SciMedCentral

Journal of Veterinary Medicine and Research

Short Communication

Lipidomics Evaluation of the Effects of Feeding a Yeast-Based Supplement Containing Algal Docosahexaenoic Acid (DHA) on DHA-Containing Glycerophospholipids in Stallion Spermatozoa

Paul L. Wood[1,2]*, Kirsten Scoggin[3], Barry A. Ball[3], Laurie Lawrence[4], Mats H. Troedsson[3], Kristen M. Brennan[5], Lauren Goedde[3], and Edward L. Squires[3]

[1]*Metabolomics Unit, Department of Physiology and Pharmacology, DeBusk College of Osteopathic Medicine, USA*
[2]*College of Veterinary Medicine, Lincoln Memorial University, USA*
[3]*Departments of Veterinary Science, University of Kentucky, USA*
[4]*Departments of Animal Science, University of Kentucky, USA*
[5]*Alltech Inc., USA*

***Corresponding author**
Paul L. Wood, Metabolomics Unit, Department of Physiology and Pharmacology, DeBusk College of Osteopathic Medicine, USA, Email: paul.wood@lmunet.edu

Submitted: 18 January 2017
Accepted: 16 February 2017
Published: 20 February 2017
ISSN: 2378-931X

Keywords
- Stallion
- Spermatozoa
- Glycerophospholipids
- Docosahexaenoic acid

Abstract

In comparison to many other species, cryopreservation of stallion sperm results in significant cellular damage which compromises sperm function in artificial reproduction efforts. It has been hypothesized that sperm membrane fragility may be due to the higher content of docosapentanoic acid (DPA; 22:5) relative to docosahexaenoic acid (DHA; 22:6) in stallion sperm. This ratioin stallion sperm appears to be the reverse of that in sperm of most other livestock species. Prvious studies have demonstrated that DHA supplementation can improve the viability of equine sperm, however, the structural lipids involved have not been examined. We therefore undertook adietary supplementation study to determine if augmented DHA levels results in increased levels of sperm glycerophospholipids containing DHA. A targeted lipidomics analysis of DHA-containing glycerophospholipids was conducted for spermatozoa obtained from stallions prior to and upon completion of 60 days of dietary yeast-based supplement containing algal DHA. Control stallions were fed a comparable diet without supplementation.These analyses demonstrate for the first time that increasing free DHA levels in spermatozoaresults in utilization of the DHA in lipid remodeling to augment levels of DHA-containing choline and ethanolamine plasmalogens and diacyl glycerophospholipids in spermatozoa. We have generated the first detailed lipidomics data of the effects of supplementation in stallions, providing the database required for evaluating the hypothesis that low DHA levels are responsible for the poor viability of cryopreserved equine sperm.

INTRODUCTION

Reproductive technologies in the equine industry, particularly as it relates to cryopreservation, are essential for international trade and efficient use of male germplasm [1]. Stallion sperm do not survive the freezing and thawing process as well as other species such as the bull. It has been suggested that this lowered freezability of stallion sperm may be due the higher content of docosapentanoic acid (DPA; 22:5) relative to docosahexaenoic acid (DHA; 22:6), a ratio that is the reverse for these polyunsaturated fatty acids (PUFAs) in most other livestock species [2-6]. Because there is limited information demonstrating that DHA supplementation augments DHA-containing glycerophospholipids in stallion spermatozoa we undertook a targeted evaluation of the equine sperm lipidome pre- and post supplementation. These glycerophospholipids are thought to be critical structural lipids that are essential for male fertility due to their roles in membrane fluidity and fusogenicity [3-5]. In particular, recent studies indicate that differences in fatty acid and plasmalogen composition of stallion spermatozoa are associated with differences in survival of sperm after cryopreservation as well as susceptibility to lipid peroxidation [3-6].

Several studies have shown that feeding a diet rich in DHA augments DHA-containing glycerophospholipids in the plasma

membrane and results in an improvement of sperm motility after cooling to 5°C or after freezing/thawing [7-11].While these studies evaluated the effects of DHA supplementation on total glycerophospholipid pools, individual lipids were not evaluated.

Since PUFAs provide fluidity and flexibility to membranes these structural alterations are thought to minimize cold shock damage. Additionally, since DHA supplementation has been reported to enhance sperm viability in stallions [7] and other species [8-11], increasing our knowledge of the effects of DHA supplementation on individual lipids in the equine sperm lipidome is essential to advance our ability to develop techniques to improve the stability and viability of stallion spermatozoa. To examine this question, we undertook a high-resolution mass spectrometric targeted lipidomics analysis of stallion spermatozoa pre- and post-60 days of supplementation with or without DHAfrom an algal source.

MATERIALS AND METHODS

Semen collection and processing (IACUC approved)

Stallions (n = 12) had semen collected with an artificial vagina on an alternate-day basis for six collections to stabilize extragonadal sperm reserves. Stallions were randomly assigned to either the control or supplementation group. Control stallions(N=6) were fed a basal diet of mixed grass hay fed at 1.8% BW and a concentrate fed at 0.4% BW for 60 days to meet the nutritional requirements for mature, idle horses (NRC). In the treatment group, stallions (N=6) received the same diet as the control group plus 60 g of a yeast-based supplement containing 2 mg selenium as selenized yeast, 1000 IU vitamin E and a 15 g algal DHA (Alltech Inc., Nicholasville, KY) for 60 days.

All horses received the basal diet during the pre-treatment collection period (12 days). Stallions were housed in individual box stalls and had access to a turnout pen every other day.

Beginning at 50 days after the initiation of supplementation, stallions again had semen collected on an alternate-day basis for six collections. For both the pretreatment and post-treatment period, semen collected at the sixth collection was processed for evaluation of sperm lipidomics. For separation of intact sperm from seminal plasma, semen was diluted (1:1) with cold PBS and centrifuged at 800xg for 20 minutes. The supernatant was removed leaving only the intact sperm pellet. The resulting pellet was resuspended in 10 mL of cold PBS and centrifuged a second time (800xg; 20 min) prior to removal of the supernatant and resupension of the pellet in 1.0 mL of PBS which was snap frozen in liquid nitrogen for storage prior to analysis. This procedure does not remove all seminal plasma components from washed spermatozoa since some seminal plasma components are adherent to ejaculated sperm.

Spermatozoa lipidomics

After thawing, 100 µL of sperm suspension (1 – 2 mg of protein) were sonicated in 1 mL of water and 1 mL of methanol containing [$^{2}H_{8}$]arachidonic acid, [$^{2}H_{4}$]hexacosanoic acid, [$^{13}C_{18}$] stearic acid,[$^{2}H_{31}$]PtdE 34:1, [$^{2}H_{54}$]PtdE 28:0, [$^{2}H_{31}$]PtdC 34:1, [$^{2}H_{54}$]PtdC 28:0, [$^{2}H_{62}$]PtdC 32:0, [$^{2}H_{31}$]SM 16:0, [$^{2}H_{31}$]PS 36:1, [$^{2}H_{31}$]PA 34:1, [$^{2}H_{62}$]PG 32:0, and bromocriptine as internal standards [6]. Next 2 mL of methy-tert-butyl ether were added and the tubes were vigorously shaken at room temperature for 30 min. Organic extracts were dried by centrifugal vacuum evaporation and dissolved in isopropanol: methanol: chloroform (4:2:1) containing 15 mM ammonium acetate.

Direct infusion lipidomics were performed utilizing high-resolution (140,000 at 200 amu; 0.2 to 3 ppm mass error) data acquisition, on an orbitrap mass spectrometer (Thermo Q Exactive) with successive switching between polarity modes [6,12-15]. In negative ion ESI, the anions of ethanolamine plasmalogens (PlsE), phosphatidylethanolamines (PtdE), lysophosphoethanolamines (LPE), phosphatidylglycerols (PG), phosphatidylinositols (PI), phosphatidylserines (PS), and fatty acids were quantitated and lipid identities validated by MS/MS. In positive ion ESI, the cations of choline plasmalogens (PlsC) and phosphatidylcholines (PtdC), were quantitated and lipid identities validated by MS/MS. The cations and anions of bromocriptine were used to monitor for potential mass axis drift. Between injections, the transfer line was washed with successive 500 µL washes of methanol and hexane/ethyl acetate (3:2) to minimize ghost effects.

Protein determination

Proteins were determined utilizing the Pierce BCA Protein Assay kit (Thermo Fisher).

Statistical analysis

R values (ratio of endogenous lipid peak area to the peak area of an appropriate internal standard) were calculated and corrected for protein. Data are presented as mean ± SEM. A paired t-test was used to determine significant differences ($p < 0.05$) between pre- and post-supplementation in the experimental group and between pre- and post-control diet in the control group.

RESULTS

Spermatozoal free fatty acids

The major polyunsaturated free fatty acids monitored in equine sperm were 22:5 (DPA, docosapentaenoic acid) and 22:6 (DHA; docosahexaenoic acid). Free DHA levels were augmented by supplementation resulting in a significantly elevated DHA/DPA ratio in sperm (Table 1).

Spermatozoal choline diacyl and alkenyl-acyl glycerophospholipids

High levels of diacyl (phosphatidylcholines, PtdC) and alkenyl-acyl (choline plasmalogens, PlsC) glycerophosphocholines, containing polyunsaturated fatty acids at sn-2 of the glycerol backbone, were measured in stallion sperm. Members of these lipid families with DHA at sn-2 were augmented by dietary DHA whilePtdC 36:5 had decreased incorporation of 22:5 at sn-2 (Figure 1). MS^2 of these lipids revealed the characteristic phosphocholine fragment (184.0738) but did not allow us to verify the fatty acid substitutions. No pre- vs. post-differences in these lipids were monitored in the control group.

Spermatozoal ethanolamine diacyl and alkenyl-acyl glycerophospholipids

Similar to choline glycerophospholipids, supplementation

Table 1: Effects of 60 days of a yeast-based supplement containing 1000 IU vitamin E, 2 mg selenium as selenized yeast, and 15 g algal docosahexaenoic acid (DHA) on the DHA/docosapentaenoic acid (DPA) ratio in stallion spermatozoa.

Group	DHA/DPA Ratio
Control – Pre	0.14 ± 0.010
Control – Post	0.12 ± 0.011
DHA Group – Pre	0.15 ± 0.011
DHA-Group – Post	0.46 ± 0.040*

DHA: Docosapentanoic acid; DPA: Docosapentanoic acid *, $p < 0.01$ vs pretreatment

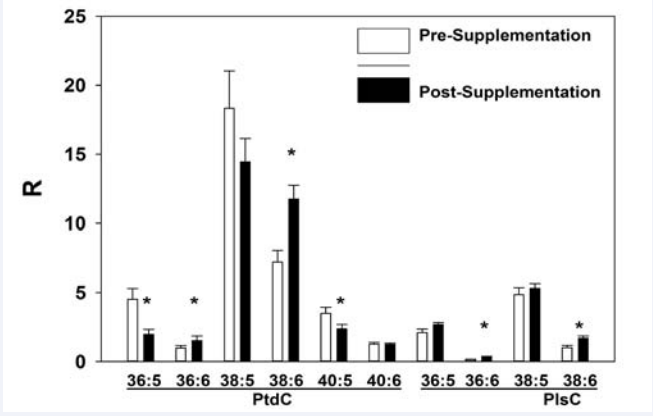

Figure 1 Augmented levels of 22:6-containing phosphatidylcholines (PtdC) and choline plasmalogens (PlsC) in stallion sperm post-supplementation. R= ratio of the peak area for the endogenous lipid to the peak area of a stable isotope internal standard. *, $p < 0.05$ vs pre-supplementation (N=6).

resulted in augmented levels of DHA-containing ethanolamine glycerophospholipids. This included phosphatidylethanolamines (PtdE) and ethanolamine plasmalogens (PlsE) as presented in Figure (2). The identities of the PtdEs were validated by MS^2 for both the sn-1 and sn-2 fatty acid substituents and the sn-2 fatty acid substituents of PlsEs (Table 2). No pre- vs. post-differences in these lipids were monitored in the control group.

Spermatozoal serine diacyl glycerophospholipids

Supplementation also resulted in augmented levels of the DHA-containing phosphatidylserine (PtdS), PtdS 40:6 (Figure 2). The identities of PtdS 40:5 and 40:6 were validated by MS^2 for both the sn-1 and sn-2 fatty acid substituents (Table 2). No pre- vs. post-differences in these lipids were monitored in the control group.

Other lipids

Supplementation was found not to alter any other glycerophospholipid not containing DHA. This included phosphatidyl cholines, phosphatidyl ethanolamines, phosphatidyl glycerols, phosphatidyl inositols, choline plasmalogens, and ethanolamine plasmalogens.

DISCUSSION

Currently there are limited lipidomics data for stallion spermatozoa in the literature [2-6]. Our data are the first to characterize the effects of a yeast-based supplement containing 15 g of algal DHA, 1000 IU vitamin E and 2 mg selenium, as selenized yeast, on individual ethanolamine, choline, and serine glycerophospholipids in equine sperm. Increases in DHA-containing phosphatidylcholines, phosphatidylethanolamines, and phosphatidylserines were observed. Also, DHA-containing choline and ethanolamine plasmalogens were shown to be augmented by supplementation. Plasmalogens are essential ether lipids for sperm motility and direct binding of sperm with the zona pellucida of the oocyte [16,17]. As ether lipids, plasmalogens require peroxisomal enzymes for generating the ether linkage at sn-1 of the glycerol backbone [17]. In this regard, since spermatozoa are devoid of peroxisomes, it appears that

Table 2: Tandem mass spectral (MS^2) analysis of diacyl and plasmenyl glycerophospholipids.

PlsE	sn-2 FA	Observed [ppm]	Observed M-(sn-2-H_2O) [ppm]
38:5	22:5	329.2484 [1.36]	436.2830 [1.82]
38:6	22:6	327.2328 [1.68]	436.2831 [1.98]
40:5	22:5	329.2484 [1.36]	464.3143 [2.18]
40:6	22:6	327.2328 [1.68]	464.3142 [2.35]

Diacyl GPL	sn-2 FA	Observed [ppm]	sn-1 FA	Observed [ppm]
PtdE 38:5	22:5	329.2485 [1.51]	16:0	255.2328 [0.27]
PtdE 38:6	22:6	327.2328 [1.55]	16:0	255.2328 [0.27]
PtdE 40:5	22:5	329.2584 [1.49]	18:0	283.2641 [0.35]
PtdE 40:6	22:6	327.2328 [1.68]	18:0	283.2642 [0.071]
PtdS 40:5	22:5	329.2484 [1.51]	18:0	283.2641 [0.35]
PtdS 40:6	22:6	327.2328 [1.68]	18:0	283.2642 [0.071]

FA: Fatty Acid; GPL: Glycerophospholipid; PlsE: Ethanolamine Plasmalogen; ppm: Parts per Million Mass Error; PtdE: Phosphatidylethanolamine; PtdS: Phosphatidylserine.

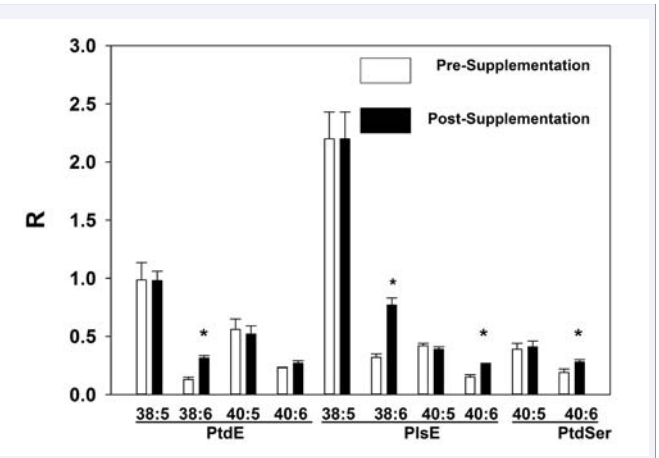

Figure 2 Augmented levels of 22: 6-containing phosphatidylethanolamines (PtdE), ethanolamine plasmalogens (PlsE), and phosphatidylserines (PtdS) in stallion sperm post-supplementation. R= ratio of the peak area for the endogenous lipid to the peak area of a stable isotope internal standard. *, $p < 0.05$ vs pre-supplementation (N=6).

peroxisomes in epithelial cells of the epididymis are probably the major source of these lipids. Epithelial cells are rich in peroxisomes in the rat [17], mouse [18], human [18], and horse [19].

While previous studies of equine spermatozoa have demonstrated the presence of C16:0 plasmalogens via quantitification of the dimethylacetyl derivative of hexadecanal [3] our studies were the first to analyze individual plasmalogens [6]. In the current study we further demonstrate that supplementation can augment the cellular levels of free DHA which in turn results in increased levels of a number of individual DHA-containing plasmalogens. A previous study [7] in stallions has demonstrated semen DHA levels increase with DHA supplementation; however, spermatozoa were not evaluated and the levels of DHA in the diet were 250 g/day, which were 16.6 times the levels in our study. DHA supplementation has been shown to improve sperm integrity, viability, and freezability in rabbits [8], chickens [9], and stallions [7].

Introduction of DHA into the glycerol backbone of glycerophospholipids occurs mainly via lipid remodeling, which involves coupled fatty acid deacylation with reacylation at sn-2 [20]. Our results demonstrate that in the case of phosphatidylcholines, deacylation coupled with DHA reacylation results in lowered levels of phosphatidylcholines containing 22:5 (PtdC 36:5, PtdC 40:5). However, in the case of choline plasmalogens, ethanolamine plasmalogens, phosphatidylethanolamines, and phosphatidylserines this was not the case. These data suggest that different enzyme isoforms may be involved in the deacylation-reacylation of sn-2 in phosphatidylcholines. The functional impact of augmented DHA-containing phosphatidylcholines, in the presence of lowered 22:5-containing phosphatidylcholines remains to be evaluated.

In summary, our study provides the first detailed analysis of supplementation on the lipidome of spermatozoa from stallions. Supplementation was found to augment the levels of free DHA which in turn resulted in increased levels of DHA-containing diacyl and alkenyl-acyl glycerophospholipids essential for male fertility. Further studies are required to assess the effects of these changes on sperm function and fertility.

CONCLUSION

Our data demonstrate that supplementation can significantly increase the DHA to DPA ratio in the sperm of supplemented horses. In addition, we also demonstrate for the first time that these increases in free DHA act to augment the levels of 22:6-containing diacyl and alkenyl-acyl glycerophospholipids. While the supplement also contained antioxidants and yeast culture, the augmented lipid changes were specific to elevations in free DHA and the associated availability of DHA for lipid remodeling of glycerophospholipids.

ACKNOWLEDGEMENTS

This work was funded by DeBusk College of Osteopathic Medicine, Lincoln Memorial University, by the Albert G. Clay Endowment and by a grant from Alltech Inc. The authors thank Dr. Juan Gallardo, Dr. Shavahn Loux, Dr. Gabriel Davolli, Lauren Goedde, Morgan Pyles for technical assistance.

REFERENCES

1. Peña FJ, García BM, Samper JC, Aparicio IM, Tapia JA, Ferrusola CO. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? Theriogenology. 2011; 76: 1177-1186.
2. Komarek RJ, Pickett BW, Gibson EW, Lanz RN. Composition of lipids in stallion semen. J Reprod Fertil. 1965; 10: 337-342.
3. Macías García B, González Fernández L, Ortega Ferrusola C, Morillo Rodríguez A, Gallardo Bolaños JM, Rodríguez Martinez H, et al. Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa. Theriogenology. 2011; 75: 811-818.
4. García BM, Fernández LG, Ferrusola CO, Salazar-Sandoval C, Rodríguez AM, Martinez HR, et al. Membrane lipids of the stallion spermatozoon in relation to sperm quality and susceptibility to lipid peroxidation. Reprod Domest Anim. 2011; 46: 141-148.
5. Parks JE, Lynch DV. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology. 1992; 29: 255-266.
6. Wood PL, Scoggin K, Ball BA, Troedsson MH, Squires EL. Lipidomics of equine sperm and seminal plasma: Identification of amphiphilic (O-acyl)-ω-hydroxy-fatty acids. Theriogenology. 2016; 86: 1212-1221.
7. Brinsko SP, Varner DD, Love CC, Blanchard TL, Day BC, Wilson ME. Effect of feeding a DHA-enriched nutriceutical on the quality of fresh, cooled and frozen stallion semen. Theriogenology. 2005; 63: 1519-1527.
8. Mourvaki E, Cardinali R, Dal Bosco A, Corazzi L, Castellini C. Effects of flaxseed dietary supplementation on sperm quality and on lipid composition of sperm subfractions and prostatic granules in rabbit. Theriogenology. 2010; 73: 629-637.
9. Cerolini S, Zaniboni L, Maldjian A, Gliozzi T. Effect of docosahexaenoic acid and alpha-tocopherol enrichment in chicken sperm on semen quality, sperm lipid composition and susceptibility to per... Theriogenology. 2006; 66: 877-886.
10. Gliozzi TM, Zaniboni L, Maldjian A, Luzi F, Maertens L, Cerolini S. Quality and lipid composition of spermatozoa in rabbits fed DHA and vitamin E rich diets. Theriogenology. 2009; 71: 910-919.
11. Alizadeh A, Esmaeili V, Shahverdi A, Rashidi L. Dietary fish oil can change sperm parameters and fatty acid profiles of ram sperm during oil consumption period and after removal of oil source. Cell J. 2014; 16: 289-298.
12. Wood PL, Shirley NR. Lipidomics analysis of postmortem interval: Preliminary evaluation of human skeletal muscle. Metabolomics. 2013; 3: 3.
13. Wood, PL, Phillipps, A, Woltjer, RL, Kaye, JA, Quinn, JF. Increased lysophosphatidylethanolamine and diacylglycerol levels in Alzheimer's disease plasma. JSM Alzheimer's Dis Related Dementia. 2014; 1:1001.
14. Wood PL. Accumulation of N-Acylphosphatidylserines and N-Acylserines in the Frontal Cortex in Schizophrenia. Neurotransmitter (Houst). 2014; 1: e263.
15. Wood PL, Bravermann NE. Lipidomics analysis of peroxisomal disorders: Discovery of deficits in phosphatidyglycerol levels in Rhizomelic Chondrodysplasia Type 1. J Data Mining in Genomics and Proteomics. 2014; S1: 001.

16. Pyttel S, Nimptsch A, Böttger J, Zschörnig K, Jakop U, Wegener J, et al. Changes of murine sperm phospholipid composition during epididymal maturation determined by MALDI-TOF mass spectrometry. Theriogenology. 2014; 82: 396-340.

17. Reisse S, Rothardt G, Völkl A, Beier K. Peroxisomes and ether lipid biosynthesis in rat testis and epididymis. Biol Reprod. 2001; 64: 1689-1694.

18. Nenicu A, Lüers GH, Kovacs W, David M, Zimmer A, Bergmann M, et al. Peroxisomes in human and mouse testis: differential expression of peroxisomal proteins in germ cells and distinct somatic cell types of the testis. Biol Reprod. 2007; 77: 1060-1072.

19. Arrighi S, Romanello MG, Domeneghini C. Ultrastructure of the epithelium that lines the ductuli efferentes in domestic equidae, with particular reference to spermatophagy. Acta Anat (Basel). 1994; 174-184.

20. Wood PL. Lipidomics of Alzheimer's disease: current status. Alzheimers Res Ther. 2012; 4: 5.

Cite this article

Wood PL, Scoggin K, Ball BA, Lawrence L, Troedsson MH, et al. (2017) Lipidomics Evaluation of the Effects of Feeding a Yeast-Based Supplement Containing Algal Docosahexaenoic Acid (DHA) on DHA-Containing Glycerophospholipids in Stallion Spermatozoa. J Vet Med Res 4(2): 1078.