins

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(Shevchenko and Simons, 2010), maintain and normalize plasma membrane function, sustain sperm viability and fertility during chilling and freezing, these fatty acids are decreased during freezing and thawing (Medeiros et al., 2002; Parks and Lynch, 1992; Watson, 2000).

Different additives like sugars, antioxidants and fatty acids have been used to decrease sperm damage during cryopreservation (Bucak et al., 2007; Memon et al., 2011, 2012; Nasiri et al., 2012). Omega-3 is important to regulate plasma membrane function, maintain sperm viability and fertility during chilling and freezing (Medeiros et al., 2002; Watson, 2000; Parks and Lynch, 1992). Gholami et al. (2010) observed dietary docosahexaenoic acid (DHA) considerably improved parameters of fresh semen of bulls. Sheikholeslami Kandelousi et al. (2013) reported that omega-3 in citrate extender decreased bull sperm motility and viability. In another study, Kiernan et al. (2013) reported that palmitic acid (PA), ALA and oleic acid (OA) added in citrate extenders improved bull sperm motility and viability during chilling for 7 days.

The variations from the previous studies in the effects of n-3 fatty acids on the quality of bull sperm may be due to biochemical damages in the lipid structure as well as lipid and protein bonds during cryopreservation (Hammerstedt et al., 1990). Previous study by Kiernan et al. (2013) have been focused on improving semen quality by adding fatty acids including ALA in citrate extenders to reduce sperm cell damage in cooled semen. Therefore, the present study was performed to determine the effect of ALA supplementation in BioXcell® extender on the quality and fatty acid composition of cooled and frozen-thawed semen.

2. Materials and methods

2.1. Experimental design

Semen samples were extended in BioXcell® (IMV Technologies, L'Agile, France) extenders which were incorporated with different concentrations of ALA (Sigma Chemical Co., St. Louis, MO, USA): 0 (control), 3, 5, 10 and 15 ng/ml. Since ALA is insoluble in water, 0.05% ethanol was added as a solvent (Nasiri et al., 2012). Only semen samples with general motility of at least 70% and normal morphology and viability ≥80% were included in the experiment (Nasiri et al., 2012; Towhidi et al., 2013; Ansari et al., 2012). This was to ensure that the treatment effects (different levels of ALA) were not confounded by differences in semen quality characteristics. The extended samples were incubated in a water bath at 37 °C for 15 min for sperm cells to uptake ALA (Ansari et al., 2012). Then, these semen samples were cooled for 2 h at 5 °C; after evaluation of the cooled samples for motility, morphology, membrane integrity, acrosome integrity, viability 0.25 ml straws were packed with sperm at concentration adjusted to 20×10^6 sperm/straw. The straws were equilibrated in a cold cabinet 5 °C for 30 min. Then the straws were arranged on a rack and placed 4 cm above the surface of liquid nitrogen vapor for 10 min contained in an expandable polystyrene box. After 10 min, the straws were immersed in liquid nitrogen for storage (Sarsaifi et al., 2013). After 24 h, the straws were thawed by placing them on a stage warmer at 37 °C for 30 s (Yoshida, 2000). Post-thawed samples were evaluated for total sperm motility by CASA, membrane functional integrity by hypo-osmotic swelling test, viability, morphology and acrosome integrity by eosin–nigrosin staining procedure, fatty acid composition (gas chromatography) and lipid peroxidation (thiobarbituric acid reactive substances, TBARS).

2.2. Animals and their management

Three fertile Brangus–Simmental cross-bred bulls were selected from the Universiti Putra Malaysia farm (2°9′18.36″N, 101°43′49.61″E). These bulls were 3 to 4 years old and weighed between 620 and 650 kg. Their body condition score (BCS) were 4, 5 and 6 (1—thin to 9—obese; Eversole et al., 2009). Each bull was kept in a separate bull pen, but under uniform management condition. The bulls were fed with *Brachiaria decumbens* and palm kernel cakes (6% crude protein and 2.6% crude fat) were given at a rate of 3 kg/bull/day while mineral licks and water were provided ad libitum.

2.3. Semen collection and samples evaluation

Semen samples were collected twice a week, at an interval of 3 to 4 days, for 4 weeks. The collection procedure was done whilst the bulls were standing. The bulls were not sedated at all. Twenty four ejaculates were collected from the three bulls (eight samples per bull), with the aid of a manually controlled electro-ejaculator, Electro Jac 5 (Ideal® Instruments Neogen Corporation, Lansing, Michigan, USA) to which a 6.5 cm diameter rectal probe, with three ventral oriented electrodes at 1 cm apart, was connected. The lubricated rectal probe was gently inserted up to about 30 cm into the rectum, with the electrodes positioned ventrally. The number of electrical stimuli was steadily increased until the bull ejaculated. Each stimulus lasted 8–10 s and paused for 2.0 s before the next stimulus was given (Sarsaifi et al., 2013). When the seminal discharge turned cloudy, a graduated tube was placed over the penis to collect the semen. Stimulation was continued as long as seminal fluid was produced and collected. After collection is completed, the graduated tube was covered with an aluminum foil to prevent exposure of semen to light. The semen samples were transported to the laboratory at 37 °C in a Coleman® cooler box for evaluation. The volume of each semen sample was recorded from the graduated tube whilst general motility and concentration were evaluated using CASA IVOS 10 system (Hamilton Thorne Bioscience, Beverly, MA, USA).

2.3.1. Sperm motility

Fresh semen samples were diluted with PBS at a ratio of 1:100. Then, 20 μl of the diluted semen was placed on a glass slide (Hamilton Thorne research 2X-CEL dual-sided sperm analysis chamber; depth: 20 μm) and loaded onto the CASA IVOS 10 system to analyze the general motility (%) of sperm. One-second tracks were captured at 60 Hz under ×4 dark-field illumination. For progressive cells, the setting of the instrument was set as follows: temperature: 37 °C, video frequency: 60, magnification: 1.92, minimum

cells size: 2 pixel, minimum contrast: 40, cell intensity: 55, VAP (path velocity): 75 μm/s and STR (straightness): 80% (Sarsaifi et al., 2013). At least 200 sperm from 10 fields were counted per reading. The software used for analysis was HTM-IVOS software, version 12.2.

2.3.2. Morphology and sperm viability

Sperm morphology and viability were assessed by using eosin–nigrosin stain (Evans and Maxwell, 1987). The smear was prepared by mixing one drop (10 μl) of semen with 3 drops (30 μl) of the stain on a warm slide. The prepared slide was examined under a light microscope at 400× magnification (Nikon Eclipse 50i, Japan) and at least 200 spermatozoa were counted in at least four different microscopic fields. Sperms that did not take up the eosin–nigrosin stain were considered alive, while those that partially or fully took up the stain were considered dead (Memon et al., 2012). Sperm morphology was determined using the same slide used for viability evaluation. The percentage of normal sperm cells were calculated from a total of 200 sperm cells examined.

2.3.3. Plasma membrane integrity

Hypo osmotic swelling (HOS) test developed by Revell and Mrode (1994) was used to assess plasma membrane integrity (PMI). One hundred microliters of semen was added to 1 ml of hypo-osmotic solution (13.51 g fructose and 7.35 g trisodium citrate dissolved in 1L distilled water; osmolarity of 150 mOsm/kg.) and incubated for 60 min at 37 °C. Then, 15 μl of the solution was placed on a pre-warmed slide covered with a cover slip and sperms were evaluated under a light microscope at 400× magnification (Nikon Eclipse 50i, Tokyo, Japan). Spermatozoa that swelled in response to the test solution were considered normal cells. Two hundred spermatozoa per slide were counted from four different microscopic fields and expressed in percentage.

2.3.4. Acrosome integrity

Acrosome integrity was determined using semen smear stained with eosin–nigrosin stain, and examined under a phase contrast microscope at 1000× magnification oil immersion (Yildiz et al., 2000). A total of 200 spermatozoa were examined for either detached or intact acrosome.

2.3.5. Fatty acid composition and lipid peroxidation (LPO) test

Fatty acids were extracted from frozen semen samples using a method developed by Folch et al. (1957) with some modifications described by Argov-Argaman et al. (2013). The frozen semen was adjusted to a concentration of 3×10^8 sperm/ml (a total of 15 straws were thawed), homogenized in chloroform: methanol (2:1 v/v), vortexed for 1 min and then kept at room temperature for 1 h. After 1 h, 4 ml of normal saline was added, vortexed and centrifuged and the supernatant was discarded while the lipid rich layer was separated and evaporated at 65 °C. The extracted fatty acids were transmethylated to their fatty acid methyl esters (FAME) using 0.66 N potassium hydroxide (KOH) in methanol and 14% methanolic boron trifluoride (BF3; Sigma Chemical Co. St. Louis, Missouri, USA) according to the methods by the Association of Official Analytical Chemists (AOAC, 1990). The FAME were separated by Agilent 7890A gas chromatography (Agilent Technologies, Palo Alto, CA, USA) using a 30 m × 0.25 mm ID (0.20 μm film thickness) Supelco SP-2330 capillary column (Supelco, Inc., Bellefonte, PA, USA). One μl of FAME was injected into the chromatograph by an auto sampler, equipped with a flame ionization detector (FID). The injector temperature was programmed at 250 °C and the detector temperature at 300 °C. The column temperature program was initially run at 100 °C for 2 min. Then heated to 170 °C at 10 °C/min for 2 min and further heated to 220 °C at 7.5 °C/min for 10 min to facilitate optimal separation.

The identification of peaks of the fatty acids was made by comparison of equivalent chain lengths with those of authentic fatty acid methyl esters (37 Component FAME mix, Supelco, Bellefonte, PA). Peak areas were determined automatically using the Agilent Gas Chromatography Chemstation software (Agilent Technologies, Palo Alto, CA, USA).

Lipid peroxidation (LPO) in semen was assessed by the malondialdehyde assay (MDA). Malondialdehyde is the end product of lipid peroxidation and is the major substrate in the thiobarbituric acid reactive substance (TBARs) test (Pryor, 1991). Lipid peroxidation was measured using TBARs according to Mercier et al. (1998). Five hundred microliter of semen was mixed with TBARs solution and then heated in a water bath at 95 °C for 60 min until the mixture appeared pink. After cooling, 1 ml of distilled water and 3 ml of *n*-butanol were added to the mixture and then vortexed. The mixtures were centrifuged at 5000 rpm for 10 min. Absorbance of supernatant was read against an appropriate blank at 532 nm using a spectrophotometer (Secomam, Domont, France). The MDA was calculated from a standard curve of 1,1,3,3-tetraethoxypropane and expressed as nmol MDA/3×10^8 sperm.

2.4. Statistical analysis

Data were checked for normality and all parameters were found to fit the normal distribution and then analyzed using parametric statistical methods. Data on the effect of different concentrations of ALA on post-cooling and frozen-thawed sperm parameters, fatty acid and MDA were analyzed using ANOVA of the general linear model procedure of SAS 9.2 version. Comparisons between different concentration means were analyzed using Duncan multiple range test following significant *F* test in ANOVA.

3. Results

From Table 1, the results showed no significant difference in sperm parameters among bulls, although two bulls had lower semen volume but higher sperm concentration compared with the other bull. The effects of different concentrations of ALA on post-cooling sperm parameters are shown in Table 2. Results show that sperm motility increases with increase in ALA concentrations up to 5 ng/ml. However, at 10 and 15 ng/ml ALA, sperm motility decreased significantly. A similar trend of increment in sperm motility with an increase in ALA concentrations was

Table 1
Sperm parameters of fresh bull semen (Mean ± SEM).

Sperm parameters	Bull 1	Bull 2	Bull 3
Volume (ml)	10.75 ± 0.8[a]	6.50 ± 0.8[b]	6.25 ± 1.0[b]
Concentration (×10^6 sperm/ml)	1626.25 ± 72.5[a]	1339 ± 77.2[a]	1426 ± 47.6[a]
Motility (%)	89.25 ± 3.5[a]	88.80 ± 2.9[a]	80.00 ± 7.0[a]
Morphology (%)	89.50 ± 1[a]	92.25 ± 1.6[a]	85.50 ± 3.3[a]
Acrosome integrity (%)	93.50 ± 0.9[ab]	92.00 ± 1.2[a]	96.00 ± 1.0[a]
Membrane integrity (%)	87.50 ± 3.4[a]	89.00 ± 0.8[a]	84.75 ± 3.0[a]
Viability (%)	87.50 ± 2.8[a]	86.00 ± 3.5[a]	87.00 ± 2.4[a]

[a,b] Mean values with different superscripts within rows are significantly different at $p < 0.05$.

Table 2
Effect of different ALA concentrations in BioXcell® extender on post-cooled sperm parameters in bulls (Mean ± SEM).

Sperm parameters (%)	ALA concentrations (ng/ml)				
	0	3	5	10	15
Motility	63.87 ± 2.1[b]	72.37 ± 2.2[a]	74.75 ± 1.9[a]	65.18 ± 1.4[b]	64.50 ± 2.1[b]
Morphology	82.19 ± 0.9[ab]	78.06 ± 2.3[bc]	86.62 ± 1.2[a]	82.93 ± 2.2[ab]	74.75 ± 2.4[c]
Membrane Integrity	82.87 ± 1.0[ab]	86.37 ± 1.2[bc]	87.87 ± 0.6[a]	82.50 ± 1.7[ab]	79.68 ± 2.3[c]
Acrosome Integrity	89.06 ± 0.4[a]	89.18 ± 0.7[a]	89.18 ± 1.0[a]	89.81 ± 0.9[a]	87.62 ± 1.1[a]
Viability	83.25 ± 1.1[bc]	86.37 ± 1.30[ab]	87.62 ± 0.7[a]	83.37 ± 1.5[bc]	80.37 ± 1.9[c]

[a,b,c] Mean values with different superscripts within rows are significantly different at $p < 0.05$.

Table 3
Effect of different ALA concentration in Bioxcell® extender on frozen-thawed sperm parameters in bulls (Mean ± SEM).

Sperm parameters (%)	ALA concentrations (ng/ml)				
	0	3	5	10	15
Motility	38 ± 0.7[c]	43 ± 0.9[b]	48 ± 1.0[a]	39 ± 0.3[c]	36 ± 0.8[c]
Morphology	64 ± 2.3[cb]	67 ± 2.1[ab]	72 ± 1.2[a]	65 ± 2.0[cb]	60 ± 1.6[c]
Membrane Integrity	69 ± 1.2[b]	71 ± 2.6[ab]	75 ± 1.6[a]	65 ± 2.2[b]	65 ± 2.2[b]
Acrosome Integrity	69 ± 1.2[b]	71 ± 1.6[ab]	75 ± 1.7[a]	71 ± 2.9[ab]	68 ± 1.9[b]
Viability	66 ± 0.9[b]	69 ± 2.1[ab]	74 ± 1.4[a]	66 ± 2.2[b]	65 ± 1.5[b]

[a,b,c] Mean values with different superscripts within rows are significantly different at $p < 0.05$.

also observed in the other parameters. However, acrosome integrity appeared to be less affected similar readings with no significant differences across all treatments (Table 3).

Table 2 shows the effects of different concentrations of ALA on frozen-thawed semen parameters. Similar to what was observed in chilled sperm, there was an increase in the quality of semen parameters, when ALA was increased from 0 to 5 ng/ml but decreased at 10 and 15 ng/ml. Unlike chilling, acrosome integrity during freezing appeared to be affected showing significant differences among treatments (3, 5 and 15 ng/ml) although the highest acrosome integrity percentage was obtained at 5 ng/ml ALA.

Sperm fatty acid (FA) composition obtained after treatment with different levels of ALA is presented in Table 4. When the amount of exogenous ALA increased from 0 to 15 ng/ml, the amount of sperm ALA recovered was also

Table 4
Comparison of fatty acid composition in different concentrations of ALA in frozen-thawed sperm (Mean ± SEM).

ALA concentration (ng/ml)					
Fatty acid (%)	0	3	5	10	15
ALA (C18:3n-3)	0.43 ± 0.05[d]	0.81 ± 0.04[c]	1.05 ± 0.11[cb]	1.20 ± 0.11[b]	1.67 ± 0.13[a]
C20:5n-3	0.92 ± 0.17[a]	1.06 ± 0.11[a]	1.15 ± 0.16[a]	1.26 ± 0.29[a]	1.32 ± 0.07[a]
C22:5n-3	0.81 ± 0.25[a]	0.82 ± 0.25[a]	0.94 ± 0.25[a]	0.85 ± 0.25[a]	1.41 ± 0.25[a]
C22:6n-3	1.30 ± 0.10[a]	1.22 ± 0.17[a]	1.26 ± 0.08[a]	1.56 ± 0.43[a]	1.65 ± 0.32[a]
N3PUFA	3.48 ± 0.24[c]	3.91 ± 0.16[bc]	4.42 ± 0.54[bc]	4.88 ± 0.26[b]	6.06 ± 0.51[a]
N6PUFA	36.03 ± 2.28[a]	34.07 ± 0.25[a]	31.73 ± 1.98[a]	37.74 ± 2.88[a]	33.44 ± 3.72[a]
PUFA	39.52 ± 2.95[a]	37.98 ± 0.22[a]	36.16 ± 1.98[a]	42.62 ± 2.62[a]	39.51 ± 3.45[a]
MUFA	24.18 ± 1.67[ab]	27.31 ± 0.21[a]	24.53 ± 1.55[ab]	27.91 ± 1.38[a]	21.88 ± 0.37[b]
SFA	36.29 ± 1.37[a]	34.69 ± 2.57[ab]	39.30 ± 0.89[a]	29.46 ± 1.91[b]	38.61 ± 3.13[a]

[a,b,c,d] Mean values with different superscripts within rows are significantly different at $p < 0.05$.
SFA: saturated fatty acids sum of (C14:0 + C16:0 + C18:0).
MUFA: monounsaturated fatty acids sum of (C16:1 + C18:1n-9).
PUFA: polyunsaturated fatty acids sum of (C18: 2n-6 + C18:3n-6 + C18:3n-3 + C20:4n-6 + C20:5n-3 + C22:5n-3 + C22:6n-3).
n-6PUFA sum of (C18:2n-6 + C18:3n-6 + C20:4n-6).
n-3PUFA sum of (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3).

Table 5
Motility characteristics of frozen thawed bull semen supplemented with ALA in BioXcell® extender.

Parameters	ALA concentration (ng/ml)				
	0	3	5	10	15
VAP (μm/s)	39.25 ± 0.25^{c}	59.65 ± 1.15^{a}	61.1 ± 0.9^{a}	44.35 ± 0.65^{b}	45.35 ± 0.65^{b}
VCL (μm/s)	51.45 ± 0.55^{d}	80.75 ± 1.25^{b}	91.35 ± 1.35^{a}	67.15 ± 0.85^{c}	69 ± 1^{c}
VSL (μm/s)	37.55 ± 0.95^{c}	55.8 ± 0.6^{a}	52.85 ± 1.15^{a}	38 ± 1^{c}	44.5 ± 0.5^{b}
ALH (μm)	7.8 ± 0^{b}	6.6 ± 0^{c}	9 ± 0^{a}	4.95 ± 0.65^{d}	4.15 ± 0.15^{d}
BCF (HZ)	0.25 ± 0.05^{b}	3.75 ± 0.25^{a}	3.55 ± 0.05^{a}	0.3 ± 0.1^{b}	0.3 ± 0.1^{b}
STR (%)	76 ± 1^{b}	78.5 ± 0.5^{b}	89 ± 0^{a}	71 ± 1^{c}	67 ± 1^{d}
LIN	57 ± 0^{c}	60 ± 0^{b}	66.45 ± 0.45^{a}	55.5 ± 0.5^{cd}	53.5 ± 1.5^{d}
Progressive motility (%)	33.6 ± 0.3^{d}	37.95 ± 0.05^{b}	45 ± 0^{a}	34.5 ± 0.5^{c}	32 ± 1^{cd}

Values in each row that do not have common superscripts are significantly different ($p < 0.05$).
VAP: average path velocity.
VCL: curvilinear velocity.
VSL: straight-line velocity.
ALH: amplitude of lateral head movement.
BCF: beat cross frequency.
STR: straightness.
LIN: linearity.

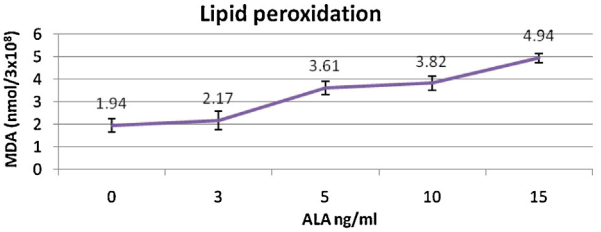

Fig. 1. MDA production in frozen thawed bovine semen treated with ALA in Bioxcell® extender.

increased. The concentrations of sperm ALA recovered at 0, 3, 5, 10, and 15 ng/ml ALA treatments were 0.43 ± 0.09, 0.81 ± 0.09, 1.05 ± 0.09, 1.20 ± 0.09, and 1.67 ± 0.09 ng/ml, respectively. Similarly, concentration of total polyunsaturated fatty acids (PUFAs) in sperm was also affected.

Table 5 shows the motility parameters observed using CASA. All parameters showed higher values for ALA at 5 ng/ml. VAP, VSL and BCF were non-significant between 3 and 5 ng/ml but other parameters such as VCL, ALH, STR, LIN and progressive motility were significantly higher in 5 ng/ml of ALA.

Lipid peroxidation assessed by the amount of MDA produced is shown in Fig. 1. From the graph the MDA production increased as ALA supplement increases.

4. Discussions

In the current study addition of ALA in semen extender exhibited positive impact on sperm parameter such as motility, membrane integrity, morphology, acrosome integrity, viability. Furthermore, at 5 ng/ml ALA group gave the highest values compared to other supplemented groups. Supplementation with 5 ng/ml ALA improved sperm cytological characteristics in chilled semen. These findings are supported by a previous similar study by Kiernan et al. (2013) who reported that addition of ALA (10 and 100 μM), palmitic acid (100 μM) and oleic acids (OA) (10 and 100 μM) in citrate based extenders with and without egg yolk improved progressive motility, linear motility and viability of bull sperm chilled for 7 days. Moreover, 5 ng/ml ALA also improved sperm cytological characteristics of frozen-thawed semen. Badr et al. (2004) and Takahashi et al. (2012) reported that palmitic acid and linoleic acid added in citrate extender increased motility and viability in frozen-thawed bull sperm. However, the findings in the present study do not echo those of Abavisani et al. (2013) and Sheikholeslami Kandelousi et al. (2013) who added n-3 fatty acids and soft gels containing n-3, n-6, n-9 fatty acids, respectively, in citrate extenders with polyethylene glycol as the solvent. These two studies reported decreased in sperm motility, morphology and viability in chilled and frozen-thawed bull semen. The disparity between the present study and the two earlier findings may be due to the types of solvent used. The polyethylene glycol used in earlier studies (Sheikholeslami Kandelousi et al., 2013; Abavisani et al., 2013) is detrimental to the sperm. In addition the effects of fatty acids on sperm quality may vary due to the sources of fatty acids, types of fatty acid and solvent used as well as the amount of fatty acids (Castellano et al., 2010; Sheikholeslami Kandelousi et al., 2013) incorporated into the extenders.

Concentrations higher or lower than 5 ng/ml ALA supplemented in the present study resulted in a lower semen quality both after chilling and freezing. This indicates that 5 ng/ml as the recommended concentration of ALA for optimum cryopreservation results. The increasing concentration from 0 to 5 ng/ml showed a progressive trend in sperm quality. This may be because these concentrations have helped in improving plasma membrane fluidity and integrity and thus reduce ice crystal formation during cooling and freezing (Nasiri et al., 2012). The higher concentrations did not produce better sperm characteristics may be because of the presence of more fatty acids that make mammalian sperm susceptible to lipid peroxidation (Aitken et al., 1993; Kothari et al., 2010).

The present study also shows an increase in ALA uptake and recovery by sperm cells with an increase in ALA supplementation. This is validated by an earlier study (Maldjian et al., 2005) that reported the occurrence of fatty

acid absorption by sperm membrane particularly at the tail region.

Lipid peroxidation (LP) is the damage of fatty acid mainly polyunsaturated fatty acids (PUFAs) which are ALA and DHA (Aitken et al., 1993; Kothari et al., 2010). As PUFAs are more vulnerable to highly reactive and short lived free radicals called reactive oxygen species (ROS) attack owing to the double bonds, the LPO was assessed by measuring melondialdehyde (MDA), a molecule released after oxidation of lipids and used as a biomarker of oxidative stress (Sanocka and Kurpisz, 2004). MDA production increases with high rate of LP process due to high production of ROS that decreases sperm motility (Sanocka and Kurpisz, 2004) and the fluidity of sperm membrane (Gharagozloo and Aitken, 2011). The decrease in quality of chilled and frozen semen in ALA concentrations >5 ng/ml may be attributed to the observed higher recovery of ALA absorbed with subsequent increased in LPO reaction and MDA production that probably may cause an oxidative stress to the sperm cells. It was observed that lipid peroxidation increased linearly with increase in ALA concentrations. In comparison to other previous studies Kiernan et al. (2013) reported that ALA delayed production of ROS in chilled semen. The results showed that cooled and frozen sperm quality were improved with 5 ng/ml. considering the results of sperm quality parameters, fatty acid uptake and lipid peroxidation of sperm cells, the optimum level of ALA to be supplemented in Bioxcell® extender so as to improve chilled and frozen bull semen quality is 5 ng/ml.

5. Conclusion

The present study revealed that adding ALA into Bioxcell® semen extender improved post-cooling and post thawed quality of bovine semen. ALA uptake was observed to be linear in relation to ALA concentration added. A concentration of 5 ng/ml of ALA was found to be the optimum level for best semen cryopreservation result using Bioxcell® extender and with tolerable LPO reactions and amount of MDA production.

Conflict of interest statement

There is no conflict of interest among authors regarding publication of this article.

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References

Abavisani, A., Arshami, J., Naserian, A.A., Sheikholeslami Kandelousi, M.A., Azizzadeh, M., 2013. Quality of bovine chilled or frozen-thawed semen after addition of omega-3 fatty acids supplementation to extender. Int. J. Fertil. Steril. 7 (3), 161–168.

Aitken, R.J., Harkiss, D., Buckingham, D.W., 1993. Analysis of lipid peroxidation: mechanisms in human spermatozoa. Mol. Reprod. Dev. 35, 302–315.

Amann, R.P., Graham, J.K., 1993. Spermatozoal function. In: Di Rienzi, D. (Ed.), Equine Reproduction. Lea and Febiger, Philadelphia, PA, pp. 715–745.

Ansari, M., Towhidi, A., Moradi Shahrbabak, M., Bahreini, M., 2012. Docosahexaenoic acid and alpha-tocopherol improve sperm cryosurvival in goat. Slovak J. Anim. Sci. 45 (1), 7–13.

AOAC, 1990. In: Herlick, K. (Ed.), Official Methods of Analysis. , 15th ed. Association of Official Analytical Chemists, Arlington, VA, p. 1230.

Argov-Argaman, N., Mahgrefthe, K., Zeron, Y., Roth, Z., 2013. Variation in lipid profiles within semen compartment—the bovine model of aging. Theriogenology 80, 712–721.

Badr, M.R., Abdel-Malak, M.G., Shaker, M.H., 2004. Influence of some fatty acids and cholesterol addition to semen extender on freezability and in vitro fertilizing potentials of ram spermatozoa. Assiut Vete. Med. J. 50, 304–318.

Bailey, J.L., Morrie, A., Cormier, N., 2003. Semen cryopreservation: success and persistent in farm species. Can. J. Anim. Sci. 83, 393–401.

Bucak, M.N., Ateşşahin, A., Varışlı, Ö., Yüce, A., Tekin, N., Akçay, A., 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram sem: microscopic oxidative stress parameters after freeze–thawing process. Theriogenology 67 (5), 1060–1067.

Castellano, C.-A., Audet, I., Bailey, J.L., Laforest, J.-P., Matte, J.J., 2010. Dietary omega-3 fatty acids (fish oils) have limited effects on boar semen stored at 17 °C or cryopreserved. Theriogenology 74 (8), 1482–1490.

Curry, M.R., 2000. Cryopreservation of semen from domestic livestock. Rev. Reprod. 5, 46–52.

Evans, G., Maxwell, W.M.C., 1987. Handling and examination semen. In: Maxwell, W.M.C. (Ed.), Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Sydney, Australia, pp. 93–106.

Eversole, D.E., Browne, M.F., Hall, J.B., Dietz, R.E., 2009. Body Condition Scoring Beef Cows. Cooperative Extension Publication, Virginia, pp. 400–795, ⟨www.pubs.ext.vt.edu/400/400-795/400-795.html⟩.

Folch, J., Lees, M., Sloane Stanely, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226 (1), 497–509.

Gharagozloo, P., Aitken, R.J., 2011. The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. Hum. Reprod. 26 (7), 1628–1640.

Gholami, H., Chamani, M., Towhidi, A., Fazeli, M.H., 2010. Effect of feeding docosahexaenoic acid-enriched nutriceutical on the quality of fresh and frozen-thawed semen in Holstein bulls. Theriogenology 74 (9), 1548–1558.

Hammerstedt, R.H., Graham, J.K., Nolan, J.P., 1990. Cryopreservation of mammalian sperm: what we ask them to survive. J. Androl. 11, 73–88.

Kiernan, M., Fahey, A.G., Fair, S., 2013. The effect of the *in vitro* supplementation of exogenous long-chain fatty acids on bovine sperm cell function. Reprod. Fertil. Dev. 25 (6), 947–954.

Kothari, S., Thompson, A., Agarwal, A., du Plessis, S.S., 2010. Free radicals: Their beneficial and detrimental effects on sperm function. Ind. J. Exp. Biol. (I.J.E.B.) 48 (5), 425–435.

Lessard, C., Parent, S., Leclerc, P., Bailey, J.L., Sullivan, R., 2000. Cryopreservation alters the levels of the bull sperm surface protein P25b. J. Androl. 21, 700–707.

Maldjian, A., Pizzi, F., Gliozzi, T., Cerolini, S., Penny, P., Noble, R., 2005. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Theriogenology 63 (2), 411–421.

Medeiros, C.M.O., Forell, F., Oliveria, A.T.D., Rodrigues, J.L., 2002. Current status of sperm cryopreservation: why isn't it better? Theriogenology 57 (1), 327–344.

Memon, A.A., Wahid, H., Rosnina, Y., Goh, Y.M., Ebrahimi, M., Nadia, F.M., 2012. Effect of antioxidants on post thaw microscopic, oxidative stress parameter and fertility of Boer goat spermatozoa in Tris egg yolk glycerol extender. Anim. Reprod. Sci. 136 (1–2), 55–60.

Memon, A.A., Wahid, H., Rosnina, Y., Goh, Y.M., Ebrahimi, M., Nadia, F.M., Audrey, G., 2011. Effect of butylated hydroxytoluene on cryopreservation of Boer goat semen in Tris egg yolk extender. Anim. Reprod. Sci. 129, 44–49.

Mercier, Y., Gatellier, P., Viau, M., Remignon, H., Renerre, M., 1998. Effect of dietary fat and vitamin E on colour stability and on lipid and protein oxidation in turkey meat during storage. Meat Sci. 48 (4), 301–318.

Nasiri, A.H., Towhidi, A., Zeinoaldini, S., 2012. Combined effect of DHA and α tocopherol supplementation during bull semen cryopreservation on sperm characteristics and fatty acid composition. Andrologia 44 (Supp: 1), 550–555.

Parks, J.E., Lynch, D.V., 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion and rooster sperm membrane. Cryobiology 29, 255–266.
Pryor, W.A., 1991. The antioxidant nutrients and disease prevention—what do we know and what do we need to find out? Am. J. Clin. Nutr. 53 (1), 391–393.
Revell, S.G., Mrode, R.A., 1994. An osmotic resistance test for bovine semen. Anim. Reprod. Sci. 36, 77–86.
Royere, D., Barthelemy, C., Hamamah, S., Lansac, J., 1996. Cryopreservation of spermatozoa: 1996 a review. Hum. Reprod. Update 2 (6), 553–559.
Sanocka, D., Kurpisz, M., 2004. Reactive oxygen species and sperm cells. Reprod. Biol. Endocrinol. 2, 12.
Sarsaifi, K., Rosnina, H.Y., Ariff, O.M., Wahid, H.A., Hani, H., Yimer, N., Naing, S.W., Abas, M.O., 2013. Effect of semen collection methods on the quality of pre-and post-thawed Bali cattle (*Bos javanicus*) spermatozoa. Reprod. Domest. Anim. 44 (6), 1006–1012.
Sheikholeslami Kandelousi, M.A., Arshami, J., Naserian, A.A., Abavisani, A., 2013. The Effect of addition omega-3, 6, 9, fatty acids on quality of bovine chilled and frozen-thawed sperms. Open Vet. J. 3 (1), 47–52.
Shevchenko, A., Simons, K., 2010. Lipidomics: coming to grip with lipid diversity. Nat. Rev. Mol. Cell Biol. 11 (8), 593–598.
Takahashi, T., Itoh, R., Nishinomiya, H., Katoh, M., Manabe, N., 2012. Effect of linoleic acid albumin in a dilution solution and long-term equilibration for freezing of bovine spermatozoa with poor freezability. Reprod. Domest. Anim. 47 (1), 92–97.
Towhidi, A., Zeinoaldini, S., Ardebili, R., Davachi, N.D., Nasiri, A.H., 2013. Combined n-3 fatty acids and α-tocopherol supplementation improved the ovine sperm cryosurvival. Iran. J. Biotechnol. 11 (4), 238–243.
Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci. 60–61, 481–492.
Wongtawan, T., Saravia, F., Wallgren, M., Caballero, I., Rodriguez-Martinez, H., 2006. Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. Theriogenology 65, 773–787.
Yildiz, C., Kaya, A., Aksoy, M., Tekeli, T., 2000. Influence of sugar supplementation of the extender on motility, viability and acrosomal integrity of dog spermatozoa during freezing. Theriogenology 54, 579–585.
Yoshida, M., 2000. Conservation of sperms: current status and new trends. Anim. Reprod. Sci. 60–61, 349–355.