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Research Article

Enhancement of Frozen-Thawed Quality of Bull Semen after Enrichment of Docosahexaenoic Acid Extended in Tris- Extender

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Abstract | The present study aimed to depict the quality of frozen-thawed bull sperm extended in Tris-buffered extender (TBE) supplemented with docosahexaenoic acid (DHA). Electro ejaculator was used to collect the semen and ejaculates having ≥ 20 × 10^9 spermatozoa with an initial motility rate above 70 %, and normal morphology and viability of ≥ 80 % were extended in TBE, to which different concentrations (0, 3, 5, 10 and 15 ng/ml) of DHA were enriched. Semen sample with supplementations were sequentially incubated for 15 minutes at 37 ˚C and cooled at 5 ˚C for 2 hour than packed in 0.25 ml straws, and cryopreserved in liquid nitrogen for 24 hours. Consequently, straws were thawed at $37C^0$ for 30 seconds and motility was accessed with the help of computer-assisted semen analyzer (CASA) and membrane integrity with the hypo-osmotic swelling test. Meanwhile morphology, viability and acrosome integrity with contrast-phase microscope (eosin-nigrosin staining) was investigated. Moreover, superoxide dismutase (SOD) assay, a fatty acid composition with gas chromatography and lipid peroxidation TBARS (Thiobarbituric acid reactive substances) content were used for oxidative stress evaluation. Frozen-thawed results showed significant ($p < 0.05$) enhancement in all sperm parameters with 10 ng/ml of DHA supplementation. DHA improved ($p < 0.05$) SOD levels in all supplemented groups compared with the control group. Level of TBARS was higher ($p < 0.05$) at 15 ng/ml as compared to other DHA supplemented and control groups. A dose-dependent increase ($P<0.05$) was found in C22:6n-3, DHA and n-3PUFA as compared to control group. In conclusion, supplementation of 10 ng/ml of DHA in a Tris-based extender improves the frozen-thawed quality of bull semen.

Keywords | Docosahexaenoic acid DHA, Fertility, Freezing, Tris-Extender, Spermatozoa.

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INTRODUCTION

A sperm membrane is an outer membrane that covers the head, midpiece, and the principal piece of sperm cells. Its constituent is mainly phospholipids, which are composites of omega3 fatty acids. Polyunsaturated fatty acids (PUFAs) are important for the function and structure of the sperm membrane and the maintenance of its fluidity

throughout freezing (Robinson et al., 2006; Gholami et al., 2010). It has been well reported that the fertilizing power of male gametes is highly interconnected with plasma membrane lipids; nevertheless, the high concentrations of PUFAs makes male gametes susceptible to peroxidation by the reactive oxygen species (Hauser et al., 1994), related to male subfertility (Ommati et al., 2013). Spermatozoa rely on extracellular components in extender for their catabolic processes to maintain the structure and function during freezing (Kaka et al., 2015a). Therefore, glycerol, carbohydrates, amino acids and fatty acids were supplemented to maintain sperm functionality and structure (Amann and Graham, 1993).

Sperm membrane of bovine contains docosahexaenoic acid (DHA), wich is the main component that contains of 55 to 60 % of total fatty acids (Kelso et al., 1997). There is a good body of literature demonstrating about the adding of DHA in animals' diets enhanced the plasma membrane integrity of mammalian spermatozoa. Supplementation of DHA in the diet of bulls improved paramenters in fresh semen, but not in post-thawed semen. It has also been reported that dietary DHA improved the quality of fresh semen and increased DHA concentration in the ovine sperm membrane. Supplementation of oils (source of DHA) in feed has been reported to improve the fresh semen quality of various species, such as boars (Castellano et al., 2010), bulls (Yuan et al., 2021), stallion (Brinsko et al., 2005), and human (González-Ravina et al., 2018). It has also been shown that the *in-vitro* adding of DHA in boar semen results in improvement of motility and membrane integrity of frozen-thawed boar spermatozoa (Kaka et al., 2017). Previous studies have been focused on the dietary effect of DHA. However, information on *in-vitro* effects, particularly on bull semen, is scarce. Therefore, the present study was carried out to evaluate the *in-vitro* additions of DHA in Tris based extender on frozen thawed bull spermatozoa.

MATERIALS AND METHODS

Semen collection and experimental design

Ejaculated semen samples were collected from three sexually mature Brangus-Simmental crossbred bulls with a history of fertility. Electro ejaculator (Electro Jac 5, 10 volts, 2 seconds on - 2 seconds off, Ideal Instruments, Neogen Co., Lansing, Michigan, USA) was used to collect the semen two times in a week (Sarsaifi et al., 2013; Kaka et al., 2016). A lubricated rectal probe was inserted into the bull's rectum. The electrodes placed ventrally and penis diverted into the collecting tube (Memon et al., 2012; Kaka et al., 2015a). After semen collection, each ejaculate was immediately transported to the laboratory to evaluate motility, morphology, and viability (Kaka et al., 2015b). Only ejaculates with motility of ≥ 70 % and normal morphology as well as viability of ≥ 80 % were diluted in 100 ml of Tris-egg yolk extender (3.51 g Tris buffer, 1.25 g fructose, 1.97 g citric acid, 7 % glycerol, and 20 % egg yolk) containing 0 (control), 3, 5, 10 and 15 ng/ml of DHA (Sigma Chemical Co., St. Louis, MO, USA) (Kaka et al., 2015b). Ethanol (0.05 %) was used to dissolve DHA in water because of its fat solubility. The extended semen were incubated at 37 °C for 15 minutes for the DHA uptake. Subsequently, the extended semen samples were chilled at 5 °C for 2 hour and filled into 0.25 ml straws with having concentration adjusted up to 20×10^6 motile spermatozoa per straw. Then 20 straws of each treatment arranged vertically on a rack, 3-4 cm above the surface of liquid nitrogen gas for 10 minutes (Sarsaifi et al., 2013). Finally, the straws were macerated and keeped in liquid nitrogen for 24 hours at -196 °C. After that, thawing of four straws from each treatment was done with the holp of water bath at 37 °C for 30 seconds (Sarsaifi et al., 2013). Frozen-thawed samples were then accessed for individual motility, membrane integrity, morphology, viability and acrosome integrity, fatty acid composition, superoxide dismutase (SOD), and thiobarbituric acid reactive substances (TBARS) content.

Semen evaluation

Computer-assisted semen analyzer (CASA, IVOS Hamilton Thorne Bioscience, Beverly, Massachusetts, USA) were used for assessment of motility using the procedure described by Sarsaifi et al. (2013). A drop of 20 µl of frozen-thawed semen was placed on a previously heated slide(37 °C, CASA 2X-cell, 20 µm), covered with a pre-warmed coverslip (20 x 20 mm) and loaded onto the CASA. An average of 10 fields was counted per reading with HTM-IVOS software, version 12.2 (Hamilton Thorne Bioscience, Beverly, Massachusetts, USA). Sperm morphology and viability were based on the eosin nigrosin staining technique (Ommati et al., 2018a). A thin smear was set by adding a drop of semen with 3 drops of the Hematoxaline and Eosin stains on a warmed slide and incubated for 3 min at 37 °C. Sperm morphology, abnormality, and viability were determined using the same slide at 1000× and 400× magnification, respectively. Abnormalities of frozen-thawed spermatozoa were monitored in duplicate (200 sperm per slide/20 high power field) after eosin-nigrosine staining. Spermatozoa with detached head, protoplasmic droplets, malformed head, double tail, coiled tail, and without tail were considered abnormal.On the other hand, spermatozoa with purple-stained heads were considered dead, while non-stained spermatozoa were considered live (Ommati et al., 2018a; Ommati et al., 2019a).

Acrosomal integrity were analysed under light microscope (Nikon Eclipse 50i, Tokyo, Japan) at 1000×magnification (oil immersion lenses). At least 200 spermatozoa were counted from different fields, moreover value was expressed

in percentage. Thickened, vesiculated, detached and absent acrosomes were classified as damaged during examinations (Yilds etal., 2000; Kaka et al., 2015a).

The hypo-osmotic swelling (HOST) test was performed to determine sperm membrane integrity (Ommati et al., 2018b; Ommati et al., 2020). 100 µL of semen were mixed with 1.0 mL of hypo osmotic solution with osmolarity 280 mOsmol/kg (fructose 13.51 g and tri-sodium citrate 7.35 g in 1L of distilled water) and incubated for 60 min at 37 ˚C. Fifteen µL of the mixed sample was placed on a warm slide, covered with a coverslip, and observed under the light microscope at 400 × magnification (Revell and Mrode, 1994; Baiee et al., 2015; Baiee et al., 2017). Sperm with the intact membrane will swell in response to the hypo-osmotic solution. A total of 200 spermatozoa were counted from 4 different microscopic fields, and the percentage of spermatozoa with curled or coiled tails were calculated.

Lipid peroxidation in frozen-thawed semen samples was measured using TBARS to determine malondialdhyde (MDA) concentration (Sarlos et al., 2002). Five hundred µL of frozen semen was added into the tube containing 2500 µL of TBARS assay solution (containing 3 mL phosphoric acid 1% w: v, 1 mL trichloroacetic acid 15% w: v, 1 mL thiobarbituric acid 0.375 w: v; pH = 2) and incubated at 95 ˚C for 45 min till the solution turned pink. Afterward, samples were allowed to cool at RT (Room Temperature). Then, 1 mL of distilled water and 3 mL of n-butanol were taken and added to mixture and the vortexed well. The mixture was runed for centrifugation at 5000 rpm for 10 minutes. Finally, the optical density of the developed color in the n-butanol phase was read against an appropriate blank at wave length of 532 nm by a spectrophotometer (Secomam, Domont, France).

The fatty acid profile of frozen semen samples was analyzed based on the method described in the literature (Ommati et al., 2013), with minor modifications. Briefly, semen samples with sperm concentration adjusted to 3×10^8 sperm/mL (a total of 15 frozen straws were thawed) were homogenized in chloroform: methanol (2:1 v/v), then vortexed (60 sec) and incubated. After 60 minutes of incubation at room temprature, Four mL of physiological saline were added, vortexed and centrifuged (3500gfor 10 minuts). Subsequently, the lipid rich layer was seprated and evaporated at 65 ˚C, meanwhile supernant was removed. The drawn out fatty acids were trans-methylated to their FAMEs (Fatty Acid Methyl Esters) using 0.66 N potassium hydroxide in methanol and 14 % of BF_3 (Methanolic Boron Trifluoride), (Sigma Chemical Co. St. Louis, Missouri, USA) as methods affirmed by the Association of Official Analytical Chemists, AOAC (26). Agilent 7890A gas chromatography (Agilent-Technologies, Palo-Alto, CA, United states of America) was employed to separate the FAMEs using 30m x 0.25mm ID (0.20µm film thickness) SP-2330 capillary column (Supelco, Inc., Bellefonte, PA, USA). One µL of FAME was injected using an autosampler into the chromatograph, equipped with a flame ionization detector (FID). The injector temperature was programmed at 250 ˚C, and the detector temperature was set at 300 ˚C. The column temperature program was initially run at 100 ˚C for 2 min, then increased to 170 ˚C at 10 ˚C/min for 2 min, and eventually increased to 220 ˚C at a rate of 7.5 ˚C/min for 10 min to facilitate optimal separation. The identification of peaks was made by comparing equivalent chain lengths with authentic FAME (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).

Superoxide dismutase is an enzymatic antioxidant test that catalyzes the dismutation of a superoxide anion radicals (O^{2-}) to hydrogen peroxides (H_2O_2). In this research, SOD was calculated with the superoxide dismutase assay kit (Cayman Chem. Co., United States of America). Semen (250 uL) was keeped in Eppendorf-tube and it was centrifuged at 1000g for 10 min. The superficial (seminal plasma) was aspirated and discarded, while the pellet was dissolved in 0.5 mili litters of 0.1 percent of Triton X100 (Fisher, Montre´al, P-Q, Canada) in phosphate-buffered saline (PBS). Then, mixed specimens were vortexed for 3 to 5 cycles of 10 seconds each and then centrifuged at 1000g for 3 min. Afterward, 100 µL of semen samples were transferred onto the sample and standard wells of an ELISA microplate (model, company, country), containing 200 µL of the radical detector and 20 µL from xanthine-oxidase. Next, the microplate was closed with a cover plate and it wass incubated for 20 min. Finally, the absorbance rate was calculated at 450 nm by a microplate reader (Tecan, Mannedorf, version 3.32.3.31.3.31 CER, Switzerland). The activity of SOD was measured by the standard regression curve.

Data analysis

Data were analyzed by using the general linear model (GLM) process of SAS 9.2 version (S.A.S Insti. Inc., S.A.S Campus Drive, Cary, North Carolina) and presented as mean ± standard error of the mean (SEM). The significant differences between means were analyzed by Duncan multiple range tests, and values were statistically significant when at $p < 0.05$.

RESULTS

The percentage of sperm motility, sperm normality, acrosome integrity, membrane integrity and viability was was the highest (P<0.05) for TBE supplemented with DHA at

Table 1: Effects of different docosahexaenoic acid (DHA) concentrations on frozen-thawed bull sperm parameters (mean % ± SEM).

Sperm parameter (%)	DHA concentration (ng/mL)				
	0	3	5	10	15
Motility	40.27±1.2^{c}	42.09±1.3^{b}	44.16±0.6^{b}	51.11±0.5^{a}	44.33±0.5^{b}
Morphology (Normal spermatzoa)	61.83±1.5^{b}	63.50±1.4^{b}	62.06±1.5^{b}	70.28±1.1^{a}	60.44±1.2^{b}
Acrosome integrity	59.17±1.8^{cb}	62.94±2.2^{b}	62.56±2.4^{b}	70.22±2.5^{a}	55.06±1.1^{c}
Membrane integrity	59.44±1.2^{b}	61.83±1.3^{ab}	60.94±1.5^{b}	67.00±1.7^{a}	58.11±2.9^{b}
Viability	58.39±1.3^{b}	62.17±1.8^{b}	63.00±1.9^{b}	70.50±1.4^{a}	56.83±3.3^{b}

^{a,b,c} Within rows, values with different superscripts differ significantly (P < 0.05).
DHA: Docosahexaenoic acid

Table 2: Comparison of fatty acid composition in different DHA concentrations in Tris® extender on frozen-thawed sperm parameters in bulls (Mean % ± SEM).

Fatty acid	DHA concentration ng/ml				
	0	3	5	10	15
C14:0	0.49±0.02	0.49±0.02	0.48±0.12	0.54±0.52	0.52±0.72
C16:0	25.14±0.62	25.24±0.72	25.02±0.82	25.23±0.02	24.85±0.92
C16:1n-7	2.93±0.13	2.96±0.33	2.96±0.63	2.94±0.38	2.93±0.93
C18:0	0.34±0.91	0.23±0.51	0.34±0.41	0.58±0.21	0.54±0.11
C18:1n-9	7.98±0.11	7.89±0.12	7.78±0.14	7.93±0.16	7.980±0.10
C18:2n-6	42.06±0.55	41.43±0.45	42.11±0.65	40.63±0.45	41.83±0.52
C18:3n-6	0.84±0.11	0.65±0.10	0.64±0.14	0.51±0.15	0.49±0.16
C18:3n-3	0.36±0.10	0.39±0.40	0.34±0.10	0.37±0.10	0.35±0.10
C20:4n-6	1.64±0.1	1.93±0.01	1.92±0.16	1.93±0.12	1.86±0.11
C20:5n-3	0.72±0.51	0.79±0.42	0.80±0.43	0.84±0.36	0.75±0.40
C22:5n-3	0.83±0.80	0.78±0.94	0.97±0.12	0.87±0.62	0.98±0.71
C22:6n-3, DHA	0.55±0.91^{d}	0.71±0.11^{c}	0.90±0.71^{b}	1.17±0.51^{ab}	1.45±0.51^{a}
SFA	33.97±0.34	33.87±0.22	33.63±0.34	4.28±0.11	33.72±0.32
MUFA	44.99±0.71	44.39±0.72	45.07±0.31	44.99±0.24	44.99±0.69
PUFA	21.03±0.66	21.72±0.60	21.29±0.37	22.13±0.47	21.51±0.41
n-6PUFA	18.55±0.69	19.03±0.90	18.26±0.30	18.85±0.70	17.96±0.40
n-3PUFA	2.47±0.90^{b}	2.69±0.83^{b}	3.03±0.81^{ab}	3.27±0.68^{ab}	3.55±0.79^{a}

^{a,b,c} Within rows, values with different superscripts differ significantly (P < 0.05).
DHA: Docosahexaenoic acid
SFA: Saturated fatty acids: (C14:0 + C16:00 + C18:0)
MUFA: Monounsaturated fatty acids: (C16:1n-7 + C18:1n-9)
PUFA: Polyunsaturated fatty acids: (C18:2n-6 + C18:3n-6 + C18:3n-3 + C20:4n-6+C20:5n-3 + C22:5n-3 + C22:6n-3)
n-6 PUFA: (C18:2n-6 + C18:3n-6 + C20:4n-6)
n-3 PUFA: (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3)

a level of 10 ng/mL in comparison with the other supplemented and control groups (Table 1). The gas chromatography findings revealed that C22:6n-3, DHA and n-3PUFA showed dose-dependent increase (P<0.05) in the supplemented groups being the highest with the highest level of DHA. However, other frozen-thawed sperm fatty acid profiles were not affected (Table 2). There were a trend of increase in TBARS and superoxide dismutase (SOD) levels in the supplemented groups as compared with those in the control group (p < 0.05) (Figure 1).

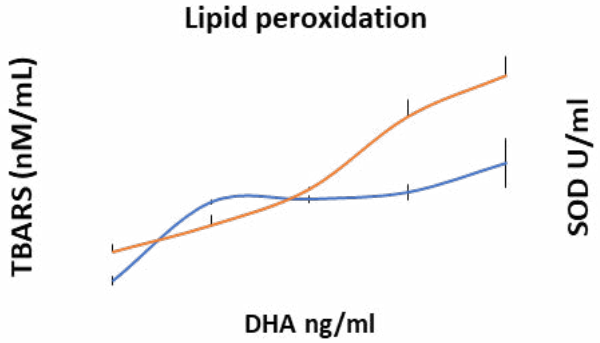

Figure 1: Seminal TBARS (nM/mL; solid blue line) and SOD (solid red line) content in frozen-thawed bovine semen supplemented with docosahexaenoic acid (DHA) in Tris based extender. TBARS: Thiobarbituric acid reactive substances

The MDA was calculated with a standard curve of 1, 1, 3 and 3-tetra ethoxy propane and presented as nmol / 3×10^8 sperm.

DISCUSSION

In the current study, DHA addition at 10 ng/ml improved frozen-thawed sperm motility, membrane integrity, morphology, acrosome integrity, and viability. Similar findings were also carried earlier by Chanapiwat et al. (2012), their obtained research findings exhibited that DHA improves motility, membrane integrity, acrosome integrity and viability in boar semen with or without the combination of *L-cysteine* in lactose based egg yolk extender. Kaeoket et al. (2010) reported an improvement in frozen/post-thawed sperm motility, membrane integrity, acrosome integrity and viability of boar sperm after supplementing DHA in the lactose based egg-yolk extender. Egg yolk, very rich in DHA, was added to the citrate based extender. It enhanced the motility, progressive motility, morphology and viability in frozen thawed goat sperm (Yimer et al., 2014). Similarly, the addition of DHA in the feed also enhanced the sperm characteristics in fresh-semen characteristics of bulls (Yuan et al., 2021; Gholami et al., 2010), boars (Rooke et al., 2001; Castellano et al., 2010), and rams (Samadian et al., 2010). These all above findings supports the result of the present study.

There was an improvement in TBARS values in supplemented groups with DHA than control. These results are in aggrement with previous findings in boars (Chanapiwat et al., 2012), and bulls (Nasiri et al., 2012). DHA plays the primary role in lipid peroxidation throughout freezing. In the present research study, peroxidation were higher in the supplemented groups. However, it didn't affected the quality parameters of frozen thawed sperm. That might be due to, DHA maintains the sperm plasma membrane fluidity, which prohibit attacks by reactive oxygen species (Hauser et al., 1994). Thus, DHA maintained the frozen thawed quality parameters of bull sperm (Chanapiwat et al., 2012). Furthermore, SOD is considered the basic enzymatic protection against lipid peroxidation in sperm for the duration of freezing. In the present experiment, SOD was measured to ensure the effect of DHA on its concentration as the SOD decreases oxidation increases, which affects sperm quality. However, DHA improved SOD concentration in frozen-thawed samples. The improvement in SOD is a positive sign of adding DHA for quality frozen-thawed bull semen. Similar findings were also stated by (Strzezek et al., 2004), who also showed that addition of PUFAs enhanced SOD in frozen thawed semen of boar.

In contrast, Castellano et al. (2010) observed DHA (fish oil) doesn't affects on the SOD actions in boar semen, enhancement in SOD amount might be due to the maintenance of membrane fluidity by DHA addition. Upgrading in SOD content limited lipid peroxidation production, furthermore, it resulted in the improvement of frozen-thawed quality parameters of bull sperm.

CONCLUSION

Supplementation of DHA into Tris extenders improved frozen thawed sperm motility, acrosome integrity, morphology, and viability at 10ng/ml in Tris based extender. Lipid peroxidation was elevated in DHA supplemented groups. Still, it did not induce damage to frozen-thawed sperm, maybe because of improvement in SOD concentration, which maintained the integrity of the sperm membrane. In end, 10 ng/ml of DHA was considered the optimum level for semen cryopreservation in Tris extender in bulls.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

NOVELTY STATEMENT

In vitro supplementation of Fatty acid and their effect on sperm is less studies most of studies were conducted on dietary supplementation.

AUTHORS CONTRIBUTION

Asmatullah Kaka, Wahid Haron , Nurhusien Yimer created the idea and conducted trial, Abdullah Channo , Ali Raza Jahejo helped in writing article and language cheking , Mahdi Ebrahimi , Dildar Hussain Kalhoro helped analysis and tabulation of data.

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