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Effect of n-3 fatty acids and α-tocopherol on post-thaw parameters and fatty acid composition of bovine sperm

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Abstract
Purpose This study was designed to determine the combined effects of adding source of n-3 fatty acids (FA) and α-tocopherol (vitamin E, VE) to semen extender on freezability and FA composition of Brown Swiss bull sperm.
Methods Semen samples were collected from 6 Brown Swiss bulls and pooled. In the first trial, semen was divided into 12 groups including 4 levels of n-3 FA (0, 1, 10, 100 ng ml^{-1}) and 3 levels of VE (0. 0.2, 0.4 mM). Motility, viability and fatty acid composition of sperm were measured.
Results The treatment of 10 ng ml^{-1} n-3 FA and 0.4 mM VE had the best post-thaw sperm characteristics ($P<0.01$). In the second trial, sperm lipid composition of this treatment and control (without FA and VE) was determined. Supplementing n-3 fatty acids during cryopreservation increased docosahexaenoic acid (DHA) and the ratio of n-3 to n-6 FA in sperm before freezing and after thawing.
Conclusions The results suggest that combining the optimal level of n-3 FA (10 ng ml^{-1}) with the highest level of VE tested (0.4 mM) in a semen extender changed the membrane lipid composition and improved freezablity of Brown Swiss bull sperm.

Capsule Bull Sperm Cryopreservation is improved by combined supplementation of n-3 PUFA, and α-tocophen.

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Introduction

Cryopreservation of bull semen is essential for its most effective use, but freezing and thawing induces detrimental effects on sperm ultrastructural, biochemical, and functional features, such as reduced motility, membrane integrity and fertilizing ability [1]. Apart from the freezing protocol, extender composition is an important factor for survival of the sperm during cryopreservation [2]. Egg yolk is used as a common component of semen extenders for domestic animals with its beneficial effect during sperm cryopreservation apparently afforded by presence of phospholipids, in association with other components that prevent damage to sperm membranes [3].

The plasma membrane is a highly dynamic structure that regulates extracellular exchange and mediates fertilization [4]. Lipid composition of the sperm plasma membrane is a major determinant of mobility characteristics, cold sensitivity, and overall viability [2]. Species differences in lipid composition of the sperm plasma membrane is a key factor for freezability of the sperm [5]. In several mammalian species, up to 60 % of phospholipid bound the total fatty acids of cells are long-chain polyunsaturated fatty acids (LCPUFA) especially, DHA [6, 7]. Presence of these LCPUFA are assumed to impart greater fluidity (less order) within the plasma membrane due in large measure to the presence of the many double bonds. These specific physical characteristics may give membranes greater resistance to damage arising from the formation of ice crystals [8]. A high proportion of DHA in both seminal plasma and sperm is positively associated with percent sperm motility in boar [9] and men [10]. DHA may contribute to the membrane fluidity required for the bending sperm tails [11]. Indeed, it

was found that the sperm obtained from asthenozoospermic men had lower levels of DHA when compared with sperm from normozoospermic men [7]. The reduction of semen PUFA concentration decreases the sperm cell number, motility and sperm fertilizing potential in ejaculated bull semen [12].

Mammalian spermatozoa are sensitive to lipid peroxidation, due to the phospholipid content of sperm membranes with their high PUFA sidechains [13]. Seminal plasma provided some protection against peroxidation via its constituent antioxidants [14]. However, dilution of semen reduces antioxidant availability for sperm. On the other hand, supplementing semen extender with PUFAs during cryopreservation increases ROS production [15]. Therefore, including an antioxidant when adding PUFAs to semen extender is beneficial [16].

Recently, we showed that combined supplementation of n-3 fatty acids and α-tocopherol in highest level (10 ng ml^{-1} and 0.2 mM, respectively) during cryopreservation of Holstein bull semen improved sperm quality after freezing and thawing. Considering that freezability of Brown Swiss sperm is usually less than for the Holstein bull, the objective of this study was to investigate the combined effect of a broad range of n-3 fatty acids (up to 100 ng ml^{-1}) and α-tocopherol (up to 0.4 mM) on both sperm fatty acid composition an cryosurvival.

Material and method

Semen collection

Six mature Swiss Brown bulls were used in this study. Ejaculates were collected from bulls twice a week for 8 weeks using an artificial vagina (total number of ejaculates =96). The neat semen samples were transferred to the laboratory and kept in a water bath at 34 °C until examination. Sperm motility was evaluated using a phase-contrast microscope at 400x and ejaculates ≥70 % progressive motility were pooled each collection day as a replicate.

Experimental design

Experiment 1

In the first trial, pooled semen was extended using Bioxcell® with different levels of n-3 FA (Viva Pharmaceutical Inc, Canada) (0, 1, 10 and 100 ng ml^{-1}) and VE (α-tocopherol, Sigma Chemical Co., St. Louis, MO, USA) (0, 0.2, 0.4 mmol). Due to the insolubility of fatty acid and vitamin E in water, lipid solution were prepared on ethanol and added as 0.05 % (v/v) to extender. Extended samples were incubated in 15 ml tubes at 37 °C for 30 min to allow uptake of fatty acids and α-tocopherol by sperm cell.

Experiment 2

Based on experiment 1, 0.4 mmol VE and 10 ng ml^{-1} n-3 FA provided optimal in vitro characteristics post-thawed, so this treatment with control group (without FA and VE) was used in Exp 2. In this experiment, fatty acid composition of sperm lipid in both groups was determined before freezing and post-thaw.

Freezing and thawing process

Diluted semen was cooled to 4–5 °C over 2 h and then frozen in 0.25 ml straws. A 46×21×19 cm Styrofoam container containing liquid nitrogen was used to cryopreserve the semen samples. A straw with the loaded straw was placed into the liquid nitrogen vapor at a height of 4 cm above the liquid for 8 min, after that, the straws were plunged into liquid nitrogen. Straws were thawed by placing them in a 37 °C water bath for 30 S [1].

Semen evaluation

a) Post-thaw sperm motility and progressive motility

A drop (6 μl) of fresh or frozen-thawed semen was placed on a pre-warmed slide and covered with a cover slip. Motility (MOT) and progressive motility (PMOT) percentages were assessed with a phase contrast microscope at 200x total magnification [17].

b) Sperm viability

Twenty μl of fresh or frozen-thawed semen was placed on a pre-warmed slide and mixed with 30 μl of the supravital stain [1 % eosin B and 5 % nigrosin (w/v) in 3 % tri-sodium citrate. Three hundred and thirty three spermatozoa in microscopic fields (33 spermatozoa per filed) were differentially counted for unstained (live) versus stained/partial stained (dead) sperm heads at 400x total magnification [17].

Fatty acid composition of sperm

Semen samples were diluted with an equal volume of 0.85 % (wt/vol) NaCl followed by centrifugation at 700*g for 20 min at 4 °C to separate seminal plasma from the cell pellet. The supernatant (seminal plasma) was transferred to a clear test tube and the sperm pellet was washed with 1 ml of 0.85 % (w/v) NaCl and recentrifuged as described above. The sperm cell pellet was resuspended in 2 ml of 0.85 % (wt/vol) NaCl. The resulting sperm pellet was washed twice with saline [3]. Total lipid was extracted from the spermatozoa after homogenization in a suitable excess of chloroform–methanol (2:1, v/v) [18]. Trans-methylation of these samples was

performed using the Metcalf method [19]. The resultant fatty acid methyl esters were analyzed by gas chromatography (HP6890 with FID detector and autosampler HP7683, Hewlett Packard, Wilmington, DE, USA) using a capillary column system Carbowax, 30 m×0.25 mm in diameter, 0.25 μm film thickness (Alltech Ltd., Carnforth, Lancashire, UK). Integration of the peaks and subsequent data handling was performed using the HP Chemstation software (Hewlett Packard), enabling determination of the fatty acid composition (proportion of total fatty acids) by comparison of the total fatty acid peak areas to that of the pentadecanoic fatty acid standard. The identities of the peaks were verified by a comparison with the retention times of standard fatty acid methyl esters. Fatty acid composition of sperm lipid from fresh and frozen-thawed samples was determined.

Statistical analysis

Data was analyzed using the GLM procedure of SAS 9.1 (SAS Institute, Cary, NC, USA). Duncan's multiple range tests were used for mean comparisons. Differences with values of $P<0.05$ were considered statistically significant. The results were expressed as mean ± SEM.

Results

Experiment 1

The mean percent post-thaw sperm characteristics of different treatments are shown in Table 1.

a) Sperm Motility

The effect of n-3 FA and VE, and their interactions on percent post-thaw sperm motility (MOT) were significant. Mean %MOT (+/− SEM) was 36.5±0.4, 41.4±0.4 and 42.1 ±0.4 for level 0, 0.2 and 0.4 mM VE, respectively with %MOT in 0.4 mM vitamin E the highest ($P\leq0.01$). The %MOT was 30.4±0.7, 35.3±0.7, 49.0±0.7 and 45.4±0.7 for 0, 1, 10 and 100 ng ml^{-1} levels of n-3 FA. Mean %MOT was significantly higher with 10 ng ml^{-1} n-3 FA than with other FA levels ($P<0.05$). The %MOT was significantly higher in combined 10 ng ml^{-1} FA and 0.4 mM VE than for other treatment combinations. In treatments including 0 and 1 ng ml^{-1} n3 FA, motility was significantly lower ($P\leq0.01$) than for ethanol-free control (Table 1).

b) Progressive motility

The effect of n-3 FA and VE and their interactions were significant on percent progressive sperm motility (%PMOT). Mean % PMOT (±SEM) was 32.7±0.4, 36.1±0.4, and 37.4± 0.4 for 0, 0.2, and 0.4 mM VE, respectively. The %PMOT with no VE was significantly lower than with 0.2 and 0.4 mM VE mM ($P\leq0.05$). Mean %PMOT was significantly higher with 10 ng/ml n-3 FA (43.6±0.7) than with lower levels (0, 1 and 100 ng/ml, 26.8±0.7, 31.8±0.7 and 39.4±0.7, respectively) ($P<0.05$). The combination of 10 ng ml^{-1} FA and0.4 mM VE had higher %PMOT ($P\leq0.05$) than other treatment groups. In groups including 0 and 1 ng ml^{-1} n3 FA, progressive motility was significantly lower ($P\leq0.05$) than ethanol- free control (Table 1).

Table 1 Post-thaw sperm characteristics for different levels of n-3 FA and vitamin E in Brown Swiss bulls

Parameter (%) Treatment	Motility (SEM = 1.2)	Progressive motility (SEM = 0.7)	Viability (SEM = 0.3)
F_0V_0 (without ethanol)	44.7 ^{bc}	39.8 ^{d}	49.6 ^{c}
F_0V_0 (with ethanol)	30.2 ^{ef}	28.5 ^{h}	37.3 ^{ef}
$F_0V_{0.2}$	31.8^{ef}	26.3^{i}	36.0^{f}
$F_0V_{0.4}$	29.2^{f}	25.7^{i}	35.5^{f}
F_1V_0	33.1^{e}	30.9^{g}	38.6^{ef}
$F_1V_{0.2}$	35.8^{de}	31.4f^{g}	40.1^{e}
$F_1V_{0.4}$	36.9^{d}	33.2^{f}	39.5^{e}
$F_{10}V_0$	40.1^{c}	35.3^{e}	44.9^{d}
$F_{10}V_{0.2}$	52.7^{a}	46.9^{b}	55.1^{ab}
$F_{10}V_{0.4}$	54.3^{a}	48.6^{a}	57.3^{a}
$F_{100}V_0$	42.7^{c}	36.0^{e}	46.6^{cd}
$F_{100}V_{0.2}$	45.4^{bc}	39.9^{d}	49.1^{c}
$F_{100}V_{0.4}$	48.0^{b}	42.2^{c}	51.8^{bc}

a, b, c) Values in each column that do not have any common letter are significantly different

F (fatty acids; 0,1,10,100 ng ml $^{-1}$); VE (Vitamin E; 0,0.2,0.4 mM)

c) Viability

Mean %viability was significantly lower with no VE (41.9±0.4) than in 0.2 (45.1±0.4) and 0.4 mM (46.0±0.4). For different levels of FA, viability was significantly higher in 10 ng ml $^{-1}$ than 0, 1 and 100 ng ml $^{-1}$ n-3 FA (52.4 %±0.7 vs. 36.3 %±0.7, 39.4 %±0.7 and 49.2±0.7, respectively). The treatment 10 ng ml $^{-1}$ FA, 0.4 mM VE had the highest ($P\leq0.05$) viability compared with other groups. In groups including 0 and 1 ng ml $^{-1}$ n3 FA, viability was significantly lower ($P\leq$\0.05) than ethanol- free control (Table 1).

Experiment 2

Sperm fatty acid composition (%), % n-3 and n-6 FA, and the ratio of n-3/n-6 and PUFA/SFA (saturated fatty acid) are shown in Table 2. The DHA percentage, n-3 FA percentage and ratio of n-3/n-6 was significantly higher ($P\leq0.05$) in FA group (treatment) before freezing and after thawing than in the group without FA (control) (Table 2).

Table 2 Fatty acid composition of spermatozoa lipid from Brown Swiss bull semen in the control group (without FA) and n-3 FA group (with FA)

Fatty acid	Before freezing		After thawing		SEM
	Without FA	With FA	Without FA	With FA	
C14:0	8.0[a]	6.1[a]	7.8[a]	7.5[a]	0.9
C16:0	34.8[a]	32.2[a]	36.5[a]	35.5[a]	1.2
C18:0	20.3[a]	19.9[a]	24.2[a]	20.7[a]	2.7
C18:1	8.8[a]	9.7[a]	9.9[a]	8.3[a]	0.4
C18:2	13.2[a]	12.9[a]	14.5[ab]	16.7[b]	0.8
C18:3	1.1[a]	1.3[a]	0.3[b]	1.2[a]	0.1
C20:4	2.9[a]	1.6[b]	0.2[c]	0.2[c]	0.1
EPA	0.3	0.4	0.2	0.2	0.1
DHA	7.9[b]	12.5[a]	3.1[c]	6.7[b]	0.9
n-3	9.3[b]	14.2[a]	3.6[c]	8.1[b]	1.0
n-6	16.1[a]	14.5[a]	14.7[a]	16.9[a]	1.5
Ratio of n-3/n-6	0.6[b]	1.0[a]	0.2[c]	0.5[b]	0.1
PUFA	25.4[a]	28.7[a]	18.3[b]	25.0[a]	1.3
SFA	63.1[a]	58.2[b]	68.5[a]	63.7[a]	2.1
PUFA/SFA	0.4[a]	0.5[a]	0.3[b]	0.4[a]	0.1

FA Fatty acids; *EPA* Ecosapantaenoic acid; *SFA* Saturated fatty acids; *DHA* Docosahexaenoic acid; *PUFA* Poly unsaturated acids

Values in each row that do not have any common letter are significantly different ($p<0.05$)

Discussion

The main objective of the present study was to determine the combined effects of adding n-3 fatty acids and α-tocopherol to semen extender on in vitro characteristics, freezability and fatty acid composition of Brown Swiss bull sperm. In the first trial, mean %MOT, %PMOT and %viability were significantly higher in 10 ng ml $^{-1}$ n-3 FA than other FA levels, with optimal results for these parameters when combined with 0.4 mM VE. Therefore, combined supplementation of fish oil (a rich source of DHA) and α-tocopherol as a biological antioxidant had a cryoprotective effect on Brown Swiss bull sperm consistent with previous work with Holstein bulls our laboratory [16]. In that study, we used 4 levels of n-3 FA (0, 0.1, 1 and 10 ng ml $^{-1}$ with 3 levels of α-tocopherol (0, 0.1 and 0.2 mM) and the best result was in group 10 ng ml $^{-1}$ n-3 FA with 0.2 mM α-tocopherol. The higher level of n-3 fatty acids (100 ng ml $^{-1}$) actually decrease post-thawed sperm quality in the present study. Therefore, these data indicate that 10 ng ml $^{-1}$ of fish oil is adequate quantity to protect sperm during cryopreservation in both Holstein and Brown Swiss bulls. A decrease in sperm quality in 100 ng ml $^{-1}$ n-3 PUFAs may be due to excess of PUFAs actually promoting oxidation by contributing reactive oxygen species (ROS) that damage sperm membranes [20]. In agreement with these data, optimal sperm quality after freezing and thawing was observed in extender that included 0.4 mM vitamin E (Experiment 1). Hence, The highest level of α-tocopherol might be more beneficial when a higher level of PUFAs is supplemented.

Oxidative stress (OS) is considered a major contributing factor to male infertility and results from an imbalance between ROS and endogenous antioxidants mechanisms. High concentrations of the ROS cause sperm pathology (ATP depletion) leading to insufficient axonemal phosphorylation, lipid peroxidation, and loss of motility and viability. Supplementing a cryopreservation extender with antioxidant has been shown to improve the cryoprotective effect on mammalian sperm quality. Extracellular antioxidants are extremely important for the protection of mammalian spermatozoa against oxidative stress because the cytoplasmic extrusion associated with sperm morphogenesis depletes these cells of endogenous antioxidant enzymes. Antioxidants such as vitamin E or C reduce membrane damage caused by excessive ROS production during cryopreservation [21].

Using egg yolk diluent enriched in n-3 fatty acids without adding antioxidant failed to improve the quality of sperm following cryopreservation [15], so that adding an antioxidants is pre-requisite to the beneficial effects observed with added n-3 FA.

In Experiment 1, sperm quality parameters in positive control (with ethanol) were lower than those in the control absent ethanol group. It might be assumed that ethanol had detrimental effects directly on sperm or indirectly due to altered cryopreservation in the presence of ethanol [22, 23]. However, the effect of ethanol as used in this trial is in contrast to some studies on sperm where no detrimental effect was seen [24, 25]. Regardless, addition of fatty acid and vitamin E in the current trial compensated for the negative effects of ethanol to some extent.

In Experiment 2, the levels of sperm DHA, n-3 FA, PUFAs and the ratio of n-3/n-6 and PUFAs/SFA with added FA were greater than extender without FA that might be reflect effective incorporation of n-3 FA into sperm

membrane. Apparently, long chain fatty acids can be transported across the plasma membrane. Once they cross the outer membrane and enter the membrane hydrophobic core where they are protonated, allowing them to partition into the inner leaflet [26]. Before semen freezing, percent DHA was higher in FA treatment than that in the group without FA, and decreased significantly in both groups after thawing. A plausible reason for this decrease could be excessive lipid peroxidation during cryopreservation of sperm in Brown Swiss and Holstein bulls that has been reported before [27]. This could also account to some extent for the decrease in LCPUFA observed in this experiment. Another explanation for the decrease in the proportion of PUFAs might be an increase in the amount of saturated FA taken up or passively bound to the sperm membranes which would reduce the proportion of the LCPUFA [15].

Increased post-thaw sperm progressive motility in this study could be a result of DHA aggregation in the sperm membrane lipids [26], especially in the sperm flagellum where increased fluidity would promote sperm motility. Analysis of fatty acids from head and tail of monkey sperm clarified that DHA composed 1.1 and 19.6 % of total fatty acids of head and tail, respectively. The difference between lipid composition of sperm head and tail may be necessary for specific functions of sperm because lipid plays a major role in integrity, fluidity, stability, and permeability of the plasma membrane. Therefore, a high proportion of DHA in the sperm tail may be necessary for increased sperm motility through biophysical effects on the membrane [28].

Another mechanism is that fatty acids may provided substrate for metabolism and sperm axoneme dynamics [29]. Moreover, it has been suggested that DHA is involved in sperm regulation of fatty acid utilization by forming fatty acyl CoAs. The action of this enzyme is necessary before free fatty acids can be utilized by cells. [30].

Improved viability in first experiment might be related to increased membrane stability in the face of intracellular ice formation and elevated solutes when the proportion of n-3 fatty acids is increased in plasma membrane [15].

PUFAs play a major role in motility, lipid metabolism, and the sperms ability to fuse with the oocyte. A decrease in %DHA in sperm lipids is accompanied by a decrease in sperm number and motility in ejaculates of aged bulls [12]. Other studies with bulls [31], goat bucks [32], rams [33] and boars [9] showed that inclusion of omega-3 source in the daily diet improved quality of sperm. These reports confirm the results of the present study.

In humans, clinical studies showed that dietary omega-3 supplementation increased sperm concentration, motility, and morphology, and also antioxidant activity in human seminal fluid. Hence, infertile men with idiopathic oligoasthenoteratozoospermia might be benefit from omega-3 fatty acids administration [34, 35]. Moreover, impaired fertility of men with oligo/asthenozoospermia could be due to the decreased fluidity of sperm membrane because of higher ratio SFA/PUFAs. Indeed membrane fluidity regulates specific functions such as the acrosome reaction and fusion with the oocyte membrane [10]. Recently, it has been indicated that DHA involves in membrane fusion of mice sperm [36]. Therefore, in vivo or in vitro administration of n-3 fatty acids may be useful for ART techniques in humans.

In conclusion, the results of the current study indicated that supplementing bull semen extender with n-3 FA and α-tocopherol improved post-thawed in vitro characteristics of Brown Swiss bull sperm. As suggested previously for Holstein bulls, incorporation of PUFA especially DHA into cell membrane before freezing where they protect and perhaps modify the cell membrane. In this study, the highest level of vitamin E (0.4 mM) was more effective. Therefore, the importance of adding an antioxidant in combination with PUFAs in bull semen extender was also reinforced.

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