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Original Research

Effects of Docosahexaenoic Acid–Rich Microalgae Supplementation on Metabolic and Inflammatory Parameters in Horses With Equine Metabolic Syndrome

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ABSTRACT

Much of the equine population is obese and therefore predisposed to the development of additional health concerns such as equine metabolic syndrome (EMS). However, pharmacologic treatments for EMS are limited. Omega-3 fatty acid supplementation is a therapeutic strategy in humans with metabolic dysfunction that improves insulin sensitivity and reduces inflammation, but the effects of omega-3 fatty acid supplementation in horses with EMS are unclear. Therefore, in this pilot study, 10 mixed-sex and mixed-breed horses with EMS were fed a docosahexaenoic acid (DHA)-rich microalgae containing 16 g DHA/horse/d or served as controls for 46 days. Inflammatory status was measured using serologic enzyme-linked immunosorbent assay and in peripheral blood mononuclear cells (PBMCs) using flow cytometry and reverse transcription polymerase chain reaction. Circulating fatty acids, triglyceride, leptin, and adiponectin concentrations were also determined. Insulin and glucose dynamics were assessed with oral sugar test (OST) and frequently sampled intravenous glucose tolerance testing. Postsupplementation, treated horses had an increase in many circulating fatty acids, including DHA ($P < .001$). Treated horses also had lower serum triglycerides postsupplementation ($P = .02$) and a trend ($P = .07$) for reduced PBMC tumor necrosis factor α. Interestingly, after 46 days, control horses had an increase in insulin responses to the OST ($P = .01$), whereas treated horses did not ($P = .69$). These pilot data indicate that DHA-rich microalgae supplementation alters circulating fatty acids, modulates metabolic parameters, and may reduce inflammation in horses with EMS.

1. Introduction

Obesity is an increasing problem in the equine population with the percentage of overweight or obese horses reportedly ranging from 20.6% to 51% [1–4], although these percentages vary based on region and may be lower in certain populations, such as in horses used for intense competition. Obesity in horses is associated with serious health concerns such as insulin dysregulation (ID) [5], both of which are two of the three defining characteristics of equine metabolic syndrome (EMS). Equine metabolic syndrome is most commonly defined by increased adiposity, ID, and a history of or predisposition to laminitis [6]. Similar to humans with metabolic dysfunction and obesity [7], horses with EMS can present with low levels of systemic [8,9] or increased levels of tissue inflammation

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[10,11]. This inflammation further promotes ID [7] and may contribute to increased susceptibility of EMS horses to the development of laminitis [12], although mechanisms underlying possibly synergy between inflammation, ID, and laminitis remain to be understood.

Equine metabolic syndrome is most often treated using dietary restriction and exercise [13,14]. Although this treatment regimen is effective, it is often a lengthy process and, because of underlying conditions such as laminitis, increased exercise is not always possible. There are two off-label pharmaceutical treatments (metformin and levothyroxine sodium) used for EMS. However, the mechanism of action for metformin is not well known, and there are conflicting reports as the long-term safety and efficacy of levothyroxine sodium [15–18]. One treatment strategy in humans with metabolic dysfunction [19,20] is supplementation with docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA), which has been shown to improve insulin sensitivity (SI) [21,22], promote fat oxidation, decrease lipogenesis, and reduce inflammation [23–25]. In horses, it improves glucose clearance [26], reduces inflammatory mediators [27–29], and changes circulating [30–32] as well as skeletal muscle [33] fatty acid concentrations. However, effects of supplementation with omega-3 fatty acids have not been investigated in the EMS animal.

Although the mechanisms of action underlying the effects of DHA on SI are not completely understood, it is thought to work, at least in part, by modulating circulating fatty acid concentrations, in particular circulating triglycerides [30,34]. This reduction in circulating fatty acids can contribute to decreases in inflammation as in obesity fatty acids can activate proinflammatory pathways. This activation of proinflammatory pathways and the resulting increase in proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) negatively impact SI by interfering with the insulin signaling cascade and promoting insulin resistance [35]. Supplementation with omega-3 fatty acids provides the building blocks for lipid-derived anti-inflammatory mediators of tissue damage recovery, such as resolvins and protectins [36], and can have direct anti-inflammatory actions [37,38]. Other mechanisms by which omega-3 fatty acids may improve SI are through their effects on metabolic transcription factors and pathways such as peroxisome proliferator–activated receptor transcription factors and the adenosine monophosphate–activated protein kinase pathway [39,40]. Therefore, supplementation with omega-3 fatty acids, in particular, DHA, may be a viable treatment option to improve metabolic and inflammatory profiles in horses with EMS. Given this, our objective was to determine the effects of DHA-rich microalgae supplementation in horses with EMS, with the hypothesis that it would improve metabolic and inflammatory responses in these animals.

2. Materials and Methods

All experimental procedures were approved by the University of Kentucky's Institutional Animal Care and Use Committee (protocol #2014-1225). Ten horses with EMS of mixed sex and mixed breed residing at the University of Kentucky's Maine Chance Farm were selected for this study. Equine metabolic syndrome was determined by the criteria in the 2010 ACVIM consensus statement [6]. To ascertain these criteria, a portable agriculture scale (model 700; Tru-Test Inc, Mineral Wells, TX) was used to establish weekly body weights. Body condition score was determined and averaged between three trained investigators using the Henneke scoring system [41]. The same trained individuals scored regional adiposity via the Carter et al. [42] cresty neck score (CNS) system. Blood collection for baseline (Day 0) serum and plasma samples was carried out after an overnight fast via jugular venipuncture, and 5–15 mL per tube was collected into tubes containing no anticoagulant for serum and ethylenediaminetetraacetic acid (EDTA) or sodium heparin containing tubes for plasma. To ascertain the presence of ID, an oral sugar test (OST) was performed after an overnight fast [6,43]. Insulin dysregulation was determined by either a fasting insulin level of >20 μIU/mL or increased insulin concentrations (>60 μU/mL) 60 minutes post administration of oral sugar [6,43]. These cut-off values were also used, as they were recommended at the time of the study by the Equine Endocrinology Group (EEG) of clinicians and researchers (http://sites.tufts.edu/equineendogroup/).

Horses were screened using the thyrotropin-releasing hormone (TRH) stimulation test [44–46], and low-dose dexamethasone suppression test was used to [47] ensure that none were affected by pituitary pars intermedia dysfunction (PPID). As recommended by the EEG, concentrations of ACTH 10 minutes after TRH injection in excess of 100 pg/mL or concentrations of cortisol exceeding 1.0 μg/dL 19 hours after dexamethasone injection were considered positive for PPID. Any animals meeting these criteria were excluded. Test to determine ID and PPID status was performed at least 2 weeks before the start of the study.

Cornell University's Animal Health Diagnostic Center Endocrinology Laboratory performed analysis of ACTH, cortisol, and insulin. The Millipore porcine insulin RIA kit (EMD Millipore Corporation, Darmstadt, Germany) was used to measure insulin concentrations [9], ACTH was measured via an automated chemiluminescent enzyme immunoassay system (Immulite, Erlangen, Germany) [48], and cortisol concentrations were determined using the Siemens Immulite Cortisol kit (Siemens, Erlangen, Germany).

2.1. Study Design and Timeline

All horses were acclimated at least 2 months before the start of the study to dry lot paddocks and to feed and feeding protocols. Two horses were randomly assigned and housed per paddock for the acclimatization period and throughout the study. Horses were provided mixed grass hay fed at 2% BW/d split between a morning (0800–1000 EST) and afternoon (1500–1600 EST) feeding. Nutrient analysis of the hay is presented in Supplemental Table 1. Horses were placed into individual pens within their paddocks and were also provided the following with their morning feeding; 1 lb/d balancer pellet (Essential K, Tribute Equine Nutrition, OH) and 1 lb/d alfalfa pellet (Hallway feeds, Farmers Feed Mill, KY) and had access to water and a mineral block ad libitum. Equine metabolic syndrome horses were randomly assigned to one of two groups, treatment group ($n = 6$; mean age 13.2 ± 4.4 years) or control ($n = 4$; mean age 11.5 ± 2.6 years). Horses were not different in age between the treated and control groups ($P = .48$). At the start of the 46-day supplementation period the treatment group received a DHA-rich microalgae supplement (FORPLUS; Alltech, Inc, Nicholasville KY) containing 16 g DHA (approximately 110 g supplement) mixed with 25 mL water, 15 mL molasses (Double S Liquid Feed Services, Inc, Danville, IL), and 4 mL anise extract (A1 Spice World, Glen Head, NY) top dressed onto their balancer and alfalfa pellets to make the supplement palatable and ensure horses received their entire daily portion of supplement. Control horses received only the vehicle of 25 mL water, 15 mL molasses, and 4 mL anise extract without the algal supplement top dressed onto their balancer and alfalfa pellets. Analysis of algal supplements nutritional and fatty acid content was performed by Eurofins Analytical Laboratories (New Orleans, LA) using high-performance liquid chromatography (HPLC) and is presented in Supplemental Table 2.

Originally, the study was intended as a cross-over design with 12 horses and a 60-day washout period. However, two control horses were dropped from the study for health reasons, one for

acute laminitis and the other for acute respiratory symptoms. In addition, the second half of the cross-over study was not completed because of significantly elevated concentrations of circulating DHA in treated horses compared with controls as far as 263 days ($P = .01$, treated horses median 0.30; interquartile range [IQR] 0.24–0.40 versus control horses median 0.06; IQR 0.06–0.172) after cessation of supplementation.

2.2. Sample Collection

Serum and EDTA plasma samples were taken after an overnight fast at baseline and 60 minutes after administration of oral sugar for the OST. This was carried out both before (Day 0; time point 1) and after the supplementation period (Day 46; time point 2). Horses were administered a frequently sampled intravenous glucose tolerance test (FSIGTT; see below) 1 day after blood collection and OST. Serum samples were used for determination of insulin, leptin, and triglyceride concentrations. Ethylenediaminetetraacetic acid plasma samples were used to determine fatty acid concentrations.

2.3. FSIGTT Tolerance Test—Optimized

At time points 1 and 2, an optimized FSIGTT was performed as previously described [49]. In brief, an indwelling, long-term intravenous catheter was placed to administer treatments and collect blood samples. After baseline blood sample collection, 50% glucose (100 mg/kg, intravenous [IV]) was administered, followed 20 minutes later by administration of insulin (20 mU/kg, IV). Blood samples were obtained at −10, −5, 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes after glucose administration. Blood samples from the FSIGTT were analyzed for glucose concentrations on an YSI analyzer (YSI Incorporated, Xylem Inc, Yellow Springs, OH) and insulin concentrations as described previously.

2.4. Endocrine and Lipid Measures

Serum samples from the OST for time points 1 and 2 and EDTA plasma from the FSIGTT were sent to Cornell University's endocrinology laboratory for determination of insulin using RIA (as abovementioned). In addition, fasting serum samples were sent to Cornell University's Clinical Pathology laboratory for leptin and triglyceride analysis via colorimetric assay using a Roche ModP analyzer (Roche Diagnostics, Indianapolis, IN) as previously described [50]. Fasting EDTA plasma samples from time points 1 and 2 were sent to Michigan State University's Diagnostic Center for Population and Animal Health for plasma fatty acid analysis using HPLC [51].

2.5. PBMC Inflammatory Cytokine Production

Heparinized blood was used to isolate PBMCs by Ficoll–Paque Plus (Amersham Biosciences, Piscataway, NJ) gradient centrifugation [52]. Cells were frozen in 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO), 40% dimethyl sulfoxide, and 50% Roswell Park Memorial Institute medium (RPMI-1640; Gibco, Grand Island, NY) until thawed for *in vitro* stimulation. For stimulation, 10^7 PBMCs were incubated in 1 mL c-RPMI (RPMI-1640 with 2.5% fetal equine serum [Sigma–Aldrich]), 100 U/mL penicillin/streptomycin (Sigma), and 55 mM 2-mercaptoethanol (Gibco) media. Cells were incubated at 37°C, 5% CO_2 with Brefeldin A (10 mg/mL; Sigma) and select wells with the positive control phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 mM; Sigma) for 4 hours. After this, determination of cytokine gene expression was carried out by separating aliquots of the cells and placing them into TRIzol (Ambion) to isolate RNA (see below). Remaining cells were assayed by flow cytometry for IFN-γ and TNF-α production. Cells were fixed in 2% paraformaldehyde (Sigma) and stored overnight at 4°C. After overnight storage, IFN-γ and TNF-α intracellular staining was carried out as previously described [53]. After the cells were stained, aliquots of cells were resuspended in FACS flow, and flow cytometric acquisition was performed on a FACSCalibur (Becton Dickinson, San Jose, CA). Determination of the percent of IFN-γ and TNF-α lymphocytes was accomplished with the use of Cell Quest (Becton Dickinson) [54].

A modified TRIzol method was used to extract total RNA from PBMC cultures (see above). One microgram of RNA was reverse transcribed into complementary DNA (cDNA), as previously described [52,54]. In brief, 1 μg of each RNA sample and a reverse transcription master mix (Promega, Madison, WI) were incubated for 15 minutes at 42°C and for 5 minutes at 95°C. Reactions included 5 μL cDNA and 20 μL of master mix. Master mix included 6.25 μL nuclease-free water (Qiagen), 1.25 μL primer/probe 20X assay mix (Applied Biosystems, Foster City, CA), and 12.5 μL TaqMan (Applied Biosystems). Equine-specific, inventoried, intron-spanning primers and probes were used with Applied Biosystems Real-Time PCR (RT-PCR; ABI 7900HT) against the following genes: housekeeping gene beta-glucuronidase (Ec03470630_m1), IFN-γ (4331182), interleukin (IL)-6 (4351372), IL-10 (4331182), IL-1β (4331182), and TNF-α (4331182). Samples were processed in duplicate and incubated for 95°C for 10 minutes. They then underwent 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Relative changes in gene expression were determined by the $\Delta\Delta C_T$ method [55], with mean ΔC_T for Week 0 set as the calibrator for all samples. Relative quantity was calculated as $2^{-}\Delta\Delta^{CT}$ and used to express results.

2.6. Serum Cytokine Analysis

Serum concentrations of TNF-α protein were determined using a commercially available equine TNF-α ELISA kit (R&D, Minneapolis, MN). This assay was optimized for equine serum samples diluted at a minimum of 1:2 [56]. All steps of the ELISA were performed according to the manufacturer's instructions, with the exception of the following which was implemented to improve the sensitivity of the assay; coating antibody provided was prepared in carbonate buffer (15 mmol Na_2CO_3, 35 mmol $NaHCO_3$, pH 9.6), and the final two incubation times were increased from 20 to 30 minutes. In addition, single absorbance was measured at A_{450}.

Serum concentrations of IL-6 were determined using an IL-6 ELISA validated for use in the horse with an analytical sensitivity of 780 pg/mL [57]. The ELISA uses a polyclonal goat anti-horse IL-6 antibody (AF1886; R&D Systems, Inc) to coat ELISA plates (Immunoplate MaxiSorp; Nalge Nunc Int, Rochester, NY). The antibody was diluted to a final concentration of 1 μg/mL in carbonate buffer (15 mmol Na_2CO_3, 35 mmol $NaHCO_3$, pH 9.6) and incubated overnight at 4°C. Afterward, the coating solution was discarded, and the plates blocked for 30 minutes at room temperature with the addition of phosphate-buffered saline (pH 7.2) containing 0.5% (w/v) bovine serum albumin. Plates were washed five times with phosphate buffer (2.5 mmol NaH_2PO_4, 7.5 mmol Na_2HPO_4, 145 mmol NaCl, 0.1% [v/v] Tween 20, pH 7.2). A recombinant equine IL-6 (1886 EL, R&D Systems, Inc) diluted in twofold serial dilutions ranging from 50 to 0.78 ng/mL was used as standard to determine IL-6 concentrations in the samples. The serum was diluted in phosphate buffer, added to the plates in triplicate wells, and incubated for 90 minutes at room temperature. After five washes, biotinylated goat anti-horse IL-6 (AF1886; R&D Systems, Inc) diluted 1:100 in phosphate buffer was added, incubated for 60 minutes, and washed again. A streptavidin–horseradish peroxidase solution

Table 1
Endocrine data, phenotypic measures, and serum leptin and triglyceride concentrations for docosahexaenoic acid–rich microalgae treated and control horses with equine metabolic syndrome both before (Day 0) and after (Day 46) supplementation period.

Endocrine and Phenotypic Measures	Control Horses Day 0	Control Horses Day 46	Treated Horses Day 0	Treated Horses Day 46
Basal insulin, μIU/mL	21.58 ± 6.89	22.85 ± 6.89	32.61 ± 5.62	29.76 ± 5.62
Insulin 60 min post oral sugar administration, μIU/mL	68.90 ± 12.29[a]	106.25 ± 12.29[b]	78.13 ± 10.04[a,b]	82.02 ± 10.04[a,b]
Change in insulin concentrations (OST 60-min basal insulin, μIU/mL)	47.31 ± 9.69	83.40 ± 9.69	45.53 ± 7.91	52.26 ± 7.91
Log BCS	0.79 ± 0.01[a]	0.84 ± 0.01[b]	0.82 ± 0.01[a]	0.86 ± 0.01[b]
CNS	2.29 ± 0.44	2.25 ± 0.44	2.97 ± 0.36	3.08 ± 0.36
Weight, kg	515.60 ± 34.54	517.19 ± 34.54	562.63 ± 28.03	568.16 ± 28.03
Leptin, ng/mL	7.23 ± 1.40	6.72 ± 1.40	7.18 ± 1.14	7.94 ± 1.14
Triglycerides, mg/dL	47.75 ± 6.62[a,b]	59.00 ± 6.62[b]	49.17 ± 5.40[b]	35.33 ± 5.40[a]

Abbreviations: CNS, cresty neck score; OST, oral sugar test.
Phenotypic measures, endocrine data, and serum leptin and triglyceride concentrations in EMS control ($n = 4$) versus DHA-rich microalgae treated ($n = 6$) horses. Results are presented as least square mean ± standard error of the mean. Within a row, differences ($P < .05$) are represented by differing superscripts.

(Jackson ImmunoResearch Lab, West Grove, PA) was added to the plates for another 30 minutes. After a final wash, substrate buffer (33.3 mmol citric acid, 66.7 mmol NaH_2PO_4, pH 5.0) was added and incubated for 20 minutes in the dark. The reaction was stopped by adding 1 volume of 0.5 M H_2SO_4, and plates were read in an ELISA reader (Bio-Tek, Winooski, VT) at 450 nm absorbance.

A commercially available equine specific kit (Kamiya Biomedical Company, Tukwila, WA) was used to measure C-reactive protein (CRP) in the serum according to manufacturer's instructions, as previously described [58].

2.7. Data Analysis

Data were analyzed via Sigma Plot 13.0 (Systat Software, San Jose, CA). A 2-way repeated measures analysis of variance was used with fixed effects set as treated versus control and time point with all possible interactions analyzed. Data not normal were log transformed and resulted in normality. Results were considered statistically significant when $P \leq .05$, and trends considered at $P \leq .10$.

3. Results

3.1. Endocrine, Phenotypic, and Lipid Measures

Oral sugar test results and phenotypic data, along with serum leptin and triglycerides, are presented in Table 1. There were no differences in fasting insulin between control and treated horses ($P = .57$). However, control horses increased in their insulin concentrations 60 minutes post oral sugar administration over time (from Day 0 to Day 46; $P = .01$). Similarly, when considering the change in insulin concentrations from baseline to 60 minutes post oral sugar administration (delta), control horses increased ($P = .01$) over time, whereas DHA-rich microalgae treated horses did not ($P = .42$). In addition, control and treated horses were not different in delta insulin concentrations at Day 0 ($P = .14$); however, controls had significantly higher delta insulin than treated horses at Day 46 ($P = .03$). Docosahexaenoic acid–rich microalgae treated horses decreased in their serum triglycerides over time ($P = .02$) and were significantly lower than controls at Day 46 ($P = .02$). There were no differences between treated or control horses for serum leptin. Although there was an overall difference between Day 0 and Day 46 for BCS ($P < .001$), with all horses increasing over time, there were no differences between treated and control horses. In addition, there were no differences seen in CNS or weight either between groups or over time.

Plasma fatty acid concentrations are presented as a percentage of total circulating fatty acids in Table 2. Docosahexaenoic acid–rich microalgae treated horses plasma DHA and C22:2n6c (docosadienoic acid) concentrations increased over time and were higher at Day 46 compared with controls. However, treated horses had a decrease over time and, at Day 46, were lower compared with controls in C18:1n9c (oleic acid; $P = .02$) and C18:3n6c (gamma-

Table 2
Plasma fatty acid results for docosahexaenoic acid–rich microalgae treated and control horses with equine metabolic syndrome both before (Day 0) and after (Day 46) supplementation period.

Plasma Fatty Acid Concentrations (% of total fatty acids)	Control Horses Day 0	Control Horses Day 46	Treated Horses Day 0	Treated Horses Day 46
C22:6n3c docosahexaenoic acid	0.05 ± 0.1[a]	0.05 ± 0.1[a]	0.01 ± 0.1[a]	1.94 ± 0.1[b]
C14:0 myristic acid	0.86 ± 0.1	0.97 ± 0.1	0.88 ± 0.1	0.89 ± 0.1
C16:0 palmitic acid	15.87 ± 0.7	16.69 ± 0.7	16.31 ± 0.6	17.22 ± 0.6
C16:1n7c palmitoleic acid	1.10 ± 0.2[a]	1.49 ± 0.2[b]	1.13 ± 0.1[a,b]	1.15 ± 0.1[a,b]
C17:0 margaric acid	0.36 ± 0.03	0.35 ± 0.03	0.44 ± 0.02	0.39 ± 0.02
C18:0 steric acid	15.38 ± 0.7	15.40 ± 0.7	15.97 ± 0.6	15.35 ± 0.6
C18:1n7c cis-vaccenic acid	1.50 ± 0.2	1.43 ± 0.2	1.55 ± 0.1	1.53 ± 0.1
C18:1n9c oleic acid	16.10 ± 1.5[b]	17.67 ± 1.5[b]	15.74 ± 1.2[b]	12.64 ± 1.2[a]
C18:2n6c linoleic acid methyl ester	43.76 ± 2.0	41.13 ± 2.0	43.49 ± 1.6	44.89 ± 1.6
C18:3n3c alpha linolenic acid	0.23 ± 0.04	0.23 ± 0.04	0.25 ± 0.03	0.18 ± 0.03
C18:3n6c y-linolenic acid	3.64 ± 0.3[b]	3.15 ± 0.3[b]	3.24 ± 0.3[b]	1.81 ± 0.3[a]
C20:2n6c eicosadienoic acid	0.15 ± 0.02	0.21 ± 0.02	0.14 ± 0.01	0.16 ± 0.01
C20:3n3c eicosatrienoic acid	0.59 + 0.1	0.75 + 0.1	0.56 + 0.1	0.89 + 0.1
C20:3n6c homo-y linolenic acid	0.19 + 0.04	0.17 + 0.04	0.12 + 0.03	0.24 + 0.03
C:22 2n6c docosadienoic acid	0.07 + 0.02[a]	0.07 + 0.02[a]	0.04 + 0.02[a]	0.22 + 0.02[b]
C22:4n6c docosatetraenoic acid	0.004 + 0.01[a]	0.009 + 0.01[a]	−0.002 + 0.01[a]	0.316 + 01[b]
C22:5n3c docosapentaenoic acid	0.12 + 0.02	0.15 + 0.02	0.09 + 0.02	0.13 + 0.02
C24:1n9c nervonic acid	0.07 ± 0.02	0.10 ± 0.02	0.04 ± 0.02	0.09 ± 0.02

Plasma fatty acid concentrations as measured by high-performance liquid chromatography in equine metabolic syndrome control ($n = 4$) versus docosahexaenoic acid–rich microalgae treated ($n = 6$) horses. Data are expressed as the percentage of total fatty acid concentrations. Results are presented as least square mean ± standard error of the mean. Within a row, differences ($P < .05$) are represented by differing superscripts.

Table 3
MinMod analysis of frequently sampled intravenous glucose tolerance test (FSIGTT) data for docosahexaenoic acid (DHA)-rich microalgae treated and control horses with equine metabolic syndrome (EMS) both before (Day 0) and after (Day 46) supplementation period.

Insulin Sensitivity Measures	Control Horses Day 0	Control Horses Day 46	Treated Horses Day 0	Treated Horses Day 46
AIRg	158.9 ± 73.8[a]	394.4 ± 73.8[b]	153.5 ± 60.2[a]	335.8 ± 60.2[b]
SI	0.442 ± 0.215	0.352 ± 0.215	0.324 ± 0.176	0.449 ± 0.176
DI	73.4 ± 77.8	106.0 ± 77.8	73.5 ± 63.5	185.8 ± 63.5
Sg	0.022 ± 0.009	0.025 ± 0.009	0.034 ± 0.007	0.018 ± 0.007

Abbreviations: AIRg, Acute insulin response to glucose; DI, disposition index; Sg, glucose effectiveness; SI, insulin sensitivity.
Measures of insulin resistance in EMS control (n = 4) versus DHA-rich microalgae treated (n = 6) horses from MinMod analysis of FSIGTT data obtained from insulin and glucose ethylenediaminetetraacetic acid plasma concentrations. Results are presented as least square mean ± standard error of the mean. Within a row, differences ($P < .05$) are represented by differing superscripts.

linolenic acid; $P = .04$) concentrations. Control horses had an increase over time in C16:1n7c concentrations (palmitoleic acid; $P = .01$) but were not different from treated horses, regardless of time point. In addition, there was an overall difference between Day 0 and Day 46 for C20:3n3c (eicosatrienoic acid; $P = .01$) and C24:1n9c (nervonic acid; $P = .03$) concentrations, with all horses increasing over time.

3.2. FSIGTT Test

As shown in Table 3, there were no treatment differences in SI, acute insulin response to glucose (AIRg), disposition index (DI), or glucose effectiveness (Sg). Nor were there any differences over time in SI, DI, or Sg. Although there was an overall increase in AIRg from Day 0 to Day 46 ($P = .01$), no treatment effects were observed.

3.3. PBMC Inflammatory Cytokine Production

Flow cytometry analysis of TNF-α and IFN-γ intracellular protein (Table 4) showed no significant differences between DHA-rich microalgae treated and control horses for the percent of lymphocytes positive (% gated) for IFN-γ or TNF-α nor for IFN-γ intensity of fluorescence per lymphocyte (mean fluorescence intensity [MFI]). There was a trend ($P = .07$) in TNF-α MFI, where DHA-rich microalgae treated horses decreased over time, whereas control horses did not change over time and were not different from treated horses. There was an overall time point difference for IFN-γ MFI ($P = .01$), with all horses at decreasing over time and an overall treatment versus control difference with TNF-α % gated higher in treated horses compared with controls ($P < .001$).

Lymphocyte gene expression of IFN-γ, TNF-α, IL-6, IL-1β, and IL-10 was not different between time points, regardless of treatment. There was an overall treatment versus control difference, with IL-1β expression higher in controls compared with treated horses ($P = .01$).

3.4. Serum Cytokine Analysis

Circulating concentrations of IL-6, TNF-α, and CRP as measured by ELISA were not different between DHA-rich microalgae treated or control horses (Table 5). There were no overall differences between Day 0 and Day 46 or any overall differences between treated or control horses.

4. Discussion

As expected, treated horses in this study had increases in circulating DHA postsupplementation, similar to what has been observed by others [32,51,59]. In addition, similar to previous studies [30], DHA-rich microalgae treated horses had a reduction in serum triglycerides after supplementation. Human studies have likewise indicated a positive effect of omega-3 fatty acid supplementation on lipids and lipid metabolism [60,61], including lipids, which are implicated in insulin resistance and inflammation, such as ceramide [62]. Indeed, changing the concentrations of dietary fatty acids can have dramatic effects on circulating fatty acid concentrations. In rats, fatty acids containing arachidonic acid, which is associated with proinflammatory effects, were increased after feeding a high-fat diet composed primarily of lard [63]. Considering that dietary supplementation with omega-3 fatty acids can affect concentrations of lipids associated with anti-inflammatory actions in ID [60,64], this supports the possibility of their use in individuals with metabolic dysfunction. In addition, given that changes in lipid composition can affect proinflammatory pathways and cytokines, which promote ID by interfering with the insulin signaling cascade [35], these studies further indicate a link between circulating fatty acids, SI, and inflammation.

Supplementation with omega-3 fatty acids in equines has been proposed as a way to improve glucose and insulin dynamics [65], similar to the improved SI seen in rats, pigs, and humans [66–68].

Table 4
Peripheral blood mononuclear cell (PBMC) inflammatory cytokine production for docosahexaenoic acid (DHA)-rich microalgae treated and control horses with equine metabolic syndrome (EMS) both before (day 0) and after (day 46) supplementation period.

Inflammatory Cytokine Measures	Control Horses Day 0	Control Horses Day 46	Treated Horses Day 0	Treated Horses Day 46
IFN-γ, % gated	20.13 ± 2.55	20.22 ± 2.55	24.51 ± 2.08	23.56 ± 2.08
IFN-γ (ibid), MFI	126.43 ± 7.98	101.86 ± 7.98	108.45 ± 6.52	94.43 ± 6.52
TNF-α, % gated	39.56 ± 2.76	40.96 ± 2.76	53.10 ± 2.25	58.79 ± 2.25
TNF-α (ibid), MFI (ibid)	57.39 ± 6.12	58.52 ± 6.12	64.11 ± 5.00	54.98 ± 5.00
IFN-γ (ibid), RQ	13,296 ± 2,192	9,383 ± 2,192	11,395 ± 1,790	9,642 ± 1,790
TNF-α (ibid), RQ	330.5 ± 136.6	315.4 ± 136.6	619.0 ± 111.6	605.3 ± 111.6
IL-6, RQ	48.41 ± 8.74	40.75 ± 8.74	42.91 ± 7.14	39.28 ± 7.14
IL-1β, RQ	3.31 ± 0.91	3.36 ± 0.91	1.86 ± 0.74	1.12 ± 0.74
IL-10, RQ	142.56 ± 34.56	144.06 ± 34.56	221.66 ± 28.22	173.84 ± 28.22

Abbreviations: INF-γ, interferon gamma; IL, interleukin; MFI, mean fluorescence intensity; RQ, Relative quantity; TNF-α, tumor necrosis factor alpha.
PBMC inflammatory data in EMS control (n = 4) versus DHA-rich microalgae treated (n = 6) horses. Flow cytometry data are represented by % gated and MFI, which characterize the percent of lymphocytes positive for IFN-γ or TNF-α protein (% gated) and the intensity of fluorescence or mean cytokine activity, per lymphocyte. Reverse transcription polymerase chain reaction data are represented by RQ of gene expression. Results are presented as least square mean ± standard error of the mean. No differences were observed between groups.

Table 5
Serum inflammatory cytokine and C-reactive protein (CRP) results for docosahexaenoic acid (DHA)-rich microalgae treated and control horses with equine metabolic syndrome (EMS) both before (Day 0) and after (Day 46) supplementation period.

Serum Inflammatory Cytokines and CRP	Control Horses Day 0	Control Horses Day 46	Treated Horses Day 0	Treated Horses Day 46
TNF-α	18.5 ± 596.6	19.1 ± 596.6	57.0 ± 487.1	1,375.1 ± 487.1
Log IL-6	0.32 ± 0.32	0.34 ± 0.32	1.05 ± 0.26	1.25 ± 0.26
CRP	272.4 ± 837.1	566.9 ± 837.1	278.5 ± 683.5	1,636.5 ± 638.5

Abbreviation: TNF-α, tumor necrosis factor alpha.
Circulating inflammatory cytokines and CRP in EMS control (n = 4) versus DHA-rich microalgae treated (n = 6) horses. Data are expressed as enzyme-linked immunosorbent assay units. Results are presented as least square mean ± standard error of the mean. No differences were observed between groups.

Algal DHA has also been shown in horses after dexamethasone administration to improve baseline glucose and insulin concentrations, as well as the modified insulin to glucose ratio [69]. In addition, marine and flax-based supplementation in horses tended to improve SI in response to an FSIGTT, but only in ID not in metabolically normal animals [65]. In this study, there was a positive effect on metabolic parameters in horses fed 16 g/d DHA from an algal source for 46 days. Specifically, treated horses did not have the same increase in insulin responses to an OST. These improved insulin responses to the OST suggest that supplementation provided a protective effect from increases in insulin responses seen in the control group. This increase in insulin measured in the control horses may be in part because of seasonal changes as the study began in the fall (early November) and ended in the winter (early January). Season can influence endocrine parameters, including higher ACTH and insulin values in the fall months in horses with PPID [70] and seasonal variations in glucose responses to IV glucose tolerance testing in healthy horses [71]. However, there have been conflicting reports regarding effects of season on insulin or glucose in groups of healthy or ID horses [65,72], indicating that more work is needed to explore possible seasonal effects on insulin and insulin responses to the OST in the EMS animal.

In humans and mice, metabolic dysfunction has long been associated with increases in inflammation [73,74]. These increases in inflammation have been proposed to play a role in metabolic dysfunction in the EMS horse or pony as well [75–77]. For this study, treated horses had a trend for a decrease in TNF-α MFI postsupplementation, indicating a potential immune-modulating anti-inflammatory effect of DHA-rich microalgae supplementation in horses with EMS. There were no differences in gene expression of PBMC inflammatory markers as measured by RT-PCR, suggesting modification occurs posttranscriptionally. Although no differences were seen in circulating inflammatory markers as measured by ELISA either pre- or post-supplementation, this is likely due in part to small sample sizes and large variability. In addition, animals may need to be challenged to see any differences in inflammatory markers, similar to previously published differences in response to OST in EMS horses versus controls [9]. Also observed was an overall effect of time on IFN-γ MFI and an overall effect of group on TNF-α % gated and IL-1β gene expression. However, there was no observed treatment by time interactions, suggesting that these overall effects were not because of treatment. Results from other work regarding the effect of omega-3 fatty acid supplementation on inflammatory parameters in the horse has been mixed. Although fish oil supplementation has not been shown to lower prostaglandin E2 (PGE2) production in lipopolysaccharide (LPS) stimulated PBMCs from yearling horses [59], there has been an effect after 14 weeks of fish oil supplementation on PGE2 production in healthy equine LPS-stimulated bronchoalveolar lavage fluid (BAL) cells compared with those supplemented with corn oil [78]. Feeding seal blubber oil as a source of omega-3 fatty acids has also been shown to have anti-inflammatory effects in the BAL of horses with recurrent airway obstruction [79]. The algal source used for this work largely contained DHA with only minor amounts of EPA, and DHA has been shown to be more effective in reducing inflammation compared with EPA [80,81]. This may have been one of the reasons for the observed trend in reduction of TNF-α MFI after supplementation contrary to negative results seen by others.

Although we did observe difference in circulating fatty acids, inflammation, and insulin responses, the major limitation of this study was small sample size. This was partly out of the investigators control, as this study was originally intended as a cross-over design. However, treated horses had significantly elevated circulating DHA concentrations 263 days after cessation of supplementation. Others have reported that 42 days [32] and 56 days [82] post-supplementation, DHA and EPA concentrations were not different from controls. This discrepancy may be explained by the fact that in the present study, horses had increased adiposity compared with presumably metabolically normal horses used in other studies. In human and horses, DHA and EPA supplementation appears to be dose responsive [32,83]. In addition, it has been shown in humans that DHA can be stored in adipose tissue for years [84]. Therefore, treated horses may have stored DHA in large amounts in adipose tissue and slowly released it over an extended period. An additional explanation may be that most other equine studies have used a fish oil source of DHA and EPA, whereas the present study used an algal source of DHA. Considering that DHA has been shown to be more readily incorporated into tissue than EPA [85], DHA versus EPA concentrations or the ratio of DHA to EPA may influence DHA incorporation into and release from tissue. Therefore, future studies with larger cohorts and with or without additional therapeutic strategies (i.e., dietary restriction) are needed to fully understand the effects of DHA supplementation in horses with EMS.

5. Conclusions

DHA-rich microalgae supplementation in this pilot study positively affected metabolic and lipid parameters and found a trend for an improvement in TNF-α MFI after 46 days of 16 g/d algal DHA supplementation in a group of horses with EMS. Although supplementation did not normalize insulin levels, given long term, it may prove useful as a nutritional therapy in addition to diet and exercise for horse with this syndrome. However, more work is needed to investigate this further and to better understand mechanisms responsible for the capacity of DHA-rich microalgae to modulate these parameters.

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Authors' contributions: S.E.E., K.M.B., and A.A.A. contributed to study design and article preparation. S.E.E., A.B., C.S., M.H.S., and V.D.B. assisted in sample collection and processing. S.B. statistically analyzed FSIGTT data and consulted on article preparation. M.M. provided animal care and assisted with sample collection.

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Supplementary data

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