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Frozen-Thawed Quality of Bull Semen after Combined Supplementation of Docosahexaenoic Acid and Alpha Linolenic Acid into Tris based Semen Extender

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Authors' Contribution
AK and HW conceived and designed the experiments. AK performed the experiments. AK, KS and ME analyzed the data and wrote the article. All others helped in experimental work and preparation of manuscript.

Key words
Omega-3, DHA, ALA, Bull Semen, Cryopreservation.

ABSTRACT

This the study was conducted to find out the effect of the addition of alpha linolenic acid (ALA) and docosahexaenoic acid (DHA) on frozen-thawed quality of bull semen. Twenty-four ejaculates (8 from each bull) were collected from three bulls. Semen ejaculates having motility ≥70% and normal morphology ≥80% were extended into Tris extender consist of equal concentration of each ALA and DHA (0, 3, 5, 10,15ng/ml). Extended semen was cooled at 5°C for 2 hours at 5°C and then packaged in 0.25ml straws and stored into liquid nitrogen for 24 hours. Successively, thawing and evaluation was performed for sperm motility, viability, membrane integrity, morphology, malondialdehyde (MDA), fatty acid composition and super oxide dismutase (SOD). Sperm characteristics such as sperm motility, viability, morphology, membrane integrity, and acrosome integrity were decreased in treated groups. DHA concentration was increased in all supplemented groups. Whereas ALA decreased in all groups except at 5ng/ml DHA was increased linearly by increasing supplementation of fatty acids. In conclusion, combination of ALA and DHA did not improve frozen-thawed bull sperm parameters when compared to control.

INTRODUCTION

Frozen semen is normally used for artificial insemination (AI) in cattle. Semen extenders are the main constituents to accomplish freezing; semen extenders provide energy, maintain osmolarity, reduce bacterial contamination and protect sperms at low temperature. Quality of frozen-thawed semen is the main factor for successful application of AI. However, it is proven that the fertility of frozen semen is lower than fresh semen in all mammals including bovine. Freezing decreases 50% sperm viability and fertility upto 70% (Lessard *et al*., 2000; Watson, 2000).

The mechanism by which cryopreservation causes sperm damage is still to be explored, however, it is proven that reduction in viability and fertility occurs due to the damage on sperm plasma membrane (Abavisani *et al*., 2013). Sperm membrane is a highly dynamic structure that controls extra and intra cellular processes and plays an important role during fertilization. Lipids are the major component of sperm membrane which forms lipid bilayer to control ionic exchanges and omega-3 fatty acids share a major portion of the membrane lipids (Towhidi and Parks, 2012).

Formation of ice crystals reorders lipids and changes in lipid-lipid and lipid-protein bonds and reduces membrane fluidity and permeability during freezing. Lipids are important for sperm maturation, viability and membrane functions. Therefore, any modification in structure of sperm membrane reduces sperm function such as viability and fertility (Kandelousi *et al*., 2013). In addition, reduction in omega-3 fatty acids occurs by lipid peroxidation (Abavisani *et al*., 2013). Omega-3 fatty acids are vital for membrane fluidity and integrity.

Nikolopoulou *et al*. (1985) stated that higher amount of PUFAs improved the feasibility of sperm. Dietary DHA enhanced motility of raw bull semen, however no affect on frozen-thawed semen (Gholami *et al*., 2010). In stallion,

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dietary omega-3 fatty acids also reduced sperm motility and morphology in raw and frozen-thawed semen (Grady *et al*., 2009). Similarly, in vitro addition of omega-3 fatty acids also decreased frozen-thawed quality of bull sperm (Abavisani *et al*., 2013; Kandelousi *et al*., 2013) while fish oil added in egg yolk- lactose extender has improved frozen-thawed quality of boar semen (Chanapiwat *et al*., 2012). Inconsistent effects of PUFAs on sperm characteristics have been reported in different species. But for bull very little data is available for *in vitro* supplementation of DHA and ALA Thus, present study was planned to investigate the effect of addition of DHA and ALA on frozen-thawed characteristics, fatty acid composition, malondialdehyde on bull sperm using Tris extender.

MATERIALS AND METHODS

Semen collection and experimental design

Semen was collected from three bulls of Brangus-Simmental crossbred cattle by an electro-ejaculator (Electro Jac 5, Ideal Instruments Neogen, Corporation, Lansing, Michigan, USA) Twenty-four ejaculates (8 from each bull) were collected two times a week. After collection, ejaculates were brought into the laboratory at 37°C in the Coleman cooler box to assess for further processing. Ejaculates which have motility ≥70% and normal morphological ≥80 % and were processed for further experiments.

Each ejaculate was extended into Tris extender consists of equal concentration of ALA and DHA (control), 3, 5, 10 and 15ng/ml concentrations (Sigma Chemical Co., St. Louis, MO, USA). Ethanol 0.05% was used to dissolve the fatty acids in water (Nasiri *et al*., 2012; Kaka *et al*., 2015a). The samples were extended in test tubes and incubated in a water bath for 15 min for absorption of fatty acids by sperm membrane (Kaka *et al*., 2015a, b). Later, extended samples were cooled at 5°C for 2 h (Kaka *et al*., 2015a) then packaged into 0.25 ml straws with 20 million sperm /straws and stored into liquid nitrogen for 24 h. The stored straws were thawed in a water bath at 37°C for 30 seconds (Kaka *et al*., 2015a, b) and then evaluated for sperm characteristics, fatty acid composition and SOD level and MDA.

Semen evaluation

Motility of sperm was determined by computer assisted semen analyzer (CASA, IVOS Hamilton Thorne bioscience, Beverly, Massachusetts USA). To evaluate motility a drop of 20µl diluted semen was placed CASA 2X-cell (20 µm) glass slide covered with a cover slip (20mm x 20mm) and loaded on the CASA for motility evaluation. A total of 200 sperm from an average of 10 fields were counted per reading (Yimer *et al*., 2011; Taşdemir *et al*., 2013) through software HTM-IVOS, version 12.2 (Kaka *et al*., 2015a).

Viability of sperm was investigated by eosin-nigrosin stain (Evans and Maxwell, 1987). Sperms were classified on the basis of colors white (alive) and red (dead) (Memon *et al*., 2012). Sperm morphology was investigated using the same slide used for viability investigation. The percentage of normal sperm was calculated from a total of 200 sperm examined per slide (Yildiz *et al*., 2000).

The membrane integrity of sperm was evaluated by hypo-osmotic swelling test (HOST; Revell and Mrode, 1994; Buckett *et al*., 1997; Rehman *et al*., 2012). The sperm swelled in the response to the solution were considered normal. Two hundred sperms were counted per slide from 4 different microscopic fields, and stated in percentage.

Malondialdehyde was determined by thiobarbituric acid reactive substances (TBARS; Mercier *et al*., 1998). Absorbance of supernatant was read against suitable blank at 532nm using a spectrophotometer (Secomam, Domont, France). The malondialdehyde (MDA) was calculated by standard curve of 1, 1, 3, and 3- tetraethoxypropane and expressed as nmol / $3x10^8$ sperm (Kaka *et al*., 2015a).

Fatty acids from frozen semen samples were determined using a method of Folch *et al*. (1957) with some modifications described by (Ebrahimi *et al*., 2012). The peaks of areas were determined using automatic Agilent Gas Chromatography Chemstation software (Agilent Technologies, Palo Alto, CA, USA).

Statistical analysis

Data on sperm characteristics, MDA and fatty acid were analyzed using the general linear model (GLM) procedure of the SAS 9.2 version. Differences within means were analyzed by the Duncan test. Statistical significant was considered when $P<0.05$. Results are shown as mean ± standard error (SEM).

RESULTS

Table I shows the percentage of sperm characteristics of frozen-thawed sperm extended in Tris extender supplemented with DHA and ALA. The findings of present study showed that all groups supplemented with DHA and ALA did not improve the sperm parameters.

Fatty acid (FA) concentration of frozen-thawed sperm found after addition of different concentrations of DHA and ALA is presented in Table II. Findings showed that ALA had no significant difference ($P>0.05$) among treatments however, DHA concentration was ($P<0.05$) higher in 10 and 15ng/ml while no significant variation ($P>0.05$) in other supplemented groups including control. Total n-3 PUFAs showed no difference ($P>0.05$) among treatment.

Table I.- Effects combination of docosahexaenoic acid (DHA) and alpha linolenic acid (ALA) in Tris extender on frozen- thawed sperm parameters of bulls (Mean%±SEM; n=24).

Sperm Parameters %	DHA ALA (ng/ml)				
	0	3	5	10	15
Motility	40.00±.4.0 ^{a}	38.00±1.7 ^{ab}	33.25±1.7^{bc}	34.00±1.9^{c}	34.00±0.9^{c}
Morphology	59.50±4.3 ^{a}	56.75±0.6 ^{ab}	53.50±2.0^{abc}	50.00±0.7^{bc}	47.25±1.7 ^{c}
Membrane integrity	55.25±3.8 ^{a}	51.50±2.5 ^{ab}	51.50±0.7 ^{ab}	49.25±2.6 ^{ab}	46.75±2.3 ^{b}
Viability	59.75±1.0^{a}	57.75±2.8^{ab}	53.00±1.7^{abc}	49.00±2.3^{bc}	45.50±4.3^{c}

^{a,b,c,d}, values with different superscripts within rows show significant difference P<0.05. DHA, docosahexaenoic acid; ALA, alpha linolenic acid; ng/ml, nanograms per milliliter; SOD, superoxide dismutase; DNA, deoxyribonucleic acid; U/ml: units per milliliter.

Table II.- Comparison of fatty acid composition with different concentrations of docosahexaenoic acid (DHA) and alpha linolenic acid (ALA) combination in Tris extender of frozen-thawed bull sperm (Mean % ± SEM, n=24).

Fatty acid	DHA ALA Concentration (ng/ml)				
	0	3	5	10	15
C18:3n-3 ALA	0.86±0.1	0.83±0.7	0.95±0.9	0.94±0.2	1.01±0.9
C20:5n-3	0.58±0.1	0.72±0.4	0.84±0.1	0.78±0.3	0.60±0.4
C22:5n-3	0.80±0.2	0.74±0.4	0.76±0.3	0.59±0.5	0.58±0.2
C22:6n-3 DHA	1.77±0.3^{c}	1.67±0.5^{bc}	1.84±0.3^{ab}	1.95±0.3 ^{a}	1.97±0.7 ^{a}
n-3PUFA	4.01±0.3	3.96±0.6	4.39±0.1	4.26±0.2	4.16±0.9
n-6PUFA	17.68±0.3 ^{a}	16.96±0.9 ^{a}	17.64±0.4 ^{a}	16.67±0.8 ^{ab}	13.23±2.3^{b}
PUFA	21.69±0.5 ^{a}	20.92±0.9 ^{ab}	22.03±0.3 ^{a}	20.93±0.9 ^{ab}	17.39±2.2 ^{b}
MUFA	44.59±1.8 ^{a}	46.11±1.8 ^{a}	42.81±1.3 ^{ab}	40.78±1.2 ^{ab}	31.62±7.3 ^{b}
SFA	33.72±1.9^{b}	32.97±0.9^{b}	35.16±1.4^{b}	38.29±1.9^{ab}	50.99±9.5^{a}

For abbreviations and statistical details, see Table I. SFA, saturated fatty acids: sum of (C14:0+C16:0+C17:0+ C18:0); MUFA, monounsaturated fatty acids: sum of (C16:1n-7+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).

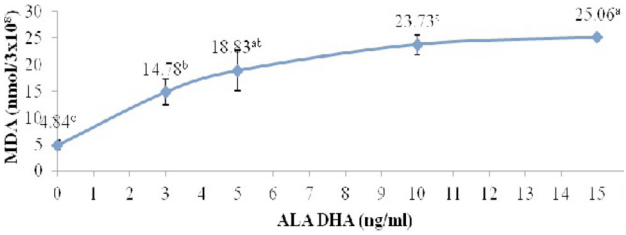

Fig. 1. Malondialdehyde (MDA) production in frozen-thawed bovine semen treated with combination of docosahexaenoic acid (DHA) and alpha linolenic acid (ALA) in tris extender. a, b, and c, significant difference at P<0.05.

Figure 1 shows that MDA production was higher (P<0.05) in groups which have DHA and ALA in comparison to control, 15ng/ml showed significantly higher (P<0.05) among all treatments.

DISCUSSION

In the present study, addition of DHA and ALA concentration produced a negative effect on frozen-thawed sperm characteristics in Tris extender. These findings are similar results reported by Kandelousi *et al*. (2013) who found that omega -3 PUFAs did not improve motility, progressive motility, morphology and viability in citrate extender in quality of frozen-thawed bull semen. Similarly, Abavisani *et al*. (2013) also reported that sperm viability, motility and morphology were significantly lower in omega-3 PUFAs supplemented groups in comparison to control.

Polyunsaturated fatty acids (PUFAs) have both positive and negative effects. However, the effects of PUFAs on fertility and sperm characteristics usually depend on only the type of PUFAs and length of carbon chain (Castellano *et al*., 2010).

Polyunsaturated fatty acids with long chains enhance the fluidity of plasma membrane of sperm and increased resistance of sperm against cold shock (Wathes *et al*., 2007). In fact, maintenance of sperm membrane fluidity and its capacity of fertilization requires important amounts of PUFA mainly DHA. The amounts of unsaturated fatty acids also have pressure on the other physical characteristics of the membrane such as permeability and the temperature related effects (Safarinejad *et al*.,

2010). Nevertheless, PUFAs also makes sperm vulnerable to attack of lipid peroxidation cascade and seriously compromise the functional integrity of the sperm. Studies have shown that vitamin E being an exogenous antioxidant has the ability to minimize the negative effects of PUFAs (Wathes *et al*., 2007).

In this study, addition of DHA and ALA increased n-3 fatty acids in the frozen-thawed sperm of bull in Tris extenders. In Tris extender DHA showed significant changes among all the treatment. Moreover, total n-3 PUFAs was not significant. This may be because in the mammalian body, ALA is converted to DHA, as ALA is considered a precursor of DHA and other n-3 fatty acids. Comparable results were reported by (Towhidi *et al*., 2013) after addition of n-3 fatty acids increased DHA concentration and total n-3 PUFAs in ovine semen. Nasiri *et al*. (2012) and Towhidi and Parks (2012) in bulls, Ansari *et al*. (2012) in goats, Rooke *et al*. (2001) in boars reported that addition of *in vivo* and *in vitro* supplementation of fatty acids improved n-3 PUFAs of frozen-thawed semen. Malondialdehyde is the main concern regarding supplementation of n-3 PUFAs which usually decreases the quality of frozen thawed bull semen. In the current experiment, the lipid peroxidation and MDA production increased due to the combined supplementation of ALA and DHA and resulted in decreased frozen-thawed quality of bull semen. Correspondingly, Kandelousi *et al*. (2013) reported that supplementation of n-3 fatty acids decreased resistance against lipid peroxidation. Moreover, Abavisani *et al*. (2013) also reported that supplementation of n-3 fatty acids decreased sperm resistance against cold shock and increased lipid peroxidation.

CONCLUSION

Conclusively, this study revealed that a combination of DHA and ALA supplementation into Tris extender decreased frozen-thawed quality of bull sperm. Frozen-thawed sperm motility, morphology, viability and morphology were decreased compared to the control. However, fatty acid, MDA and SOD were increased.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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